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POSSIBILITIES FOR THE HEALTHY AND NUTRITIONAL IMPROVEMENT OF CONFECTIONERY AND SWEET PRODUCTS

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LIST OF ABBREVIATIONS

AACC: American Association of Cereal Chemists; ABTS: 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid); AOPP: advanced oxidation protein product; ARE: antioxidant-responsive element; AX: arabinoxylans; BHA: butylated hydroxyanisole; BHT: 3,5-di-tert-4-butylhydroxytoluene; b.w.: body weight; C: control; CAP: principal components; CC: control *gelato* obtained from a mixture developed using a cold method; CD: celiac disease; *C/EBPα*: CCAAT-enhancer binding protein-α; CP: control *gelato* obtained from a pasteurized mixture; DBS: donor bovine serum; DCFH: 2',7'-dichlorofluorescein; DCFH-DA: 2',7'-dichlorofluorescein diacetate; DHA: docosahexaenoic acid; DMEM: Dulbecco's modified Eagle's medium; DOX: doxorubicin; DMSO: dimethyl sulfoxide; 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; FBS: fetal bovin serum; FID: free induction decays; GEN: genistein; *GLUT4*: glucose transporter type 4 gene; GM: DMEM/F12 Glutamax I added of D-glucose; GPx: glutathione peroxidase; GR: glutathione reductase; GRAS: generally recognized as safe; GSH: reduced glutathione; HEPES: N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; HDL: high-density lipoprotein; HPLC-DAD-ESI-MS: high-performance liquid chromatography with diode array detection coupled to electrospray and mass spectrometry; *HPRT1*: hypoxanthine phosphoribosyltransferase 1 gene; IBS: irritable bowel syndrome; IC50: inhibitory concentration of 50%; IL-8: interleukin 8; IL-10: interleukin 10; KL_{IT}: cookies made with Italian Kamut® wheat fermented using *S. Cerevisiae* and LAB; KL_{US}: cookies made with USA Kamut® wheat fermented using *S. Cerevisiae* and LAB; KP: Kamut® wheat pasta; KS_{IT}: cookies made with Italian Kamut® wheat fermented using *S. Cerevisiae*; KS_{US}: cookies made with USA Kamut® wheat fermented using *S. Cerevisiae*; LAB: lactic acid bacteria; LC: liquid chromatography; LDH: lactate dehydrogenase; LPS: lipopolysaccharides; MC: experimental *gelato* obtained from a mixture developed using a cold method; MDA: malondialdehyde; MES: 2-(N-morpholino)ethanesulfonic acid; MP: experimental *gelato* obtained from a pasteurized mixture; MRP: Maillard reaction products; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NADH: reduced β-nicotinamide adenine dinucleotide; $NADP^+$: nicotinamide adenine dinucleotide phosphate; NADPH: reduced nicotinamide adenine dinucleotide phosphate ND: non differentiated cells; NDC: non-digestible carbohydrates; NMR: nuclear magnetic resonance; Nrf2: nuclear factor

erythroid 2-related factor 2; NSP: non-starch polysaccharides; ORAC: oxygen radical absorbance capacity; PCR: polymerase chain reaction; *PPARγ*: peroxisome proliferator-activated receptor gamma; RFU: relative fluorescence units; ROMs: reactive oxygen metabolites; ROS: reactive oxygen species; RPMI: Roswell Park Memorial Institute; SBM: sugar beet molasses; SCM: sugar cane molasses; SD: standard deviation; SDS: sodium dodecyl sulfate; SFN: sulforaphane; TAA: total antioxidant activity; TAC: total antioxidant capacity; TBA: 2-thiobarbituric acid; TBARS: thiobarbituric acid reactive substances; *TBP*: TATA box binding protein gene; TC: α-tocopherol; TCA: trichloroacetic acid; TE: trolox equivalent; TNB: 2-nitro-5-thiobenzoic acid; TPC: total phenolic content; TrxR: thioredoxin reductase; TQ: *talis quails*; TSP: 3-(trimethylsilyl)propionate-2,2,3,3-d4; US: unsupplemented; WG: whole grains; WL_{IT}: cookies made with Italian whole-grain durum wheat fermented using *S. Cerevisiae* and LAB; WP: modern durum wheat pasta; WS_{IT}: cookies made with Italian whole-grain durum wheat fermented using *S. Cerevisiae*.

PREFACE

Why do modern people need healthy confectionary and sweet products?

Confectionery products are complex foods, often intrinsic to our cultural identity, characterized by a sweet taste that consumers associate with pure enjoyment.

The sense of taste and the ability of detecting and responding to various stimuli provide animals with critical sensory input and valuable information about the quality and nutritional value of food: bitter receptors elicit aversive behavioral reactions to noxious and toxic substances, while sweet receptors allow recognition of high-caloric food sources [1].

Moreover sensory perceptions and preferences for the taste, aroma, and texture of foods affect not only food preferences but also eating habits [2], factors that are generally established during early childhood [3]. Children especially love all that tastes sweet [4], but palatable high-sugar and high-fat foods are universally preferred and often in response to emotional pressure to manage stress [5].

The human body requires sugar because it is a good energy source used for the maintenance of human metabolism [6] and, with a few exceptions and unlike any other tissue, glucose is the obligatory energy substrate of the brain [7].

However, excessive consumption of sugar is correlated with several diseases including obesity, cardiovascular diseases, diabetes mellitus type 2 and certain types of cancer [8-10].

Indeed, despite genetic factors and aging are important in determining the overall risk of many chronic diseases, a substantial proportions of these diseases occur in conjunction with a series of modifiable risk factors susceptible to lifestyle modifications, including diet [11].

The rising number of incurable diseases, despite the ground-breaking developments in modern medicine, the high incidence of obesity all over the world, and the dietary guidelines suggesting more appropriate eating habits have led people to consider confectionary products negative from a nutritional point of view.

Highly rewarding sweet foods may temporarily be a major source of pleasure and ease stress, but, in longer term, are likely to have negative impact on well-being.

Some years ago the attentions of researchers and the media has included the excessive promotion and consumption of low calorie foods with artificial sweeteners and fat replacers.

However, firstly, intense sweeteners can increase appetite for sweet foods, promote overeating and may even lead to weight gain [12], secondly consumers should realize that if

they increase the consumption low-fat/calorie foods, they may compensate for the lowered energy intake of these products [13]. This important issue should be addressed not only to consumers and industry but also, and firstly, to food science research.

As it is true that food is so frequently implicated in a variety of maladies it is also true that it can exert a positive life-long environmental impact on human health. Modern nutrition research focuses on improving health and wellness, preventing or delaying diseases, and optimizing performance. The approach of preventive food compounds has gained considerable support since people prefer to consume a food with positive effects on health rather than no longer eating what they like most and it encouraged companies and science to search for new compounds able to improve human health.

Many naturally-occurring compounds in dietary plants and animals products possess a variety of physiological functions which contribute to reduce the risk of diet-related disease. These compounds, known collectively as bioactives possess biological activity in addition to their nutritional value and are normally present at very low concentrations in foods [14].

Most of these bioactives are non-nutrient low-molecular-weight components produced by plants for their protection against pests, for the regulation of their growth, or as pigments or odor. Scientists have identified them as phytochemicals and they are a heterogeneous group of molecules, including phenolic compounds, glucosinolates, phytates, phytoestrogens, phytosterols, difficult to define and classify. A large variety of phytochemicals that are present in the daily human diet in vegetables, spices and herbs, fruits, grains and legumes have been found to possess a large range of beneficial health properties as antioxidative, anti-inflammatory and anticarcinogenic [15, 16].

Bioactive compounds effects on human health are being studied intensively. Studies concerning phytochemicals are often in disagreement because of the biological system chosen (cell line, primary culture, animals, humans), the markers considered, the dose and the time of supplementation. In any case the concentration and the chemical form of the molecules that reach human tissues after digestion should be taken into account. Another variable can be the use of pure molecules, extracts or whole foods. The interaction between bioactives and the whole food matrix could aid or hinder the bioaccessibility and bioavailability of the actives molecules. The effective dose of the isolated compound could change if administrated as part of a specific food. Others components already present in the food matrix could exert an additional positive or negative effect on the bioactive final effect. Despite the importance of this factor most of the studies do not consider it.

Furthermore the mechanism of action of bioactive compounds imply that the healthy properties that are associated with fruits, vegetables and other healthy products consumption are complex and could arise from the synergistic combination of several distinct molecules, not only within a given food but also from the overall composition of the diet. In addition, the concentration of the active compounds can be different among foods of the same category and both agronomic conditions and post-harvest operations, including food processing, have a major influence on the levels of phytochemicals in vegetable products [17-19].

Functional compounds are frequently added to foods that naturally do not contain the specific bioactives. However, a relatively easy and practical strategy to increase the nutritional value of complex systems as confectionary products could be the substitution of traditional ingredients with others of the same category that naturally contain healthy constituents. For example, it is well known that byproducts of plant food processing not only represent a disposal problem for the industry concerned, but they also represent an important source of sugars, minerals, organic acid, dietary fiber and phenolics which may be used because of their favorable nutritional properties [20, 21]. The employment of byproducts in food formulations could be an innovative and sustainable strategy that meets current and future expectations of consumers about environmental impact, ethical issues, human health and safety, maximizing the net benefit to society.

Confectionary products are poorly studied and only recently few attempts have made to improve sweet products functionality by modifying their composition [22, 23].

Identifying ingredients naturally rich in bioactives that could be exploited in the confectionary fields and establishing their health effects alone and inside the food matrix is an important scientific inquiry that could take potential societal benefit.

The overall objective of the project of my PhD thesis was to investigate the possibility to increase the nutritional value of confectionary and sweet products by the use of natural ingredients with healthy functions.

First I focused on the possible substitution of the most characteristic components of confectionary products, i.e. refined sugar, with another ingredient having a higher nutritional value. Molasses, a sugar byproduct that is still rich in vitamins, minerals, and phytochemicals is an interesting alternative to refined sugar, devoid of these healthy components. In the study reported in **Chapter 1** the antioxidant activity and the effectiveness in oxidative stress counteraction of molasses has been investigated in cultured liver cells.

Another main ingredient of confectionary products is wheat flour. To obtain health-valued products refined flour could be partially substituted by another food byproduct, durum or soft wheat bran. Bran, is a rich source of valuable health-promoting compounds that can be appropriately exploited for the production of antioxidant food ingredients; **Chapter 2** reports the results obtained evaluating the antioxidant capacity of different wheat milling fractions.

As alternative, modern wheat flour could be substituted by flours from ancient grains, that are supposed to have a higher nutritional value. **Chapters 3** deals with the healthy characteristics of an ancient grain, Kamut® khorasan, which antioxidant and anti-inflammatory activity have been evaluated and compared to whole durum wheat both *in vitro* and *in vivo*.

High consumption of confectionary products is a risk factor for obesity. A new possible strategy for the counteraction of this disease considers the effects of some bioactives on adipogenesis. In fact, compounds able to regulate size, number and function of adipocytes could contribute to treat or to prevent obesity, and could potentially be used for the formulation of functional foods, included confectionery products. **Chapter 4** focuses on three bioactives and their effectiveness in inhibiting adipocytes differentiation by evaluating both lipid accumulation and the modulation of adipogenic markers expression.

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Chapter 1
Sweetening agents as functional components of confectionery products: the case of molasses

Current attention to reducing refined sugar intake translates into replacement by artificial sweeteners (sucralose, aspartame, etc.). However, natural whole sweetening alternatives could represent a way to increase the antioxidant and nutritional content of a confectionary product, mainly considering that some recommended dietary changes involve increasing the intake of antioxidant-rich foods. Many alternatives to refined sugar are available, though not widely used. In this chapter the substitution of refined sugar with molasses is discussed and molasses antioxidant activity is demonstrated both as sweetener alone and as ingredient inside a sweet food formulation. Actually, the choice of the byproduct of sugar production as sweetening agent has a double added value because it is not only healthy, as we demonstrated, but also sustainable.

Sugar cane and sugar beet molasses, antioxidant-rich alternatives to refined sugar

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ABSTRACT

Molasses, the main byproduct of sugar production, is a well-known source of antioxidants. In this study sugar cane molasses (SCM) and sugar beet molasses (SBM) were investigated for their phenolic profile and *in vitro* antioxidant capacity, and for their protective effect in human HepG2 cells submitted to an oxidative stress. According to its higher phenolic concentration and antioxidant capacity *in vitro*, SCM exhibited an effective protection in cells, comparable to or even greater than that of α-tocopherol. Data herein reported underline the potential health effects of molasses and the possibility of using by-products for their antioxidant activity. This is particularly important for consumers in developing countries, as it highlights the importance of consuming a low-price, yet very nutritious, commodity.

INTRODUCTION

Sugar cane (*Saccharum officinarum* L.) and sugar beet (*Beta vulgaris* L. *ssp. saccharata*) are the most important crops for the production of sugar. Molasses, the thick, dark syrup obtained as a byproduct from the processing of sugar cane and sugar beet into sucrose, consists of fermentable carbohydrates (sucrose, glucose, fructose) and several nonsugar organic materials (betaine and other amino acids, mineral and trace elements, vitamins especially of the B-group, etc.). Although molasses is mainly used as a supplement for livestock feed and as a source of carbon in fermentation processes, e.g. for the production of ethanol [1], by tradition it also serves as a sweetener and colorant substitutes in cakes. Molasses is considered to be generally regarded as safe (GRAS) by the U.S. Food and Drug Administration, and people believe molasses has health benefits beyond its special taste and flavor due to it being rich in minerals. In addition, several studies evidenced that molasses is a rich source of phenolic compounds [2, 3] having possible role in the prevention of several chronic diseases involving oxidative stress [4-6]. Maillard browning carbohydrate-amino acid condensation products, formed during sugar processing, are also in very high concentration in molasses and range from low organic compounds to complex aromatic polymers. They are strongly involved in the color and aroma of molasses and they have been reported to have antioxidant activities [7-10]. In the light of the recommendation of increasing the intake of antioxidant-rich foods [11-14], the substitution of sugar with molasses could represent a potential extra-source of antioxidants.

In this study we assessed the *in vitro* antioxidant capacity and phenolic composition of molasses from sugar cane (SCM) and from sugar beet (SBM), comparing them to other common sweeteners. Then, to go further in demonstrating the oxygen free radical inhibition by molasses, the biological activity of SCM and SBM was verified supplementing HepG2 cells with two different molasses concentrations. HepG2 cells, a human hepatoma cell line considered to be a good model to study *in vitro* cytotoxic agents [15, 16], were chosen as model system given that the liver is the organ mainly involved in xenobiotic metabolism [17]. SCM and SBM protection from the oxidative damage induced by cell exposure to hydrogen peroxide (H_2O_2) was assessed by measuring cell viability, reduced glutathione (GSH) and reactive oxygen species (ROS) intracellular contents, cytosolic total antioxidant capacity (TAC), and lactate dehydrogenase (LDH) release and thiobarbituric acid reactive substances (TBARS) content in the media. To compare the effect of molasses to the effect of a well-known antioxidant, some cells were supplemented with 8.6 μg/mL (20 μM) α-tocopherol (TC), considered the most important endogenous lipophilic antioxidant in cells [18].

Our results emphasize the potential health effects of molasses, adding functional properties and nutritional value to a sweetening agent and sustaining its use as refined sugar substitute. Considering that refined sugar is the most common form of sugar in North America as well as in Europe [19], the use of molasses as alternatives to refined sugar could increase antioxidant intake similar to replacement of refined grains with whole grains [20].

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). Ethanol, and 1-propanol were supplied by Carlo Erba (Milan, Italy), while HPLC-grade solvents acetonitrile, water, methanol and acetic acid were purchased from MERCK KGaA (Darmstadt, Germany). All other chemicals were purchased from Sigma-Aldrich (Milan, Italy).

Sweeteners: White refined beet sugar, brown raw cane sugar, sugar cane molasses, sugar beet molasses, acacia honey, maple syrup, and fructose were purchased from local markets. Glucose was purchased from Sigma-Aldrich (Milan, Italy) and grape sugar and rebaudioside (60% and 98% purity) were a kind gift of Eridania Spa (Bologna, Italy).

Methods

1. *In vitro* antioxidant capacity (TAC) and phenolic composition

***In vitro* total antioxidant capacity (TAC) of different sweeteners using the ABTS assay**

One gram of each sweetener was dissolved in 10 mL of water. TAC was measured using the method of Re *et al.* [21], 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) ($ABTS^{\bullet+}$). The decolorization of $ABTS^{\bullet+}$ 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) per gram.

***In vitro* total antioxidant capacity (TAC) of different sweeteners using DPPH assay**

SBM and SCM TAC were also evaluated using the 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging capacity assay according to Cheng *et al.* [22] with some modifications. Solutions of molasses were prepared in ethanol/water 70:30 at different concentrations (0.1, 0.2, 0.5, 1, 2, 5, 10.0 mg/mL), and 100 μL 0.208 mM DPPH in ethanol/water (70:30 v/v) was added to 100 μL of each solution. The obtained mixtures were left to stand in the dark for 60 min and the absorbance at 515 nm was measured with a Tecan Infinite M200 microplate

reader (Tecan, Männedorf, Switzerland). A blank with a mixture of 100 µL ethanol/water 70:30 and 100 µL 0.208 mM DPPH was also determined for absorbance. The radical scavenging activity was calculated by the following formula: Inhibition = [(Abs $_{blank}$ - Abs $_{sample}$)/Abs $_{blank}$] x 100. The concentration required to obtain a 50% radical scavenging activity (IC_{50}) was calculated based on a dose-response curve correlating the concentration of molasses solution to the average inhibition percentage [23].

HPLC with diode array detection coupled to electrospray and mass spectrometry (HPLC-DAD-ESI-MS) analysis of phenolic compounds

Liquid chromatography (LC) analyses were performed using an Agilent 1100 series LC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a degasser, a binary pump, an autosampler, a column heater, a diode array detector (DAD) and a quadrupole mass spectrometer. Separation was carried out on a fused core type column Kinetex^{TM} C18 (100 mm x 4.6 mm, 2.6 µm) (Phenomenex, St. Torrance, CA, USA). The gradient elution was programmed using as mobile phase A acidified water (1% acetic acid) and as mobile phase B 60% of phase A and 40% of acetonitrile. The program was developed as follows: from 5% phase B to 7% B, 0-2 min; from 7% B to 9% B, 2-4 min; from 9% to 12% B, 4-7 min; from 12% to 15% B, 7-8 min; from 15% to 16% B, 8-9 min; from 16% to 18% B, 9-12 min; from 18% to 20% B, 12-14 min; from 20% to 22% B, 14-15 min; from 22% to 25% B, 15-16.5 min; from 25% to 28% B, 16.5-18 min; from 28% to 30% B, 18-19 min; from 30% to 31% B, 19-20 min; from 31% to 32% B, 20-21.5 min; from 32% to 34% B, 21.5-23 min; from 34% to 35% B, 23-24 min; from 35% to 40% B, 24-25.5 min; from 40% to 50% B, 25.5-27 min; from 50% to 100% B, 27-30 min; 100% B, 30-33 min; and from 100% to 5% B in 2 min. The flow rate was constant at 0.8 mL/min and the column temperature was maintained at 25°C. The injection volume was 2.5 µL and UV spectra were recorded from 200 to 600 nm, whereas the chromatograms were registered at 280 and 330 nm.

MS analyses were carried out using an electrospray (ESI) interface using the following conditions: drying gas flow, 9.0 L/min; nebulizer pressure, 35 psig; gas drying temperature, 350°C; capillary voltage, 3000 V; fragmentor voltage, 80 V.

2. Biological protective activity of molasses

HepG2 cells culture and supplementation

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

6-well plates and after 24 h (75-80% confluence), they were randomly divided into two groups (supplemented and unsupplemented). Supplemented cells were grown in serum-free DMEM containing TC (8.6 µg/mL) or molasses (SCM or SBM) at two different concentrations (10^2 and 10^3 µg/mL medium); unsupplemented (US) cells received a corresponding amount of sterile water. Prior to supplementation, SCM and SBM were dissolved in water and filtered with sterile 0.2 µm membrane. The total volume of the added molasses solution was less than 1% of the medium total volume.

Twenty-four hours after supplementation, cells were washed twice with warm DPBS. To cause an oxidative stress, cells were exposed to 0.2 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 $MgSO_4$) for 1 h. Non-stressed US cells instead received EBSS without H_2O_2. After 1 h, EBSS was removed, centrifuged at 400g for 3 min and used for thiobarbituric acid reactive substances (TBARS) assay and lactate dehydrogenase (LDH) release determination as described below. Cells were washed twice with cold DBPS and immediately used for further analysis.

Measurement of intracellular reactive oxygen species (ROS) concentration

Intracellular ROS concentration was monitored spectrofluorometrically according to Jiao *et al.* [24] with slight modifications. DCFH-DA (2 mM) in absolute ethanol was kept in dark at -20 °C until used, and 10 µL DCFH-DA/mL medium was added to HepG2 cells 30 min prior to H_2O_2. 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 and centrifuged at 14,000g for 15 min. DCF fluorescence intensity was detected (λ_{ex} = 485 nm, λ_{em} =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 non-stressed US cells.

Thiobarbituric acid reactive substances (TBARS) concentration

TBARS, the end-products of lipid peroxidation, were assayed in EBSS as reported [25]. One hundred microlitres of EBSS buffer were added to a mixture containing 100 µL of TCA (30% in 0.25 N HCl), 100 µL of TBA (0.75% in 0.25N 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 (λ_{ex} = 535 nm, λ_{em} = 595 nm) [26]. TBARS level was

expressed as relative fluorescence units (RFU) and normalized for mg of proteins in each well.

Lactate dehydrogenase (LDH) release

LDH is a soluble cytosolic enzyme converting pyruvic acid to lactic acid through NADH oxidation. Because loss of membrane integrity causes LDH leakage, the level of enzyme activity in extracellular fluids is used as an indicator of membrane damage. LDH activity in the EBSS buffer was assessed spectrophotometrically at 340 nm for 1 min by measuring the rate of NADH oxidation [27]. The assay mixture contained 100 µL of 1.4 mM NADH, 100 µL of 10 mM pyruvate, and 600 µL of DPBS; the reaction started with 200 µL of sample. Enzyme activity was calculated using the extinction coefficient of NADH (6.22 $mmol^{-1}$ cm^{-1}), expressed as mU/mL medium and normalized for mg of protein in each well.

Cell viability

Cell viability was evaluated by measuring the conversion of the tetrazolium salt to its formazan product as previously reported [28]. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was dissolved in RPMI-1640 medium without phenol red (final concentration 0.5 mg/mL) and 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. Cell viability in stressed cells was expressed as percent of non-stressed US cells.

Cytosolic TAC

Cells were lysed with 1 mL of cold Nonidet P-40 (0.25% in DPBS), incubated for 30 min on ice and centrifuged at 14,000g for 15 min. Cytosolic TAC was measured on the supernatant using the method of Re *et al.* [21], as described above. Values were normalized for protein content in the sample and expressed as µmol of trolox equivalents (TE)/mg protein.

Total thiols content

Cytosolic thiol content was determined as previously described [29] and calculated as GSH. Cells were lysed with 700 µL of cold Nonidet P-40 (0.25% in DPBS), incubated for 30 min on ice and centrifuged at 14,000g for 15 min. One hundred microlitres of the supernatant was incubated with 100 µL of reagent buffer (80 mM sodium phosphate, pH 8.0, 2 mM EDTA, 2% SDS and 250 µM DTNB) for 30 min. Thiols were 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 nmol of thiols calculated as GSH/mg protein.

Protein content

Protein content was determined according to Bradford [30], using bovine serum albumin in water as standard.

Statistical analysis

Data on *in vitro* antioxidant activity and phenolic profile are reported as means ± SD (n = 3); data obtained in cell cultures are reported as means ± SD of at least six samples derived from three independent cell cultures.

The evaluation of DPPH and HPLC-MS data statistical significance was carried out by the Student's t test. All other data were analyzed for statistical significance by the one-way ANOVA, using Dunnett's post-hoc test.

RESULTS

1. *In vitro* TAC and phenolic composition

The *in vitro* TAC of SCM and SBM was from 20 to 100 and from 5 to 20 fold higher than the other tested sweeteners respectively (Figure 1).

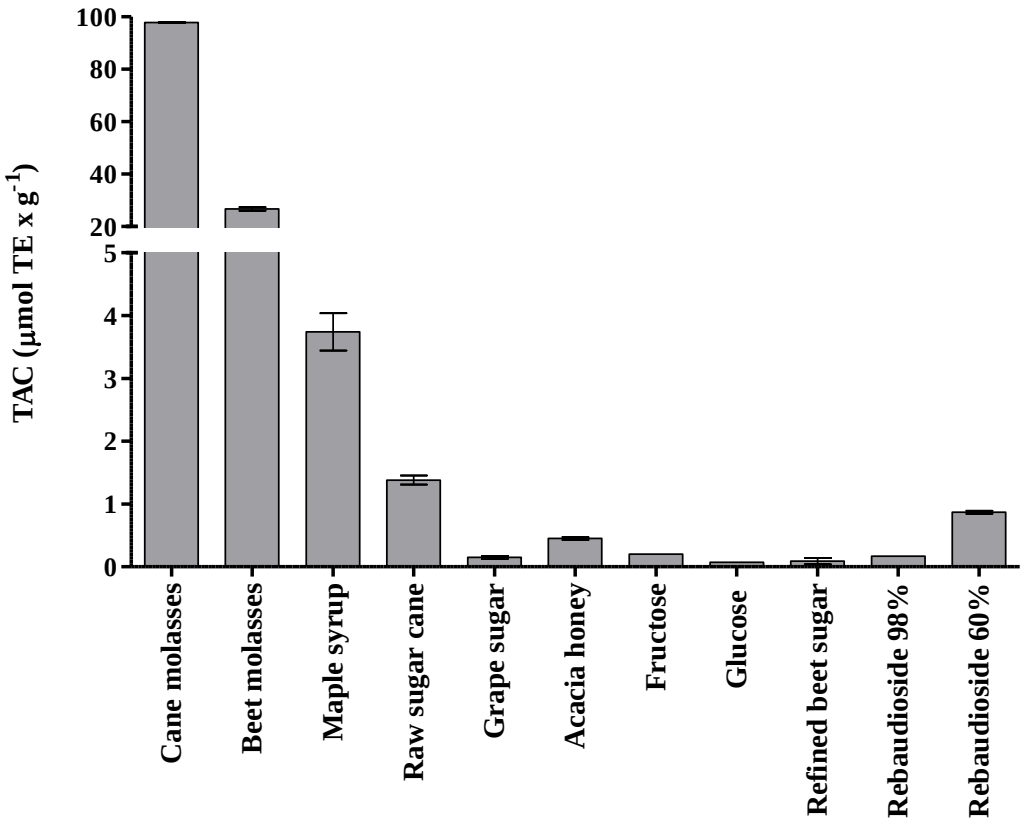

Figure 1. Total antioxidant capacity (TAC) of the different sweeteners.

TAC is expressed as µmol of Trolox equivalents (TE)/g of sweetener. Data are means ± SD**.** Statistical analysis was by the one-way ANOVA ($p < 0.001$).

The higher antioxidant capacity of SCM than SBM was confirmed by the DPPH assay, because the molasses concentration needed to reduce oxidation by 50% (IC_{50}) were 7.25 mg/mL for SBM and 1.47 mg/mL for SCM ($p < 0.001$).

Total phenolic content and SCM and SBM profiles showed significant differences between the two molasses, SCM possessing not only a 6 times higher total phenolic content but also a more complex and different profile (Table 1).

Table 1. SBM and SCM phenolic profile[a]

	Phenolic compounds	RT	$[M-H]^-$	µg/g SCM	µg/g SBM
1	**5,7-Dihydroxyflavanone**	4.6	255	9.71 ± 1.05	
2	**Catechin**	5.0	289	16.42 ± 0.20	
3	**4-hydroxyphenylacetic acid**	6.9	151	5.83 ± 0.14	
4	**Dicaffeoylquinic acid glucoside**	7.4	677	2.08 ± 0.33	
5	**Vanillic acid**	7.7	167	30.07 ± 0.20	
6	**Syringic acid**	8.5	197	85.53 ± 1.38	2.26 ± 0.07
7	**Quercetin 3-O-glucosyl-xyloxide**	9.7	515	25.27 ± 1.94	
8	**Vanillin**	9.9	151		17.41± 0.51
9	**Feruoylquinic acid**	10.0	367	5.32 ± 0.06	
10	**Diferuoylquinic acid**	10.4	735	5.23 ± 0.20	
11	**Tricin 7-O-glucoside**	10.8	491	16.45 ± 1.02	
12	***p*-coumaric acid**	10.9	163	9.18 ± 0.91	
13	**Apigenin-hexoside-pentoside**	11.6	563	53.66 ± 3.02	
14	**Ferulic acid**	12.3	193	6.25 ± 0.63	14.83 ± 0.29
15	**Hydroxybenzaldehyde**	13.4	121		2.93 ± 0.10
16	**7-methylapigenin-6-C-glucoside**	13.8	445	22.28 ± 0.96	
17	**Hydroxybenzoic acid**	13.9	137		1.12 ± 0.11
18	**Caffeoyl-O-malonyl-O-coumaroylquinic acid**	15.2	585	4.19 ± 0.41	
19	**6,8-Dihydroxykaempferol**	15.8	287	22.35 ± 1.67	
20	**Tricin-7-O-b-(6-*p*-methoxycinnamate)-glucoside**	16.6	651	15.52 ± 0.38	
21	**Luteolin/Kaempferol**	19.8	285		17.24 ± 0.49
22	**Caffeoylquinic acid**	20.0	353	10.45 ± 0.71	
23	**Feruloyl-arabinose-arabinose**	20.3	307	35.99 ± 1.31	4.51 ± 0.47
24	**Caffeoyltartaric acid**	25.8	311		1.95 ± 0.15
	Total			381.62 ± 6.82	62.25 ± 1.72

[a]Phenolic compound concentration is expressed in µg analyte/g. Data are means ± SD. Student's t test was used to determine the statistical differences for peak 6 ($p < 0.001$), peak 14 ($p < 0.001$), and peak 23 ($p < 0.001$).

Figure 2 shows the UV chromatogram at λ = 280 nm of the SCM and SBM phenolic compounds identified using UV and MS data. Syringic acid, the major phenolic component of SCM, was present in small amounts in SBM, whereas vanillin, luteolin/kaempferol, and ferulic acid, the major components of SBM, were absent or present in smaller amounts in SCM.

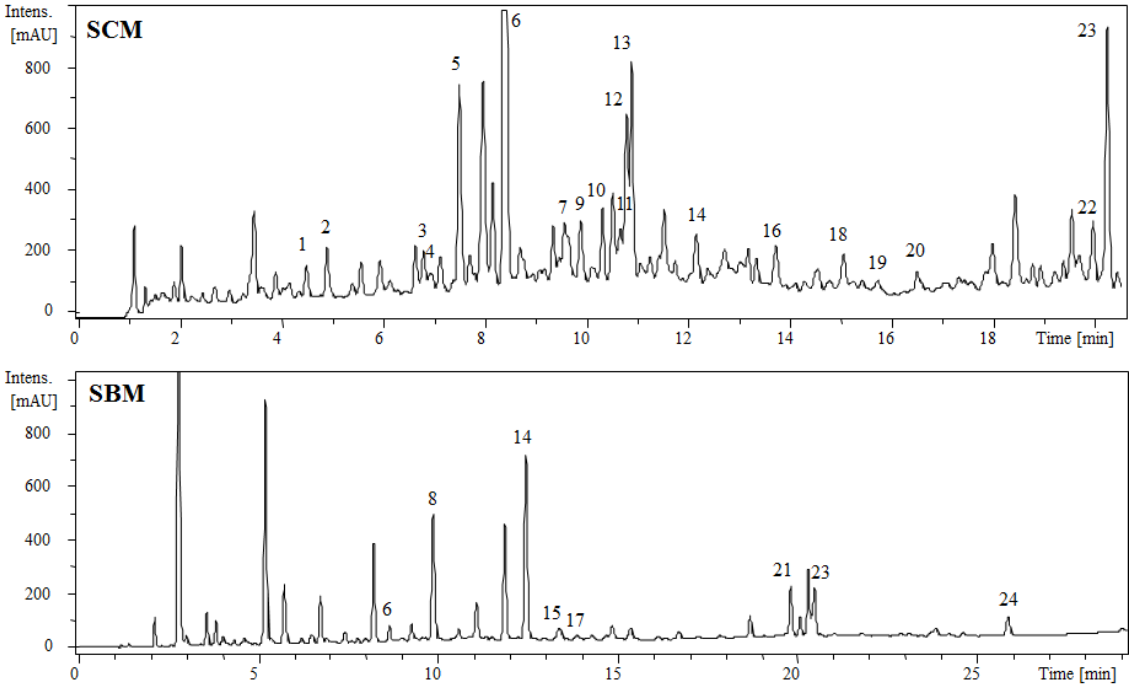

Figure 2. Chromatograms of the phenolic compounds of SCM and SBM at λ = 280 nm. Peaks were identified as: 1, 5,7-dihydroxyflavanone; 2, catechin; 3, 4-hydroxyphenylacetic acid; 4, dicaffeoylquinic acid glucoside; 5, vanillic acid; 6, syringic acid; 7, quercetin 3-O-glucosyl-xyloxide; 8, vanillin; 9, feruoylquinic acid; 10, diferuoylquinic acid; 11, tricin 7-O-glucoside; 12, p-coumaric acid; 13, apigenin-hexoside-pentoside; 14, ferulic acid; 15, hydroxybenzaldehyde; 16, 7-methylapigenin-6-C-glucoside; 17, hydroxybenzoic acid; 18, caffeoyl-O-malonyl-O-coumaroylquinic acid; 19, 6,8-dihydroxykaempferol; 20, tricin-7-O-b-(6-p-methoxycinnamate)-glucoside; 21, luteolin/kaempferol, 22, caffeoylquinic acid; 23, feruloyl-arabinose-arabinose; 24, caffeoyltartaric acid.

2. Biological protective activity of molasses

The biological activity of SBM and SCM was verified using HepG2 cells as model system. In preliminary experiments cells were supplemented with SCM and SBM at 10^{-2}-10^{5} µg/mL medium concentration, and possible cytotoxic effects were assessed by MTT and LDH assays. Neither SCM nor SBM up to the 10^{4} µg/mL medium concentration caused modifications in the tested parameters (*data not shown*), while the highest molasses

concentration (10^5 µg/mL medium) caused cell death.

The microscope observation of 10^4 µg/mL supplemented cells highlighted appreciable changes in morphology, supplemented cells appearing less in number than US cells, mainly in clusters and with a well-rounded shape. For this reason the 10^2 and 10^3 µg/mL medium concentrations were used for supplementation in the following experiments.

In order to verify the onset of an oxidative stress due to H_2O_2 treatment and its possible counteraction by SBM and SCM, intracellular ROS production and TBARS concentration were detected (Figure 3).

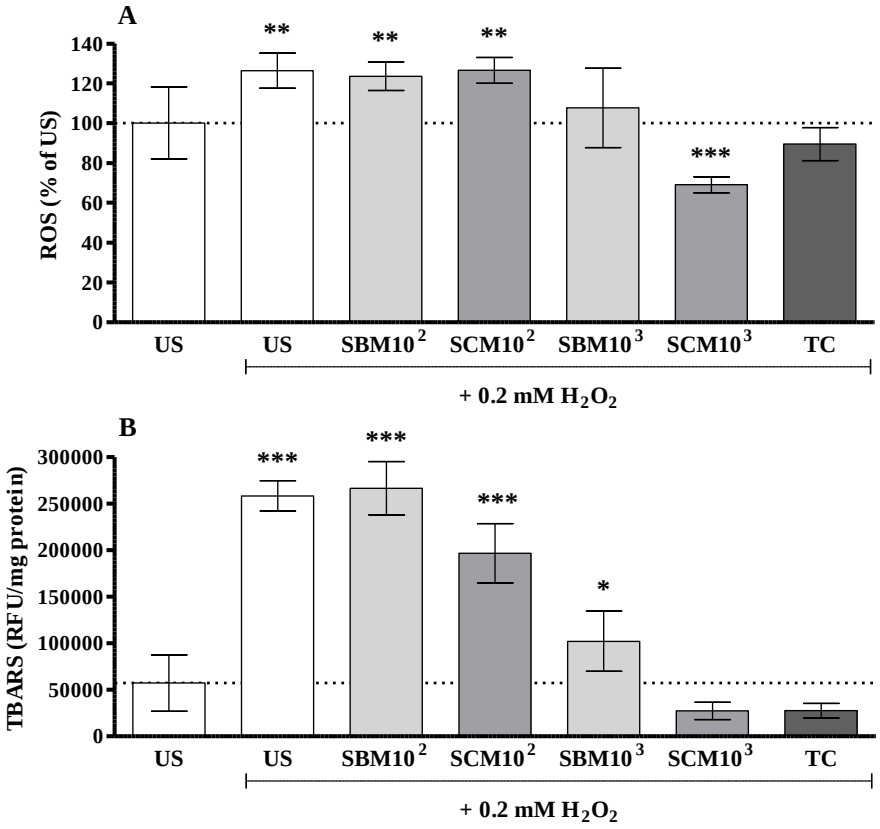

Figure 3. Cellular ROS (A) and TBARS (B) concentration in unsupplemented and supplemented cells.

ROS concentration (panel A) is expressed as percent of the concentration determined in non-stressed, unsupplemented (US) cell (assigned as 100%). TBARS concentration (panel B) is expressed as RFU/mg protein in the corresponding well. Data are means ± SD. Statistical analysis was by the one-way ANOVA ($p<0.001$) with Dunnett's post-hoc test: *$p<0.05$, **$p<0.01$, and ***$p<0.001$ vs non-stressed US cells.

As reported in Figure 3A, incubation with 0.2 mM H_2O_2 resulted in a significant increase in ROS production in unsupplemented and 10^2 µg/mL SBM and SCM supplemented cells. Intracellular ROS concentration was unchanged in TC and 10^3 µg/mL SBM supplemented cells and decreased in 10^3 µg/mL SCM supplemented ones with respect to non-stressed unsupplemented HepG2. Treatment with H_2O_2 caused a significant increase of TBARS concentration in all tested conditions except for TC and SCM at the highest concentration (Figure 3B).

The strong increase in LDH leakage induced by the oxidative stress in US cells was completely prevented by SBM and SCM even at the lower concentration used (Figure 4A).

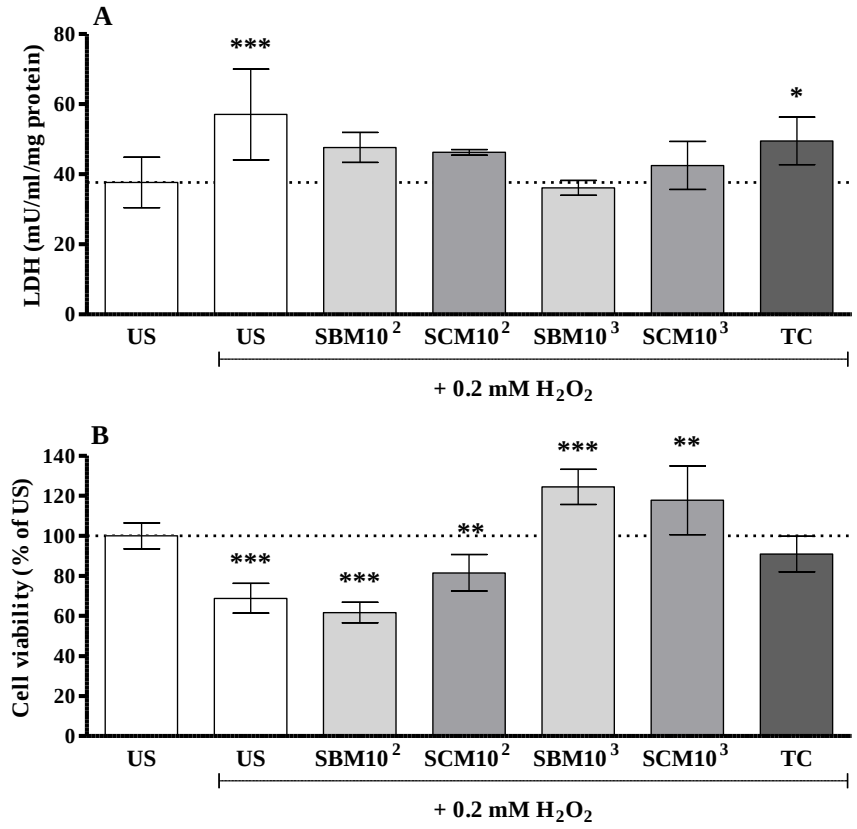

Figure 4. LDH release (A) and cell viability (B) in unsupplemented and supplemented cells.

LDH activity in the medium (panel A) is expressed as mU/mL medium/mg protein in the corresponding well. Cell viability (panel B) is expressed as percent of non-stressed, unsupplemented (US) cells (assigned as 100%). Data are means ± SD. Statistical analysis was by the one-way ANOVA ($p<0.001$) with Dunnett's post-hoc test: *$p<0.05$, **$p<0.01$, and ***$p<0.001$ vs non-stressed US cells.

Exposure to H_2O_2 evoked a reduction of cell viability in unsupplemented and 10^2 SBM supplemented cells, whereas 10^2 SCM supplementation was slightly protective. TC and the higher SBM and SCM concentration completely protected HepG2 cells, viability being even higher in molasses supplemented than in non-stressed US ones (figure 4B).

As shown in Figure 5A, cytosolic TAC did not change in any of the tested conditions compared to non-stressed US cells; similarly no modification in GSH content was observed in US cells or in cells supplemented with SBM and SCM at the lowest concentration. On the contrary TC and the highest SBM and SCM concentration caused an increase of the antioxidant thiols (Figure 5B).

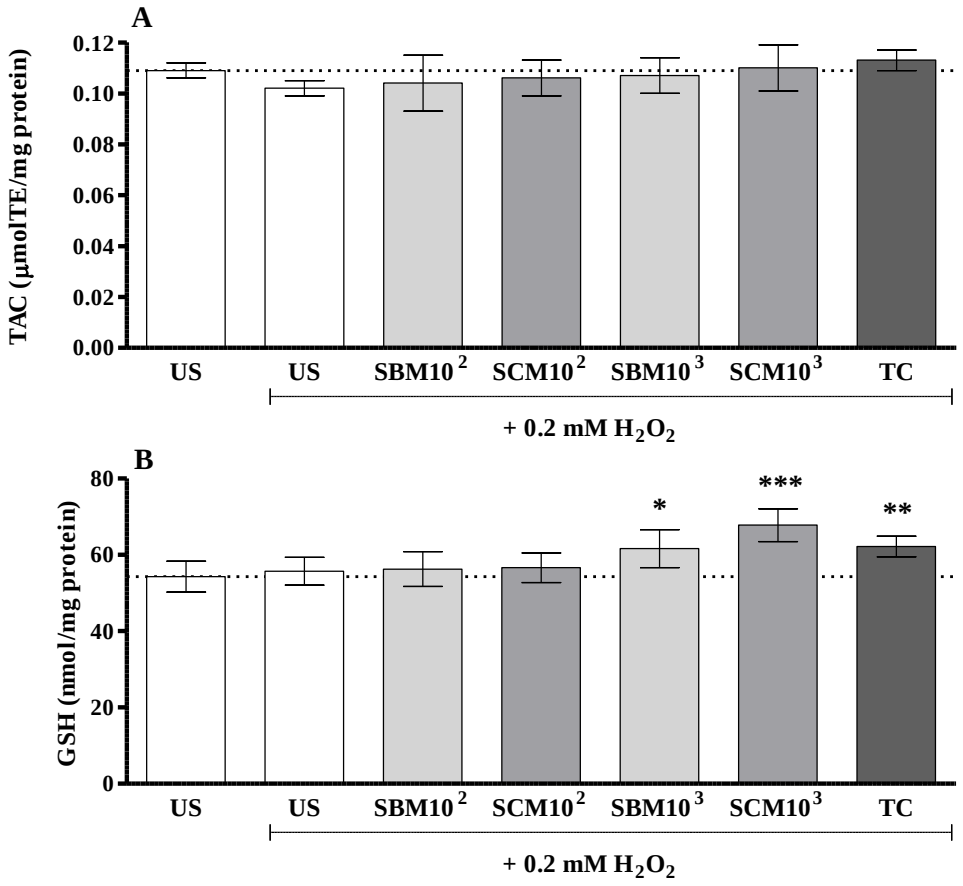

Figure 5. Cytosolic TAC (A) and reduced glutathione (B) concentration in unsupplemented and supplemented cells.

Cytosolic TAC (panel A) is expressed as µmol TE/mg protein in the corresponding well, and GSH concentration (panel B) as nmol/mg protein in the corresponding well. Data are means ± SD. Statistical analysis was performed by the one-way ANOVA ($p<0.001$) with Dunnett's post-hoc test: *$p<0.05$, *$p<0.01$, and ***$p<0.001$ vs non-stressed US cells.

DISCUSSION

Byproducts of plant food processing represent a major disposal problem for the food industry, but they are also promising sources of compounds which may be used because of their favorable technological or nutritional properties [1]. Special attention has already been paid to agricultural byproducts such as rice hulls, almond hulls, potato peel waste, olive mill waste water, grape and citrus seeds and peels, green-vegetable byproducts that have been proven to be effective sources of antioxidants [31, 32].

The presence of phytochemicals in sugar is often undesirable, as they influence the quality and the color of the final product; hence these phytochemicals are removed through various purification procedures in the sugar industry [19]. Thus, molasses, the byproduct of sugar refining, is a very good source of residual antioxidant components from the plant and of antioxidants molecules formed during the cooking of the juice [20].

In this study cane and beet molasses were firstly evaluated *in vitro* for their antioxidant capacity and compared with other sweeteners. Our results are in accordance with those obtained by Phillips *et al.* [20] who reported substantial differences in the TAC of several sweeteners, SCM having the highest one. In our study maple syrup, for which a high content of phenolics has been already reported [33], showed a quite good TAC, while sugar cane had a low TAC. Dissimilarities among brown sugars have been reported [34] and are related to differences in cane varieties, in the maturity of the cane plant at harvest time, in the processing procedures, and mainly in the techniques used to remove color and impurities that affect the amount of volatiles and polyphenols that end up on the surface of the crystal. The observed low TAC of acacia honey is in agreement with Ghedolf and Engeseth [35], who found a wide range of antioxidant capacity in honey from different sources, acacia honey having the lowest one. The higher TAC of rebaudioside 60% than rebaudioside 98% can be accounted to the lower purity of the former sweetener. TAC of other sweeteners was negligible.

Since the *in vitro* TAC of foods is only an approximate reflection of their biological protective activity, chemical assays and cell-based methods giving often contradictory results [36, 37], we evaluated the protective effect of SCM and SBM supplementation against an induced oxidative stress in HepG2 cells. α-tocopherol, a well-known potent antioxidant acting as peroxyl radical scavenger that terminates chain reaction [38], was used as positive control. In preliminary experiments possible cytotoxicity was evaluated using different SCM and SBM concentrations, and further experiments were performed using the highest molasses concentrations causing no sign of cell toxicity.

The effectiveness in the protection of HepG2 cells from the induced oxidative stress, as indicated by the different markers considered, was greater for SCM than SBM and dependent on the concentration used: at the highest concentration SCM protection was equal to or higher than TC effect.

The higher biological effectiveness of SCM is in agreement with data on *in vitro* TAC and phenolic composition, which were higher for SCM than for SBM, emphasizing the importance of phenolic concentration and profile for molasses protective action. It is conceivable that molasses antioxidant proprieties are mainly ascribable to the phenolic content, although other molecules such as the Maillard reaction products (MRP) could contribute to the overall effect. Indeed, MRP effective antioxidant protection against oxidizable substrates has already been evidenced in cell culture systems [17, 39, 40]. This strongly suggests the implication of MRP in the observed protective effects of molasses.

Extensive work has been carried out for the identification and quantification of the mayor macromolecules (including colorants) in cane and beet sugar processing at all stages [41-43]. In general the colorants are believed to be produced during Maillard reaction, alkaline degradation reactions and sugar degradation [43]. Godshall *et al.* [41] evidenced that beet and cane colorants are fundamentally different: beet colorants tend to be produced during processing, mainly from alkaline degradation of invert and melanoidin formation, while cane colorants enters the process in the cane juice as plant pigments associated with polysaccharide, and changes very little in process. In addition, cane polysaccharides involved in the color formation have been shown to be associated with polyphenolic acids [44]. These differences could have contributed to the higher activity of SCM than SBM.

In this study we supplemented cells with the whole molasses, and not with molasses-derived compounds, so it was not possible to define which components were the most protective ones. Although it could appear a limitation of our study, to our aim, that is, the evaluation of the possible protective effect of molasses as food/food ingredient, it was the best approach, and it allowed us to consider the synergism between the different antioxidant molecules and the importance of the food matrix, well-recognized factors of the overall antioxidant effectiveness [45] that are ignored in studies evaluating the effect of pure compounds.

Few data are available in the literature on molasses bioactivity in *in vivo* or *ex vivo* systems: sugar molasses have been reported to have immunomodulatory activity in human whole blood cell cultures [46], to raise HDL cholesterol level in rats [47], and to have inhibitory effects on mutation and nitric oxide production in lipopolysaccharide stimulated macrophages [48]. To our knowledge this work is the first one evidencing molasses effectiveness in the

counteraction of the oxidative damage in cultured cells. We acknowledge that results in whole organisms may diverge from those in the cultured cells because of bioavailability and metabolism of ingested phytochemicals mixtures and agents, and therefore results in the cultured cells could be misleading if taken in isolation. After ingestion the most of dietary (poly)phenolics appear in the circulatory system not as the parent compounds, but as phase II metabolites [49]. Although in our study the use of human hepatic cells able to metabolize the parent compounds reduced in part the distance between our approach and the physiological situation in humans, further investigation *in vivo* are needed before drawing conclusion. Further *in vitro* mechanistic studies are also needed to understand how molasses bioactive molecules interact with human physiological and pathological processes, particularly considering that it is becoming clear that the mechanisms of action of polyphenols go beyond the modulation of oxidative stress [50]. Particularly Guimarães *et al.* [3] demonstrated cane molasses protection against DNA oxidative damage besides radical scavenging capacity.

Although the variability due to agronomical and technological factors among the different molasses must be taken into account, our results support a greater exploitation of molasses as food ingredient considering it as a tasty extra source of antioxidants. In this light the broad quality of molasses sources must be carefully considered, since some impurities (particularly plant growth regulators, pesticides, clarification polymers such as polyacrylamide, heavy metals, and plant electrolyte salts) concentrated in the sugar syrups could be present in the magma from which molasses originates [51]. Consequently, the relative quality of the molasses must be assessed before marketing to the public.

Besides all these considerations, data herein reported underline the potential health effects of molasses, adding functional properties and nutritional value to a sweetening agent, and sustaining its use as refined sugar substitute. Furthermore, they emphasize the possibility of using byproducts for their antioxidant activity. This is particularly important for consumers in developing countries, as it highlights the importance of consuming a low-price, yet very nutritious, commodity.

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Evaluation of the total antioxidant capacity of gelato [§] prepared with cane molasses as refined sugar substitute

INTRODUCTION

The antioxidant potential of foods is dependent on the synergistic interactions of many bioactive compounds, phenolics being one of the major contributors [1]; for this reason it is often evaluated as total antioxidant capacity (TAC), and it is not based on the concentration of single antioxidant molecules.

A number of factors, including genetics and growing conditions (cultivar, maturity at harvest, soil and water state, climate, and postharvest treatments) are known to affect the concentration of plant phytochemicals having antioxidant activity, therefore modulating their TAC [2].

Food processing, as cooking or freezing, storage, and preparation are other factors that can impact TAC, together with the interaction between the antioxidant bioactive molecules and other compounds/ingredients in the food.

To evidence a high TAC in one food ingredient is not sufficient to claim high antioxidant potential of the whole food once ready to eat. Therefore, after having evidenced the potent antioxidant activity and the high phenolic content of cane molasses as single ingredient [3], in this study we have evaluated the TAC of a *gelato* prepared with sugar cane molasses, and compared it with the same product conventionally prepared with refined sugar.

METHODS

Gelato preparation

Two types of *gelato* were prepared by Optima Srl (Rimini, IT) according to the recipe reported in Table 1.

[§] *Gelato simply means "frozen" and is not just the Italian word for ice cream. The difference between gelato and ice cream (or industrial gelato) mainly depends upon three factors: fat, air and temperature. I) Ice cream, according to American laws, must have at least a 10% fat content, whereas Italian artisan gelato generally contain only from 3 to 10% fat; II) Gelato has less overrun than ice-cream, that is the air added to frozen desserts to increase volume and obtain softer products; III) Gelato is served at a higher temperature than ice cream because of the less overrun.*

	Molasses	**Control**
Milk	900 g	900 g
Cream	100 g	100 g
Skim milk powder	30 g	30 g
Refined sugar	187.5 g	250 g
Cane molasses	62.5 g	
Stabilizer (E417)	3 g	3 g

Table 1 *Gelato* recipe

The experimental *gelato* was prepared using 25% of cane molasses instead of refined sugar compared to the standard recipe. Usually, ice cream is pasteurized at 85°C for few minutes and this procedure could modify the TAC. So, both the experimental and traditional *gelati* were also developed using a "cold" procedure.

Thus, the samples developed were of 4 kinds:

- Experimental *gelato* obtained from a pasteurized mixture (MP)
- Experimental *gelato* obtained from a mixture developed using a cold method (MC)
- Control *gelato* obtained from a pasteurized mixture (CP)
- Control *gelato* obtained from a mixture developed using a cold method (CC)

Extracts preparation

All chemicals, reagents and solvents were purchased from Sigma-Aldrich Co. (St Louis, MO, USA) unless otherwise stated.

The *in vitro* TAC was evaluated after three different complementary methods of extractions, since a single procedure could not accurately reflect all the antioxidants in a complex system [4].

A) Extraction performed using combined solvents, ethanol/water (70:30, v/v) acidified with HCl 0.1%, according to the method of Rababah *et al.* [5] as optimized in our laboratory. Briefly, 1 g of *gelato* was extracted with 6 mL of 70% acidified ethanol (20 min at 40 °C with shaking) and centrifuged at 3,000g for 5 min. The residue was extracted again with 3 mL of the hydro-alcoholic solvent (20 min at 40 °C with shaking) and centrifuged at 3,000g for 10 min. The supernatants from both extractions were filtered and mixed. Two independent extractions were performed for each sample.

B) Extraction performed using methanol. Briefly, 1 g of gelato was extracted with 6 mL of methanol (20 min at 40 °C with shaking) and centrifuged at 3,000g for 5 min. The residue was extracted again with 3 mL of the same solvent (20 min at 40 °C with shaking) and

centrifuged at 3,000g for 10 min. The supernatants from both extractions were filtered and mixed. Two independent extractions were performed for each sample.

C) Extraction performed using ultrapure water (Milli-Q; Millipore, Bedford, CT, USA). Briefly, 1 g of *gelato* was extracted with 9 mL of water (20 min at 40 °C with shaking) and centrifuged at 4,500g for 10 min. The supernatants from both extractions were filtered and mixed. Two independent extractions were performed for each sample. All extracts were preserved at -20 °C until analysis.

Total Antioxidant Capacity (TAC) assay

TAC was measured using the method of Re *et al.* [6], 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). Values obtained were compared to the concentration-response curve of the standard Trolox solution, and expressed as µmol of Trolox equivalents (TE)/g of product.

RESULTS AND DISCUSSION

As shown in Figure 1, the TAC of the experimental *gelato* was significantly higher than that one of control, independently from the kind of extraction considered.

No significant differences were detected between the products developed with the pasteurized mixture and those ones prepared following the "cold" procedure. Thus, pasteurization does not affect the TAC of *gelato*, at least in the reported conditions.

Comparing the different extraction methods, the one performed using ethanol/water (70:30, v/v) acidified with HCl 0.1% proved to be the most exhaustive one, as it is shown by the higher TAC values of the hydroalcoholic extracts. This result indicates that the best solvent for a complex food as *gelato* should include an hydrophilic phase and a lipophilic phase. Indeed, on the basis of their solubility antioxidants can be roughly classified into two groups: i. hydrophilic antioxidants, comprising vitamin C and many of the polyphenolic compounds, and ii. lipophilic compounds, predominantly consisting of vitamin E, carotenoids and chlorophylls.

In addition, antioxidants have different degree of solubility; as example, the solubility of polyphenols varies according to their molecular weight and the degree of glycosylation, acylation (e.g. galloyl groups) or esterification; in particular, water solubility increases with increasing glycosylation [7].

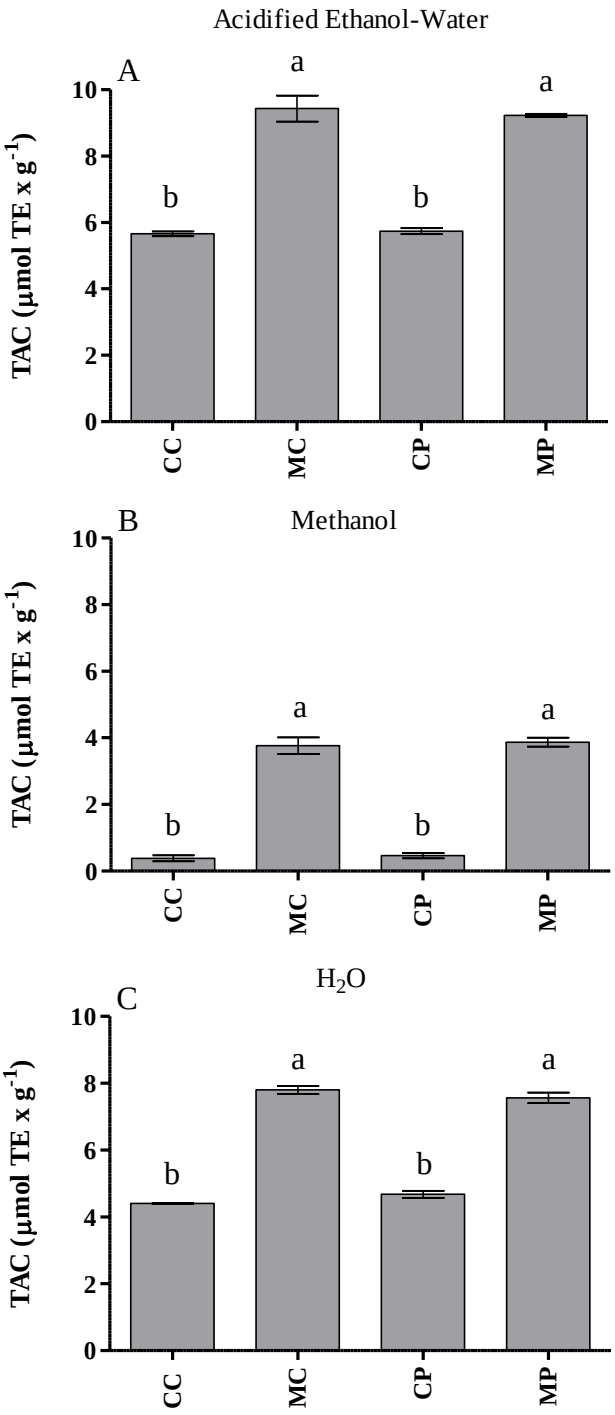

Figure 1. Total antioxidant capacity of gelato extracts expressed as µmol TE/g.

Data are means ± SD (n=4). Statistical analysis was carried out by one-way ANOVA ($p<0.001$ in all cases) using Tukey's as post-test. Different letters indicate significant differences.

Regardless the method of extraction used, the partial substitution of refined sugar with molasses was able to significantly increase the TAC of the final product. It is worth noting that the tested *gelati* were prepared by an industry producing ingredients for artisan *gelato*, and were totally comparable to products on the market.

The observed increase in TAC is therefore extremely positive, since it could help increasing the intake of dietary antioxidants together with a product that people generally consider negatively from a nutritional point of view.

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Chapter 2
Durum and soft wheat bran, valuable health-promoting ingredients for the amelioration of confectionary products

V. Valli, F. Danesi, J. Robertson, K. Waldron, F. Fava, L. Vannini, A. Bordoni - Potenziale bioattività di sottoprodotti dell'industria cerealicola per la produzione di nuovi alimenti - Riunione Nazionale SINU "Comprendere e applicare i LARN" - Florence, 21-22 October 2013 (ISBN: 978-88-97843-09-2).

Wheat is an important agricultural commodity and dietary component across the world. It is the main ingredient of bread, pasta, and many sweet baked goods as cookies and cakes.
The exploitation of soft and durum wheat byproduct is another strategy for a sustainable and healthy enrichment of confectionery products. In this chapter the isolation of oligosaccharides with bioactive functions form different fraction of the milling stream is shown and the new extracted ingredients are readily applicable to sweet foods providing them dietary fiber and antioxidants.

ABSTRACT

Bran is an underutilized byproduct of the milling process, generally discarded as waste or used in animal feeding. However, wheat bran represents an inexpensive and abundant rich source of valuable health-promoting compounds, including phytochemicals and antioxidants, of high interest for the food sector. The objective of this study was to evaluate the exploitation of different wheat milling fractions by enzymatic treatments for the production of bioactive ingredients. The sugar composition and the total antioxidant capacity were evaluated before and after a hydrolysis with xylanase. The enzymatic digestion was designed to release oligosaccharides from component arabinoxylans, these possibly having a high potential antioxidant activity.
Whilst further analyses are required, the results of this study clearly indicate that ingredients with bioactives properties can be selectively and sustainably prepared from wheat byproducts

INTRODUCTION

The production of waste is an important issue and an increasing problem for the food and drink industry. A relevant challenge for the sector could be to convert food processing byproducts into ingredients acceptable for incorporation into food systems. Indeed, plant food

residues normally consists of high amounts of polyphenols and their glycosides, carotenoids, steroids, and lipids which can be extracted through appropriate treatments and be converted in specific components of high interest for food formulations [1, 2].

Wheat bran is the byproduct of the flour milling process. It is obtained from the outer cereal layers, so mainly comprises the pericarp and the aleurone layers of the wheat grain. It is generally discarded or used in animal feeding. However, it is an inexpensive and abundant source of natural value-added molecules and biomaterials. In fact, bran contains health-promoting compounds, including phytochemicals, that correlate well with the benefits associated with the consumption of whole grain as the reduced incidence of certain chronic and inflammatory diseases [3].

There are several studies showing a marked antioxidant activity of wheat bran [4-6]. This activity is mainly due to phenolic compounds that can be either the same contained in fruits and vegetables, or unique of the specific cereal [7].

Synthetic additives as BHT and BHA are more and more rejected by consumers and natural antioxidant originating from natural sources would be in great demand nowadays [8]. This is particularly valid for phenolic compounds which, in contrast to most carotenoids and vitamins, are not chemically synthesized and need to be extracted from plant material [9].

Cell wall material might provide the source for a range of functional valuable components and for this reason it currently deserves more detailed attention. Oligosaccharides derived from wall hydrolysis are associated to the so called dietary fiber [5] and are currently considered prebiotics because they reach the colon undigested and are fermented mainly by bifidobacteria and lactic acid bacteria; this fermentation produces positive health effects, for example as improvement of glucose control and modulation of triglycerides metabolism [10].

Not all the dietary fiber sources have health promoting characteristics and attempts to determine functionality of different fibers types would markedly contribute to the proper targeting of product development.

Exploitation of modern processing methods can chemically and physically alter carbohydrate-based food materials and add substantial value to residues and co-products which can provide a reliable source of functional dietary fiber [11].

The major objective of this work was to characterize ingredients from wheat bran in terms of composition and bioactive properties. In particular, different fractions taken randomly from the flour milling, a mechanical gradual reduction process where the endosperm is separated from the bran layers, were considered.

The sugar composition and the total antioxidant capacity (TAC) were evaluated before and after an enzymatic hydrolysis with a specific xylanase. The xylanase digestion allowed the release of the oligosaccharide fractions which may have an enhanced antioxidant potential.

This work has been undertaken within the EU project NAMASTE, aimed at the development and assessment of laboratory-scale experimental protocols for the economically and environmentally sustainable conversion of several vegetable processing co-products into ingredient for new healthy foods [12].

MATERIALS AND METHODS

Materials

All chemicals, reagents, and solvents were purchased from Sigma-Aldrich Co. (St Louis, MO, USA) unless otherwise stated.

Samples of wheat bran were obtained as milling fractions produced from the flour milling stream *en route* to production of the total unsieved bran (Bulk bran). The samples were provided as random samples and were labeled by the miller. 12 bran samples were examined. Fractions obtained from the hard wheat milling (HW) were: B2 Germ, A F, G Coarse, G Fine, FBF, CBF; fractions obtained from the soft wheat milling (SW) were: B K, B F, BF3, BF4, 4MD/B2, Bulk (total bran).

Methods

Fiber analysis and concentration

Dietary fiber was assayed as total dietary fiber using the Megazyme Kit assay (Megazyme K-TDFC) (Bray, Ireland). To remove starch and protein, wheat bran samples were digested using a thermostable α-amylase (Megazyme:E-BLAAM) and a protease (Megazyme:E-BSPRT). Starch digestion was completed using amyloglucosidase (Megazyme; E-AMGDF).

Samples were solubilized in MES-TRIS buffer (0.05 M, pH 8.2) and heated in a water bath at 90-100°C to gelatinize starch, then incubated with heat-stable α-amylase for 35 min to digest starch. After cooling to 60°C protease was added, and samples were incubated at 60°C for 30 min. Following pH adjustment to 4.1-4.8, 100 μL amyloglucosidase were added to each tube; after 30 min incubation at 60°C absolute ethanol (40 mL) was added to each tube to precipitate fiber components. After 16 h at room temperature the fiber pellet was recovered by centrifugation.

Xylanase digestion of fiber concentrates

The fiber concentrates prepared from each sample were treated with a xylanase (Pentopan® Mono (Sigma, UK)), Pentopan® is recombinant xylanase from *Thermomyces lanuginosus*

(≥2500 units/g). In duplicate, 100 mg samples were weighed into 15 mL centrifuge tubes and dispersed in 3 mL of deionized water. After equilibration in a water bath (15 min at 50 °C), 50 µL of Pentopan® Mono (stock prepared in 0.5 mg/mL BSA to give 7.5 Units Pentopan®/g sample) were added to each sample. Samples were incubated for 5 h at 50 °C with mixing, then boiled to inactivate enzyme activity, and centrifuged. The pellet was washed three times; oligosaccharides-containing supernatants were recovered by centrifugation, combined and freeze dried.

Sugar analysis

Fiber residues and oligosaccharide fractions produced through xylanase treatment were analyzed for component sugars using Gas Chromatography (GC) [12]. To distinguish cellulosic and non-cellulosic glucose Saeman hydrolysis (72% H_2SO_4; 3 h; room temperature), followed by dilution to 1 M H_2SO_4 and incubation at 100°C for 1.5 h was used. For oligosaccharide samples only 1 M H_2SO_4 and incubation at 100°C for 1.5 h was used. Samples of each hydrolysate were derivatized to alditol acetates and quantified using GC.

Extracts preparation

The preparation of extracts was performed according to Danesi *et al.* [13] with slight modifications. Two different procedures were used to extract antioxidant compounds: sequential use of solvents (water and ethanol) and combined use of solvents. Briefly, 1 g of sample was extracted with 10 mL water under shaking for 20 min at 40 °C, centrifuged at 3,000*g* for 10 min and the supernatant collected. The extraction was repeated with 5 mL water and the two supernatants were filtered and combined. The residues were re-extracted with 10 mL ethanol (20 min at 40°C with shaking) centrifuged at 3,000*g* for 10 min and the supernatant collected. The extraction was repeated with 5 mL ethanol and the two supernatants were filtered and combined. Two independent extractions were performed for each sample.

Extraction with the combined solvents was performed using ethanol/water (70:30 v/v). Briefly, 1 g of lyophilized food was extracted with 10 mL of 70% ethanol (20 min at 40°C with shaking) and centrifuged at 3,000*g* for 10 min. The extraction was repeated with 5 mL of 70% ethanol (20 min at 40 °C with shaking); the supernatants were filtered and combined. Two independent extractions were performed for each sample.

Determination of Total Antioxidant Capacity (TAC)

TAC was measured using the method of Re *et al.* [14], 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), so causing the decolorization of $ABTS^{\bullet+}$, 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.
The antioxidant capacities of aqueous and ethanolic extracts were measured separately and the obtained values summed to obtain the final TAC.

Statistical analysis

The reported data are means of at least three replicates. Statistical analysis was by one-way ANOVA, using Tukey's post-hoc test. The Pearson correlation test analysis was applied to analyze the correlation between TAC value obtained with the different extractions.

RESULTS

1. Results on bran

The fiber content of the original milling fractions was variable, ranging from 57.1 to 20.2% and from 52.9 to 13.0% in the different fractions of hard and soft wheat, respectively (Table 1). The major component of the fiber matrix was non starch polysaccharide (NSP). NSP represents over 50% of the total fiber in all samples, apart from B2 Germ (hard wheat) and BF (soft wheat). NSP represents more than 70% of the fiber in the soft wheat fractions BF3 and Bulk.

Samples	Fiber (%original sample)	NSP (% original sample)	NSP (% fiber)	Samples	Fiber (% original sample)	NSP (% original sample)	NSP (% fiber)
Hard wheat (HW)				**Soft wheat (SW)**			
CBF	57.1	33.7	59.0	**BF**	44.5	21.0	47.2
AF	39.5	21.1	53.4	**4MD/B2**	13.0	8.1	62.3
B2 Germ	28.5	13.5	47.4	**BK**	46.7	32.0	68.5
G Fine	20.2	11.3	55.9	**BF3**	52.9	40.0	75.6
G Coarse	49.7	30.2	60.8	**BF4**	36.4	19.9	54.7
FBF	47.3	26.0	55.0	**Bulk**	53.1	39.6	74.6

Table 1. Fiber content and NSP contribution from bran milling fractions

The fiber and NSP content in the different milling fractions is expressed as % original sample (w/w). NSP content is also expressed as % fiber in the sample (w/w).

Composition analysis for sugars in the fiber residue (Figure 1A) showed that arabinose, xylose and glucose accounted for over 90% of the NSP sugars present, hence that

arabinoxylans (AX) and cellulose were considered the major polysaccharides. When account is taken of the fiber content in the original sample, then it is apparent that whilst some samples are relatively poor in AX, *e.g.* 4MD/B2, with nearly 5% AX, other fractions are rich, *e.g.* BF3 with over 20% AX (Figure 1B).

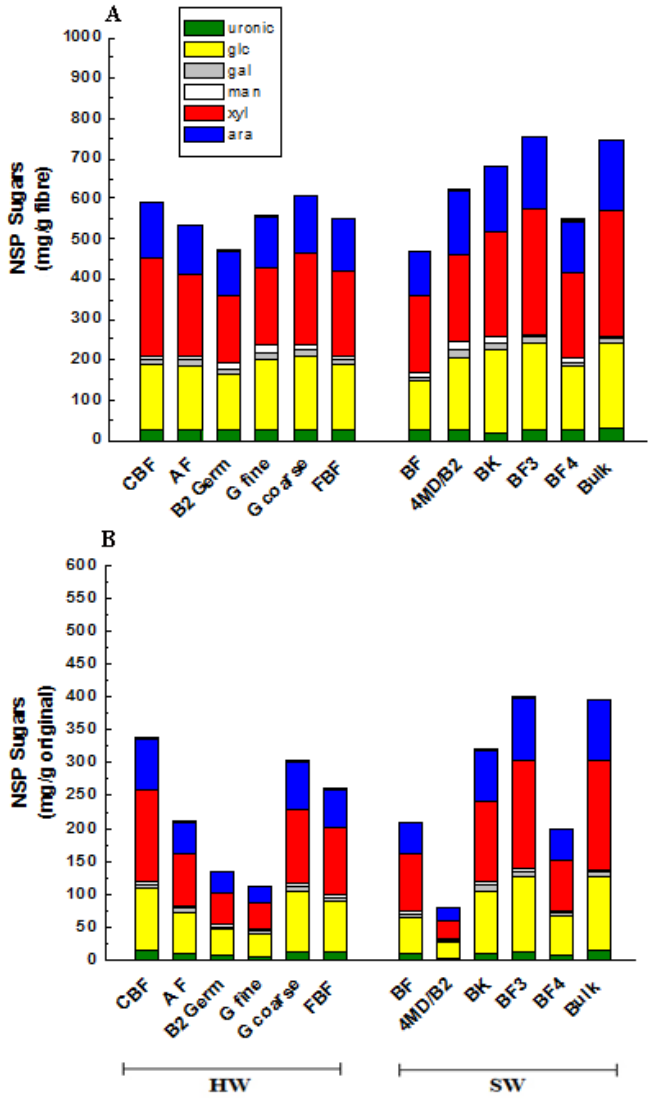

Figure 1. NSP Composition of bran.

Results are expressed per g fiber concentrate from wheat milling fractions (panel A) and per g original milling fraction bran sample (panel B). ara = arabinose, xyl = xylose, man = mannose, gal = galactose, glc = glucose, uronic = uronic acids.

Figure 2 shows the TAC of bran extracted with both the sequential use of water and ethanol (Figure 2A), and the combination of the two solvents (ethanol/water 70:30 v/v) (Figure 2B).

Using the sequential extraction (panel A), the TAC was different in the milling fractions, and in all samples the aqueous extract contributed more to total TAC than the ethanolic extract. Wheat fractions, AF, G coarse and BF, showed the highest TAC, whilst the lowest TAC was detected in 4MD/B2 soft wheat sample. These results were mostly confirmed using the combined extraction (Figure 2B) which evidenced the highest TAC in BF sample and the lowest in 4MD/B2 and G Fine ones. A significant correlation was observed between the TAC results obtained with the two extraction procedures (Pearson correlation coefficient: r^2=0.68; $p<0.05$).

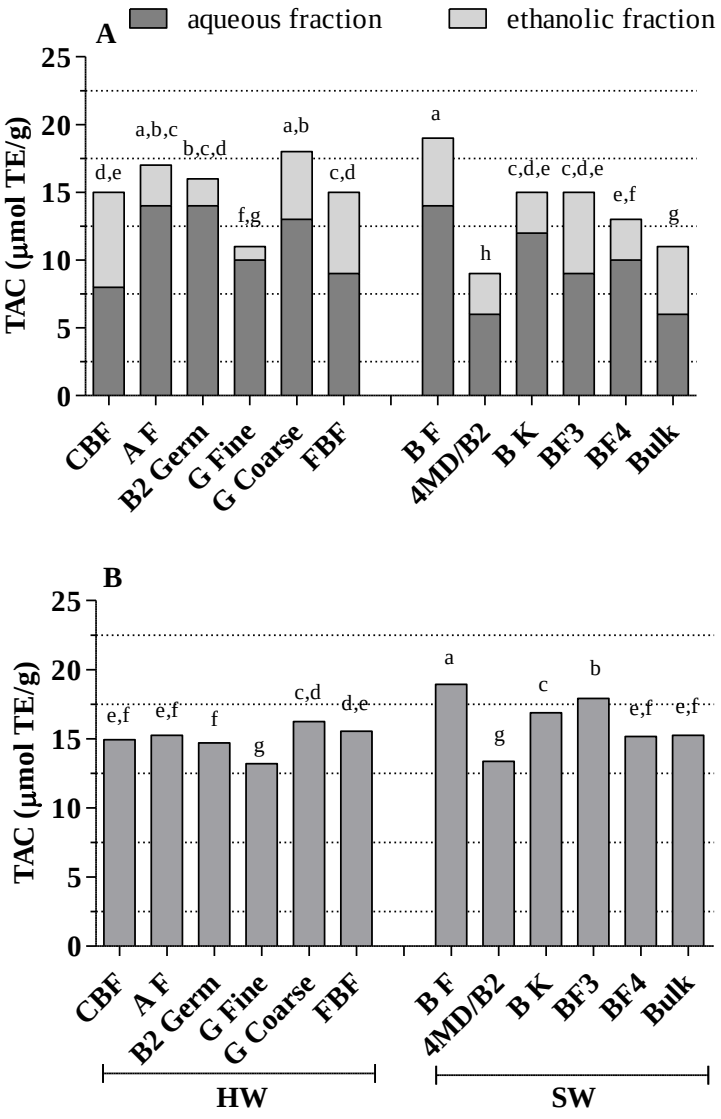

Figure 2. TAC of bran both extracted with the sequential use of water and ethanol (panel A) and with the combination of the two solvents (panel B).

Statistical analysis was by the one-way ANOVA ($p<0.001$) with Tukey's post-hoc test. Different letters indicate significant differences (at least $p<0.05$).

2. Results on the oligosaccharide fractions

Sugars analysis of the xylanase-solubilized fractions (Figure 3) confirmed that each fraction was enriched in arabinose and xylose, though yield varied from ~60 – 90% among milling fractions.

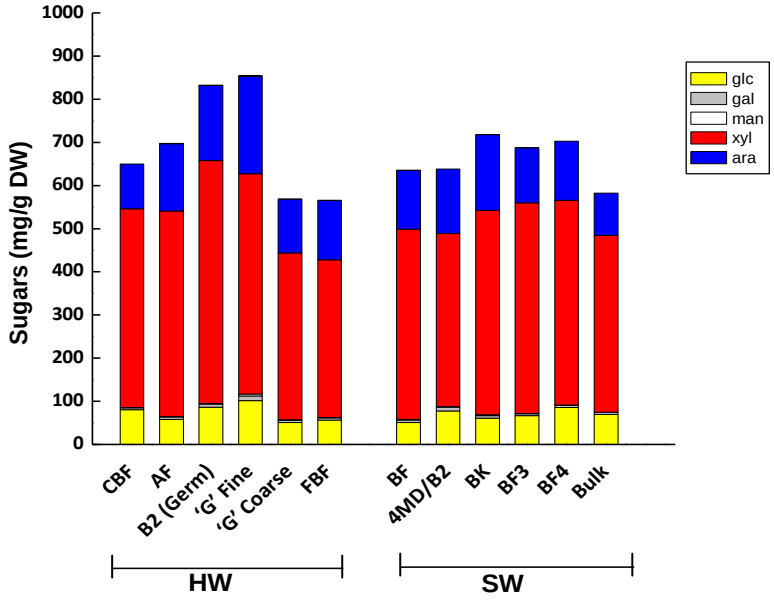

Figure 3. Content and composition of Pentopan-solubilized wheat bran.

ara = arabinose, xyl = xylose, man = mannose, gal = galactose, glc = glucose, uronic = uronic acids.

As illustrated in Figure 4, oligosaccharides TAC ranged approximately from 90 and 140 µmol TE/g, whereas bran TAC ranged approximately from 9 and 19 µmol TE/g (Figure 2). Thus, the enzymatic treatment with the xylanase greatly enhanced the wheat bran TAC.

Samples showing the highest TAC before the xylanase digestion were also among the most antioxidant oligosaccharides; similarly 4MD/B2 and G Fine were among the samples with the lowest TAC both before and after the enzymatic treatment. However, differences among fractions were reduced after Pentopan® usage, as shown by multiple comparisons.

In the oligosaccharide fractions the contribution of the ethanolic extract to the total TAC was negligible (Figure 4A). A significant correlation was observed between the TAC results on the two kinds of extractions (Pearson correlation coefficient: $r^2=0.79$; $p<0.01$).

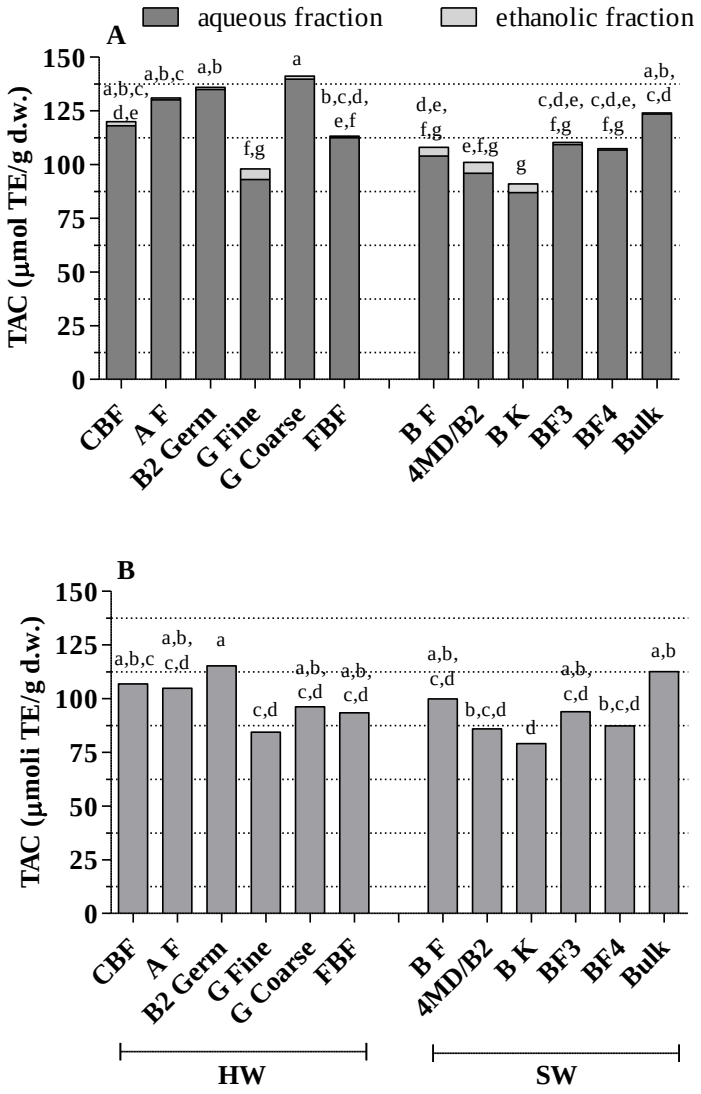

Figure 4. TAC of bran both extracted with the sequential use of water and ethanol (panel A) and with the combination of the two solvents (panel B).

Statistical analysis was by the one-way ANOVA ($p<0.001$) with Tukey's post-hoc test. Different letters indicate significant differences (at least $p<0.05$).

DISCUSSION

Milling of wheat generates byproducts, which can be used to improve the technological performance and/or to integrate foods with healthy compounds [5].

Wheat bran, from the outer tissues of wheat kernel, is mainly composed of non-starch polysaccharides (NSP); in the cell wall of Gramineae like wheat and other cereals and grasses. Arabinoxylans (AX) rate as the principal component of the NSP [15]. AX are polymers of mainly pentose sugars, based on a xylose backbone with arabinose side chains. Uronic acid

and phenolic acids, mainly ferulic acid, may also be substituents in AX and these may promote the antioxidant properties of AX.

Results herein described for fiber analysis are in agreement with previous findings [16, 17], and evidence that AX are a major components of all the different milling fractions of both hard and soft wheat. Since the NSP content was significantly different among the milling fractions, AX content was also diverse and depending on grain tissue composition then AX composition would also be expected to vary.

AX are considered important bioactive molecules not only because they exert the benefits of the dietary fiber, but also because ferulic acid, either in its monomeric or dimeric form, is ester linked to arabinosyl residues [18]. Ferulic acid is the main phenolic acid in wheat bran and it is particularly interesting for its antioxidant activity [19].

Arabinoxilans may be degraded to oligosaccharides by acidic or enzymatic hydrolysis [20]. Due to the interest in their technological and functional applications [21], the isolation of feruloyl oligosaccharide from wheat cell wall by treatment with polysaccharide hydrolyzing enzymes has been performed in many studies [18, 22].

The potential yield of oligosaccharides available through specific xylanase activity and the related bioactivity can be influenced by the NSP content and AX structure. In this study, different and random milling fractions were considered in order to evidence the ones that are richer in yield of components of interest for extraction and exploitation as bioactive agents. Structural constraints of AX on oligosaccharide yield have not been considered in this study.

The heterogeneous distribution of chemical components in the kernel and the different technological passages of the flow sheet followed in the mill can cause not only a different NSP composition but also a different antioxidant activity in the milling samples [23].

Since analytical procedures can significantly affect the antioxidant activity of a sample because of the variable contents and types of phytochemicals that can be obtained through different preparation procedures [24], in this study two kinds of extractions for the TAC evaluation were performed. Correlation analysis between the results obtained from the two extraction methods suggests that the procedures are both suitable for a TAC evaluation in this kind of matrix.

Our results clearly indicate that the most antioxidant agents in the milling fractions were aqueous rather than ethanol soluble. This is in agreement with Adom *et al.* [4], who showed that different milled fractions of wheat have different profiles of both hydrophilic and lipophilic phytochemicals. Hydrophilic antioxidant activity contributed mainly to the total antioxidant activity of analyzed wheat samples.

Once fiber concentrates from bran were submitted to an enzymatic digestion through xylanase in order to break down the component arabinoxylans and release oligosaccharides with a bioactive activity, an increase in TAC was observed. This agrees with the results of Katapodis *et al.* [25] and Yuan *et al.* [21] who showed that feruloylated oligosaccharides from wheat bran have a strong antioxidant activity.

The most promising fractions regarding TAC, before the enzyme hydrolysis, were BF, AF and G Coarse. However this difference leveled out after the treatment. Xylanase treatment impacted on either the overall TAC or the solubility of antioxidants that were extracted. Indeed, the contribution of the ethanolic extract to total TAC was not significant, since probably water soluble oligosaccharide were the major component in the samples. However, the results obtained on samples extracted with the combination of solvents (ethanol/water 70:30 v/v) suggest that hydroalcholic extraction also recovers a certain percentage of non-oligosaccharide components. Since the sugars analysis on the oligosaccharide fractions evidenced not only that arabinose and xylose were the major component, but also that some glucose was present in all fractions, it could be speculated that other components, e.g. β-glucans, are solubilized during oligosaccharide preparation as well.

Further research is warranted to optimize yield and purity of oligosaccharides, and more studies are necessary to further elucidate which milling fraction has the highest bioactive potential.

However, the obtained results represent a first step for the evaluation of the antioxidant properties of bran demonstrating that ingredients with healthy properties can be selectively prepared from wheat byproducts. This is particularly interesting because some antioxidants from natural origins, such as herbs extracts from rosemary or thyme, have strong herb flavors which restricts their application in food use [1].

Finally the new value-added ingredients would be appreciated by consumers not only for the functional effects but also for the sustainable and ethical concept that drive their production.

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Chapter 3

Could ancient grains be functional components of sweet products?

Considering that consumers are changing their purchase and eating habits around meal opportunities like breakfast and snacking, product developers should consider the addition of ancient grains flour as an appealing way to add variety, nutrients, and healthy characteristics to confectionary products. In particular this chapter focuses on the possibility of substituting whole durum wheat with Kamut® Khorasan; the antioxidant and anti-inflammatory effects of the modern and ancient grain were evaluated and compared both *in vivo* and *in vitro*. While in the first study *in vivo* we preferred to feed rats with pasta in order to avoid interferences of many ingredients, in the second study we focused on the possible utilization of Kamut® flour in a confectionary product (cookies), evaluating its effect in cultured cells.

Role of Kamut® brand khorasan wheat in the counteraction of non-celiac wheat sensitivity and oxidative damage

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ABSTRACT

It has been suggested that ancient grains show weaker immunogenic properties and therefore can be introduced in the diet of non-celiac wheat-sensitive people. In the present study we investigated the possible difference in inflammation caused by feeding ancient Kamut® wheat pasta (KP) compared to modern durum wheat pasta (WP) to rats. The effect of the two experimental diets on the oxidative status was also compared under basal condition and after inducing an exogenous oxidative stress. In rats fed WP the histological evaluation of the duodenum morphology evidenced a flattened mucosa, an unusual shape and shortening of the villi, and a high lymphocyte infiltration, while no modifications were detected in KP fed animals. The fecal metabolite profiling was differently modified by the two diets, suggesting significant changes in the gut microflora. Furthermore, the results confirmed previous data on the antioxidant protection in rats by Kamut® wheat foods. It is conceivable that Kamut®

components can act through an hormetic effect, eliciting an adaptive response that protects the organism against both oxidative stress and inflammation.

INTRODUCTION

Cereal-based food products have been the basis of the human diet for a long time. Cereals contain all the macronutrients (proteins, fats, and carbohydrates), and they are an excellent source of minerals, vitamins, and other micronutrients required for adequate health. Nowadays in Western countries most cereals are consumed after milling, that involves the removal of the outer layers of the grain (bran and germ) and the preservation of the starch-rich white endosperm. In so doing, milling takes out a significant amount of the key nutritional components from cereals.

There is an increasing amount of evidence showing that consumption of whole grains (WG) and whole-grain-based products is associated with a reduction of the risk of developing many diseases, including cardiovascular diseases [1], hypertension [2], metabolic syndrome and type 2 diabetes [3], and different types of cancer [4]. WG cereals are a rich source of fiber and bioactive compounds, such as n-3 fatty acids, sulfur amino acids, oligosaccharides, minerals, B vitamins, phytosterols, and antioxidants. Different mechanisms have been proposed for explaining the protective role of WG, all based on studies in which one component is isolated and tested, but the protective effects of WG consumption may go beyond what would be estimated by considering the addition of the effects of each individual component, suggesting that synergistic effects and interactions between these components may be as important (or perhaps more important) than the individual effects [5].

The concentration of WG bioactive components has been reported as higher in ancient crops and/or minor cereals (e.g. Kamut® wheat, barley, spelt, rye, einkorn, millet, oats, sorghum), thus increasing the interest in the use of ancient grains because of their better health-related composition [6]. In addition, the use of ancient grain blends has been evidenced as suitable to make highly nutritious, modern and innovative baked goods meeting functional and sensory standards in terms of nutritional added value, palatability (high sensory scores), convenience (extended shelf life) and easy handling during processing [7].

In two recent studies [8, 9], we demonstrated the protective effect of WG bread, particularly when made from Kamut® brand khorasan wheat, in rats submitted to an exogenous oxidative stress due to the intraperitoneal injection of doxorubicin (DOX). Furthermore, the histologic evaluation of the hepatic tissue of these same rats showed a complete protection from the onset of the DOX-induced inflammation by a diet of Kamut® bread compared to a diet of

modern durum bread. The hypothesis of an anti-inflammatory action of Kamut® khorasan bread is intriguing in the light of the controversial hypothesis suggesting ancient grains might show lower immunogenic properties and therefore opening the possibility to introduce them in the diet of non-celiac wheat sensitive people.

Therefore, in the present study we have investigated in healthy, unstressed rats the possibility that there is less inflammation caused by a diet of ancient Kamut® whole wheat compared to a diet of modern durum whole wheat. The duodenum, spleen and lymph nodes were chosen as target organs for study since the duodenal mucosa is in direct contact with the potential inflammatory agents, and there is a large number of cells especially sensitive to inflammatory agents in the spleen and lymph nodes. Furthermore, since the influence of a food or diet on the health of the host may be affected by changes occurring in the composition and metabolism of the gut microbiota, the fecal metabolite profiling was also compared.

In this study, rats were fed durum or Kamut® cooked pasta, since our aim was to compare real cereal food products commonly used in the human diet; pasta was chosen in order to exclude fermentation as a variable of the potential differential protective effect. In fact, the study by Coda *et al.* [10] demonstrated the capacity of sourdough lactic acid bacteria to release peptides with antioxidant activity through the proteolysis of native cereal proteins. The use of sourdough fermentation could therefore be considered as an adjuvant to enhance the recovery from intestinal inflammation of coeliac patients at the early stage of the gluten-free diet [11]. Rats were fed modern whole wheat pasta (WP) or ancient whole wheat Kamut® pasta (KP) for 7 weeks, and then half animals in each group were submitted to an exogenous oxidative stress by intraperitoneal injection of doxorubicin (DOX). DOX is an anthracycline antibiotic, widely used as an anticancer agent. Despite its high antitumor activity, its use in clinical chemotherapy is limited because of diverse toxicities. Oxidative damage to membrane lipids and other cellular components is believed to be a major factor in the DOX toxicity, which is caused by the formation of an iron-anthracycline complex that generates free radicals [12]. Besides the histological analyses in the duodenum and spleen which indicated the inflammatory potential of WP and KP, the modifications which occurred in the fecal metabolite profiling were assessed by ^{1}H-NMR spectroscopy. All the same analyses already reported on rats fed modern durum and ancient Kamut® bread [8, 9] were carried out on pasta fed rats. This allowed us to check if the superior antioxidant protective effect already observed in Kamut® bread fed animals was also seen in animals fed Kamut® pasta.

MATERIAL AND METHODS

Chemicals and reagents

Doxorubicin was a kind gift of Ebewe (Rome, Italy). Hematoxylin-Eosin and PAS stains were purchased from Kaltek Italia (Padua, Italy). All chemicals and solvents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless differently stated, and were of the highest analytical grade.

Pasta preparation and composition

According to the Italian guidelines of organic pasta processing, the process parameters accounted for a long (more than 6 hours) and low temperature (around 55°C) desiccation cycle. The final water content was lowered to less than 12.5%. The cooking process of pasta was performed by a semi-industrial pasta cooker in boiling water (10% w/w pasta/water ratio) for 8 minutes. Pasta was frozen in single dose packages immediately after cooking, defrosted at room temperature before use and administered to rats without any further heating.

Moisture, ash, protein, fat and carbohydrate content of the cooked pasta were evaluated according to the standard AACC methods [13]; soluble and insoluble dietary fiber content was estimated according to the method described by Asp *et al.* [14]. The cooked pasta content of carotenoids, folic acid, vitamin E, Se, and total polyphenols was estimated as described previously [9].

Animals

Twenty-four male Wistar rats, aged 30 days, were used. Animals were housed in individual cages in strictly controlled conditions of temperature (20 ± 2°C) and humidity (60-70%), with a 12 hour dark-light cycle. After a 7 day acclimation period animals were randomly divided into two groups, each receiving one of the following diets: 1. wheat pasta (WP); and 2. Kamut® khorasan pasta (KP). Water and pasta were provided *ad libitum*; food consumption was measured every day, and rat body weight (b.w.) every week. The dietary treatment lasted for 7 weeks and then rats of each group were randomly divided into two subgroups. The first one received intraperitoneally 10 mg/kg b.w. of DOX in a single dose, the second one similar volumes of NaCl 0.9% (w/v) in distilled, apyrogenic water solution. Forty-eight hours later, after 12 hours of fasting, rats were anaesthetized and sacrificed. Blood was sampled by intra-cardiac withdrawal. Plasma was immediately separated by centrifugation and stored in separated aliquots at -20 °C until analysis. The liver was quickly excised, washed in phosphate buffered saline, weighed, and immediately frozen at -80 °C. The duodenum, lymph nodes, and spleen were also excised and immediately fixed in formalin.

Stool samples were collected from rats at the beginning of the experimental feeding (T0) and the day before DOX administration (T1), and immediately frozen at -80 °C until analysis. Stools were not examined after DOX administration, since it was an acute treatment, and the time between treatment and sacrifice was too short to evidence modifications in the microflora.

The Animal Care Committee of the University of Bologna approved the study (Prot. 50932-X/10).

Histologic evaluations

Portions of the excised tissues were fixed in 4% formalin. Specimens were then embedded in paraffin, and tissues were cut to obtain 3- to 4-µm sections. Sections were stained with hematoxylin and eosin and periodic acid-Schiff and microscopically (20 X) evaluated using a digital microscope D-Sight (Menarini Diagnostics-Nikon, Florence, Italy).

In the duodenum, the morphology, the villi length, and the intraepithelial lymphocyte number were evaluated according to the diagnosis criteria for celiac disease in humans [15, 16]. In the lymph nodes and in the spleen the morphology and diameter of lymphatic follicles were evaluated.

Fecal metabolite profiling

To study the water soluble fraction of the feces by means of ^{1}H NMR spectroscopy, 40 mg of thawed fecal mass was thoroughly homogenized by vortex-mixing with 400 µL of cold deuterated water at pH 7.4±0.02, containing 1 mM sodium 3-(trimethylsilyl)propionate-2,2,3,3-d4 (TSP) as internal standard. The mixtures were centrifuged at 14,000 rpm for 5 minutes and the supernatant was collected. To ensure the complete recovery of the water soluble species and highly reproducible spectra, this extraction procedure was repeated twice, the supernatants were combined and their pH was finally adjusted to 7.4±0.02 [17]. NMR spectra were then registered at 300 K on a Mercury-plus NMR spectrometer from Varian, operating at a proton frequency of 400 MHz. Residual water signal was suppressed by means of presaturation. ^{1}H NMR spectra were processed by means of VNMRJ 6.1 software from Varian. To minimize the signals overlap in crowded regions, all free induction decays (FID) were multiplied by an exponential function equivalent to a −0.5 line-broadening factor and by a Gaussian function with a factor of 1. After manual adjustments of phase and baseline, the spectra were scaled to the same total area, in order to compare the results from samples of different weight and water and fiber content. The spectra were referenced to the TSP peak, then digitized over the range of 0.5–10 ppm. The residual water signal region, from 4.5 to 5.3 ppm, was excluded from the following computations by means of R [18] scripts developed in-

house. To compensate for chemical-shift perturbations, the remaining original data points were reduced to 128 by integrating the spectra over 'bins', spectral areas with a uniform size of 0.038 ppm. A 23×128 bins table was finally obtained for univariate and multivariate statistical analysis, as one sample was lost. As some parts of the spectra were very crowded, some bins could contain peaks pertaining to different molecules. In order to consider this potential source of error the bins containing peaks ascribed to the same molecules were not summed up.

Analyses in plasma

Total antioxidant activity (TAA)

TAA was measured in plasma using the method of Re *et al.* [19], on the basis of the ability of the antioxidant molecules in the sample to reduce the radical cation of 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), determined by the decolorization of $ABTS^{\bullet+}$, and measured as the quenching of the absorbance at 734 nm. Values obtained for each sample were compared to the concentration-response curve of the standard trolox solution and expressed as μmoles of trolox equivalent (TE)/mL.

Concentration of reactive oxygen metabolites (ROMs)

ROM level in plasma was measured by applying the d-ROMs test (Diacron, Grosseto, Italy) as reported by Danesi *et al.* [12]. This test is based on the ability of transition metals to react with peroxides by the Fenton reaction. The reaction produces free radicals that, trapped by an alchilamine, form a colored compound detectable at 505 nm. Values obtained for each samples were compared to standard (H_2O_2), and expressed as μg H_2O_2/mL.

Plasma glucose estimation

Plasma glucose level was determined by the glucose oxidase enzymatic method [20]. Briefly, glucose present in the sample is oxidized by the enzyme glucose oxidase to gluconic acid with the liberation of hydrogen peroxide (H_2O_2), which reacts by peroxidase with 4-aminophenazone and phenol giving a colored compound which can be measured at 515 nm. Values obtained for each samples were compared to a standard curve obtained using glucose serial dilutions, and were expressed as mg/dL.

Analyses in liver

Liver glutathione peroxidase (GPx) activity

One hundred milligrams of liver were homogenized in 1 mL of cold buffer (50 mM Tris HCl, pH 7.5, 5 mM ethylenediaminetetraacetic acid and 1 mM dithiothreitol) and centrifuged, and the GPx activity was measured in the supernatant using a commercial kit as proscribed by the

manufacturer (Cayman Chemical Co, Ann Arbor, MI, USA) [21]. GPx catalyzes the reduction of hydroperoxides, including hydrogen peroxides, by reduced glutathione and functions to protect the cell from oxidative damage. The kit measures GPx activity indirectly by a coupled reaction with glutathione reductase (GR). Oxidized glutathione, produced upon reduction of an organic hydroperoxide by GPx, is recycled to its reduced state by GR and NADPH. The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm. The rate of decrease in the A_{340} is directly proportional to the GPx activity in the sample.

Results were adjusted for the protein content in the sample and expressed as units per milligram of protein.

Liver thioredoxin reductase (TrxR) activity

One hundred milligrams of liver were homogenized in 1 mL of cold buffer (25 mM potassium phosphate, pH 7, containing 2.5 M ethylenediaminetetra-acetic acid) and centrifuged, and the TrxR activity was analyzed in the supernatant with a commercial kit as proscribed by the manufacturer (Sigma-Aldrich) [22].

Thioredoxin reductase is an ubiquitous enzyme that is thought to be involved in many cellular processes such as cell growth, p53 activity, and protection against oxidation stress. The kit uses a colorimetric assay for the determination of thioredoxin reductase activity. It is based on the reduction of 5,5'-dithiobis(2-nitrobenzoic) acid (DTNB) with NADPH to 5-thio-2-nitrobenzoic acid (TNB), which produces a strong yellow color that is measured at 412 nm.

Results were adjusted for the protein content in the sample and expressed as units per milligram of protein.

Intracellular glutathione (GSH) level

One hundred milligrams of liver were homogenized in 1 mL of cold buffer (25 mM HEPES, pH 7.4, containing 250 mM sucrose) and centrifuged, and GSH levels were analyzed in the supernatant using a commercial kit from Sigma-Aldrich as proscribed by the manufacturer [23]. Briefly, the glutathione content of the sample is evaluated using a kinetic assay in which catalytic amounts of glutathione cause a continuous reduction of 5,5′-dithiobis-(2-nitrobenzoic) acid (DTNB) to TNB. The oxidised glutathione formed is recycled by glutathione reductase and NADPH. The product, TNB, is assayed colorimetrically at 412 nm.

Results were adjusted for protein content in the sample and expressed as nanomoles of GSH per milligram of protein.

Liver α-tocopherol and β-carotene content

One hundred milligrams of tissue were homogenized in 1 mL of cold phosphate buffered saline (pH 7.4) and deproteinized with ethanol. Liposoluble antioxidants were then extracted

with hexane and analyzed by reversed-phase high-performance liquid chromatography as described previously [24]. Results for α-tocopherol and β-carotene were expressed as micrograms per gram of tissue.

Liver malondialdehyde (MDA) level

One hundred milligrams of tissue were homogenized in 1 mL of cold buffer (0.25M Tris, 0.2M sucrose, 5mM dithiothreitol, pH 7.4). After centrifugation, the supernatant was derivatized with thiobarbituric acid and the MDA-thiobarbituric acid complex extracted with butanol. Samples were then analyzed by reverse-phase high-performance liquid chromatography as previously described [25]. Results were adjusted for protein content in the sample and expressed as nanomoles of MDA per milligram of protein.

Liver advanced oxidation protein product

One hundred milligrams of tissue were homogenized in 1 mL of cold buffer (20 mM phosphate buffered saline, pH 7.4) and centrifuged, and advanced oxidation protein product (AOPP) levels were measured in the supernatant by colorimetric detection at 340 nm as previously described [26]. Results were adjusted for protein content in the sample and expressed as nanomoles per milligram of protein.

Protein concentration

The protein concentration in all samples was determined according to the method of Bradford [27].

Statistical analysis

The point by point comparisons of the NMR spectra were performed by means of the non-parametric statistical test set up by Wilcoxon [28]. Canonical analysis of principal components (CAP) [29] and the consequent leave-one-out test were performed by employing the software CAP freely available through the author's web page. All other data are reported as mean ± standard deviation (SD), and statistical significance was evaluated by the Student's t test and the one-way ANOVA using Tukey's as post-test.

RESULTS

The two experimental pastas provided similar energy, fats, carbohydrates and fiber, while protein content was higher in KP than in WP (Table 1). The concentration of antioxidant compounds appeared different in the two experimental pasta: selenium was almost 20 times higher in KP than in WP, and also total polyphenols were higher in the former, while vitamin E, total carotenoids, and folic acid presented higher values in the latter (Table 1).

Table 1. Composition of the experimental pasta.

	Wheat Pasta	**Kamut® Pasta**
Energy (kcal/100 g)	174 ± 12	190 ± 14
Energy (kJ/100g)	734 ± 51	803 ± 59
Protein (g/100 g)	5.1 ± 0.3	7.1 ± 0.6 **
Fat (g/100 g)	4.3 ± 0.2	4.3 ± 0.3
Carbohydrates (g/100 g)	28.7 ± 1.01	30.8 ± 1.47
Soluble fiber (g/100 g)	0.8 ± 0.2	0.7 ± 0.1
Insoluble fiber (g/100 g)	3.8 ± 0.3	3.8 ± 0.24
Water (g/100 g)	56.7 ± 3.31	52.5 ± 2.67
Ash (g/100 g)	0.61 ± 0.05	0.79 ± 0.07 **
Selenium (µg/100 g)	0.031 ± 0.002	0.541 ± 0.031***
Vitamin E (µg/100 g)	1500 ± 111	1060 ± 87 ***
Carotenoids (µg/100 g)	23.7 ± 1.0	16.1 ± 0.6 ***
Folic acid (µg/100 g)	40.5 ± 2.2	29.5 ± 1.9 **
Total polyphenols (mg/100 g tannic	20.1 ± 0.94	23.4 ± 1.0 *

The composition of the experimental pasta was determined as previously reported. Statistical analysis was by the Student's t test: * $p<0.05$; **$p<0.01$; ***$p<0.001$.

In the end of the experimental dietary period all animals appeared in a fair state of health, having normal reactivity and behavior and no symptoms of malnutrition, although smaller in size compared to age-matched standard rats. No significant differences in b.w. gain were observed between the two dietary groups (Figure 1A). In basal conditions, no significant differences in plasma glucose concentration were detected between the two dietary groups; DOX administration caused a significant increase in glycemia in WP rats only (Figure 1B).

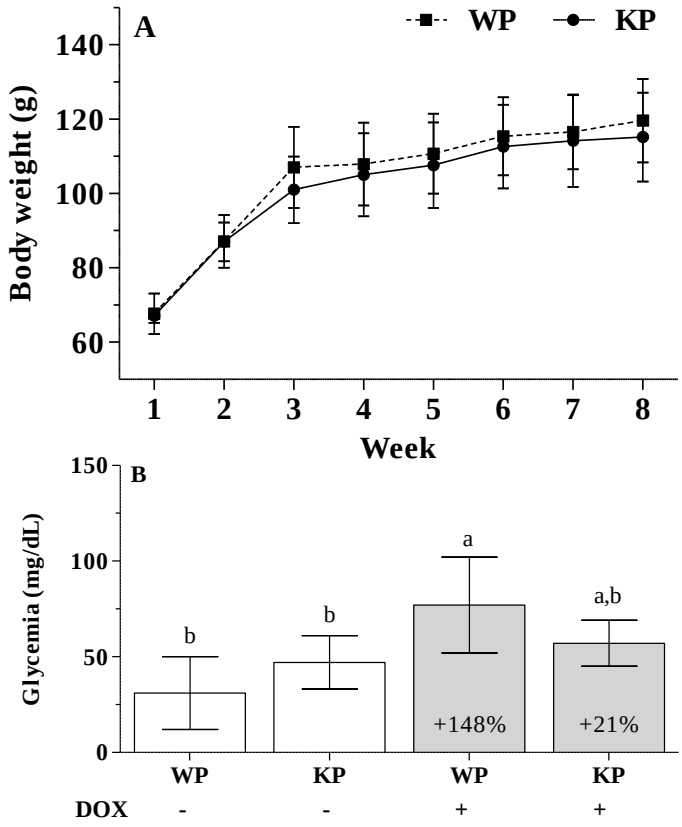

Figure 1. Body weight gain (panel A) and glycemia (panel B) in rats fed the two experimental diets.

Body weight was measured once a week during the dietary treatment. Data are means ± SD of 12 rats in each group. Glycemia was measured in basal (white bars) and stressed (grey bars) conditions, and is reported as mean ± SD of 6 rats in each group. Statistical analysis was performed by the Student's t test for the body weight (not significant) and by the one way ANOVA ($p<0.01$) using Tukey as post-test for glycemia: different superscript letters indicate statistical significance (at least $p<0.05$). In stressed condition percent variations of the corresponding basal values are reported within the corresponding bar.

In both basal and stressed conditions, the histological evaluation of the duodenum morphology evidenced in WP rats a flattened mucosa and an unusual shape of the villi (figure 2A), confirmed by the measurement of the villi length (figure 2B). Furthermore, a higher lymphocyte infiltration was observed in WP animals (figure 2C).

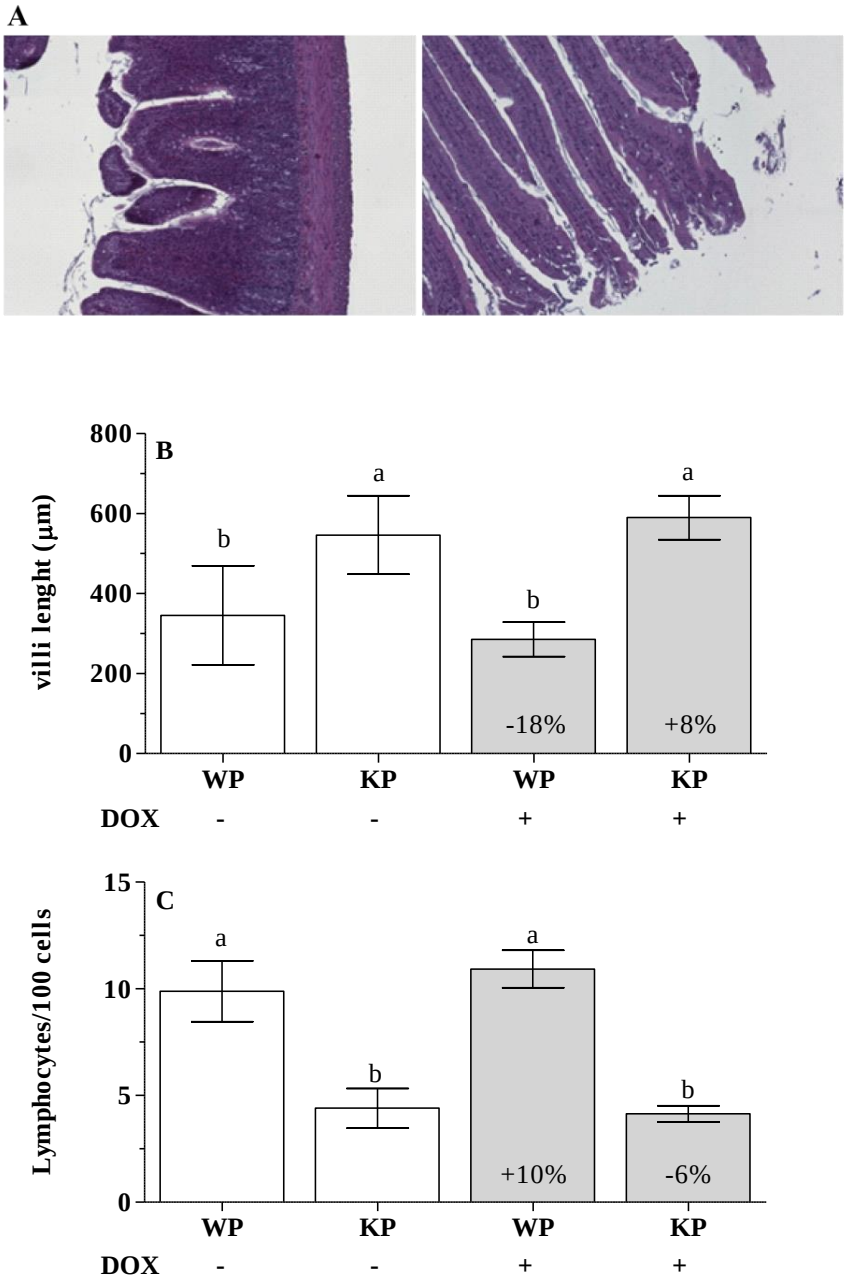

Figure 2. Histological evaluation of the duodenum in rats fed the different experimental diets.

In panel A, the different morphology of duodenum in WP (left side) and KP (right side) under basal condition is reported. Villi length (panel B) was measured in basal (white bars) and stressed (grey bars) conditions. Lymphocyte infiltration of the mucosa (panel C) was evaluated in basal (white bars) and stressed (grey bars) conditions as the number of lymphatic cells/100 cells. Data in graphs are the mean ± SD of 6 rats in each group. Statistical analysis was performed by the one way ANOVA (villi length $p<0.001$; lymphocytes $p<0.001$) using Tukey as post-test: different superscript letters indicate statistical significance (at least

p<0.05). In stressed condition percent variations of the corresponding basal values are reported within the corresponding bar.

In the spleen and lymph nodes of WP animals, a significant enlargement of the lymphatic follicles was observed (Figure 3), and no modification occurred within each group after DOX administration.

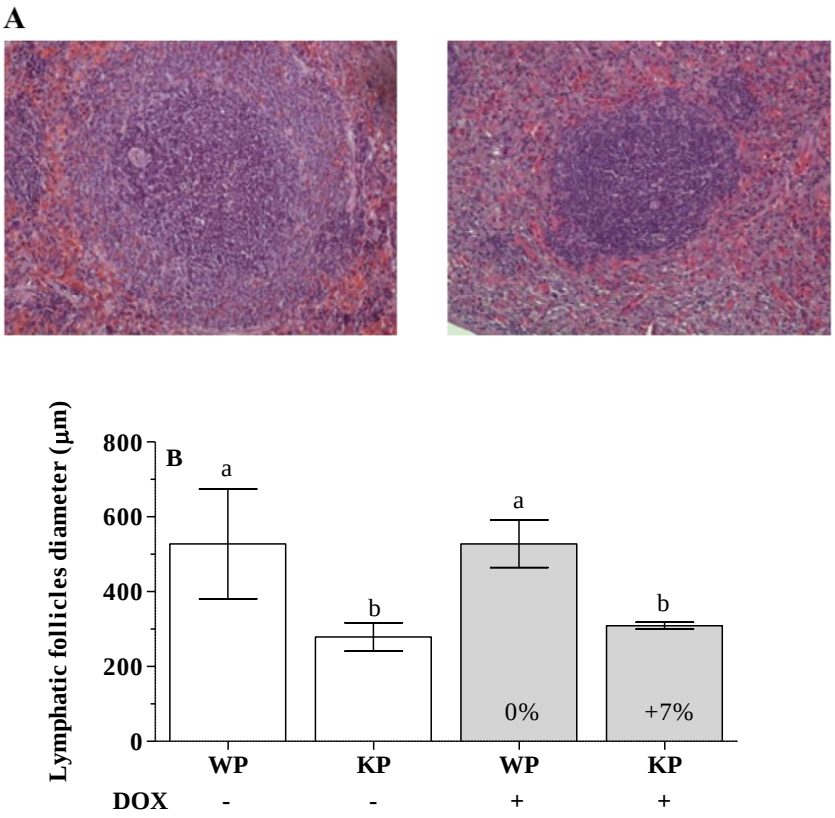

Figure 3. Histological evaluation of the spleen and lymph nodes in rats fed the different experimental diets.

In panel A, the different morphology of the spleen lymphatic follicles in WP (left side) and KP (right side) is reported. In these images the different thickness of the mantle around the follicles, that is composed of activated B lymphocytes and indicates a greater immune response, as well as the different diameter of the follicles are clearly evidenced. The diameter of the lymphatic follicles (µm) was measured in basal (white bars) and stressed (grey bars) conditions (panel B). Data are means ± SD of 6 rats in each group. Statistical analysis was performed by the one way ANOVA (p<0.001) using Tukey as post-test: different superscript letters indicate statistical significance (at least p<0.05). In stressed condition percent variations of the corresponding basal values are reported within the corresponding bar.

No differences related to the dietary treatment or to DOX administration were detected in plasma TAA (Figure 4A), while plasma ROM concentration appeared significantly lower in KP than WP animals regardless of the DOX treatment (Figure 4B).

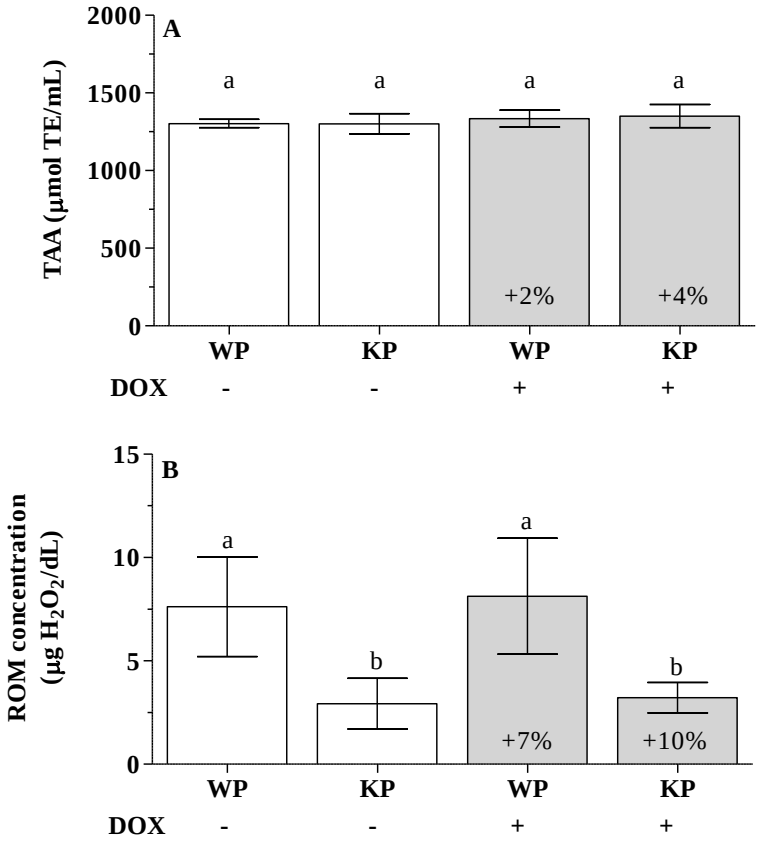

Figure 4. Plasma TAA and ROM concentration in rats fed the different experimental diets.

The plasma TAA (panel A) and ROM concentration (panel B) were measured in basal (white bars) and stressed (grey bars) conditions. Data are means ± SD of 6 rats in each group. Statistical analysis was performed by the one way ANOVA (TAA n.s.; ROMs $p<0.01$) using Tukey as post-test: different superscript letters indicate statistical significance (at least $p<0.05$). In stressed condition percent variations of the corresponding basal values are reported within the corresponding bar.

In basal conditions significant differences were detected in liver GPx and TxR activities, which appeared extremely lower in WP than KP rats (Figure 5A and 5B, respectively). Compared to the corresponding basal condition, the oxidative stress increased GPx activity in WP rats, having no effect in KP ones.

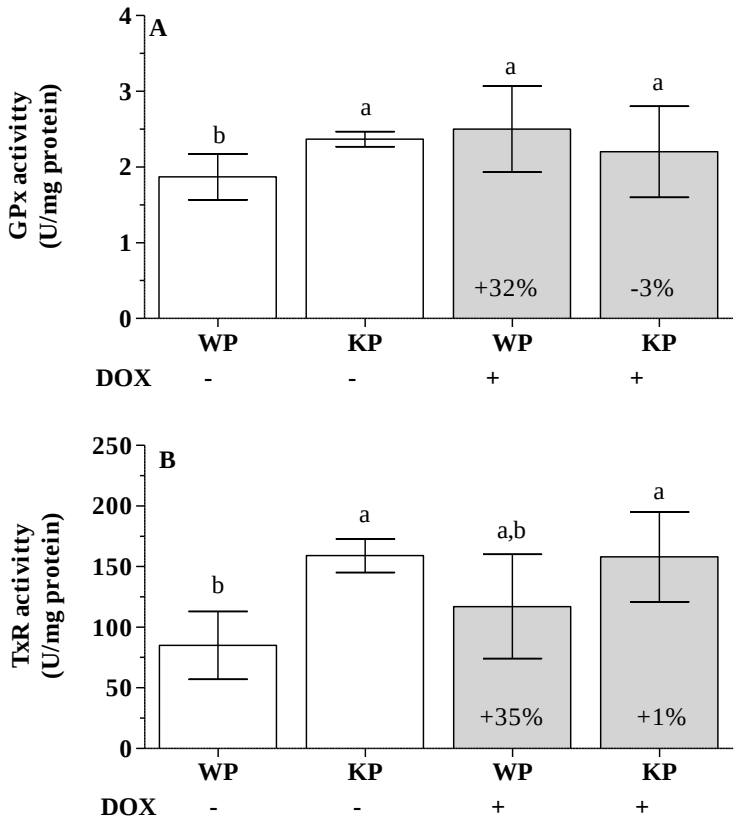

Figure 5. Liver GPx (A) and TrxR (B) activities in rats fed the different experimental diets.

The antioxidant enzyme activity in liver of rats fed the experimental diets was measured in basal (white bars) and stressed (grey bars) conditions. Data are means ± SD of 6 rats in each group. Statistical analysis was performed by the one way ANOVA (GPx $p<0.05$; TxR $p<0.001$) using Tukey as post-test: different superscript letters indicate statistical significance (at least $p<0.05$). In stressed condition percent variations of the corresponding basal values are reported within the corresponding bar.

In basal conditions, liver intracellular GSH, α-tocopherol, and β-carotene concentrations were the same in the two experimental groups (Figure 6A, 6B, and 6C, respectively). In WP rats DOX administration caused a significant decrease in α-tocopherol and β-carotene level, while only the latter significantly decreased in KP stressed rats compared to their basal counterpart.

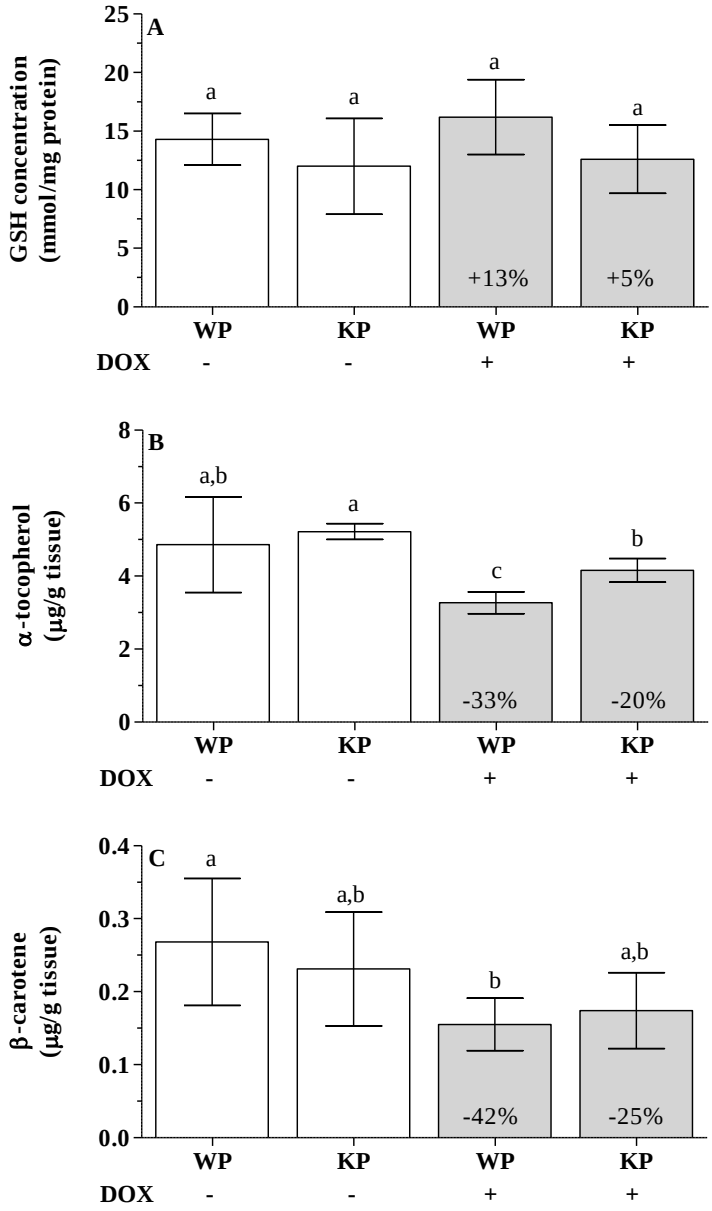

Figure 6. Liver GSH, α-tocopherol, and β-carotene concentrations in rats fed the different experimental diets.

Liver GSH (A), α-tocopherol (B), and β-carotene (C) concentrations measured in basal (white bars) and stressed (grey bars) conditions. Data are means ± SD of 6 rats in each group. Statistical analysis was performed by the one way ANOVA (GSH n.s.; α-tocopherol $p<0.001$; β-carotene $p<0.01$) using Tukey as post-test: different superscript letters indicate statistical significance (at least $p<0.05$). In stressed condition percent variations of the corresponding basal values are reported within the corresponding bar.

Liver MDA and AOPP levels were also comparable in the two experimental groups in basal conditions (Figure 7A and 7B, respectively); after DOX administration, MDA and AOPP levels significantly increased with respect to the corresponding basal value in WP fed rats, while no changes were detected in KP group.

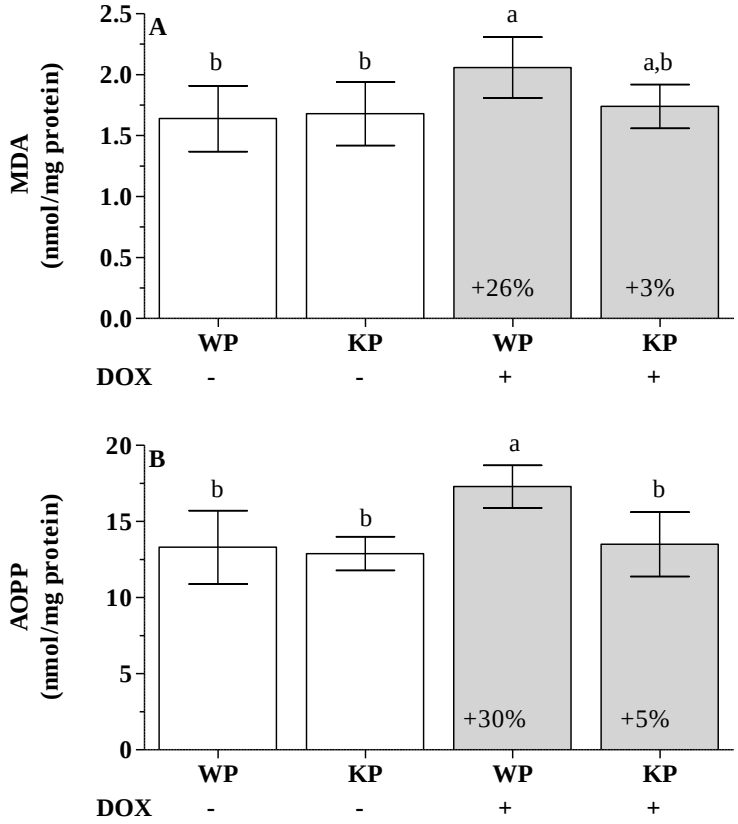

Figure 7. Liver MDA and AOPP levels in rats fed the different experimental diets.
Liver MDA (A) and AOPP (B) levels were measured in basal (white bars) and stressed (grey bars) conditions. Data are means ± SD of 6 rats in each group. Statistical analysis was performed by the one way ANOVA (MDA $p<0.01$; AOPP $p<0.001$) using Tukey as post-test: different superscript letters indicate statistical significance (at least $p<0.05$). In stressed condition percent variations of the corresponding basal values are reported within the corresponding bar.

A typical ^{1}H-NMR spectrum obtained on the fecal masses analyzed during the present investigation is represented in Figure 8, together with the assignments of the main peaks, obtained through comparisons with the literature [17] and with the addition of pure compounds to the samples.

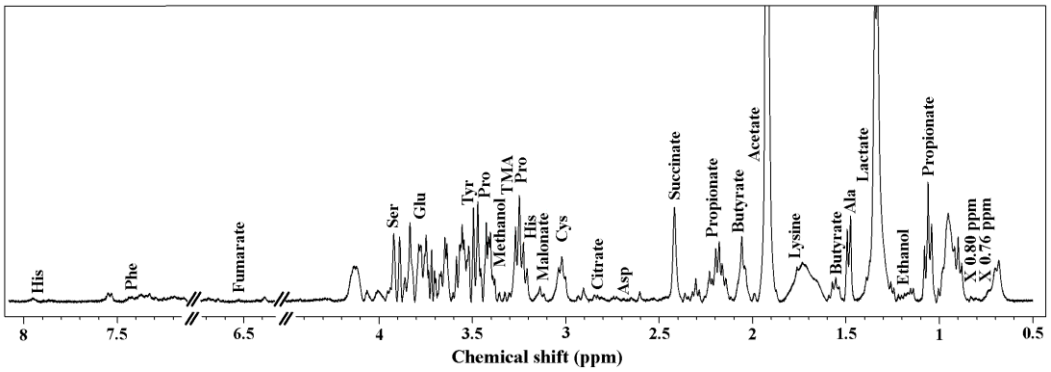

Figure 8. A typical ^{1}H NMR spectrum obtained during the present investigation.

The suggested assignments are based on the literature and/or by adding pure standard compounds.

To emphasize the main differences between the samples under investigation, a multidimensional space was built with the points forming each spectrum, so that the Euclidean distance between rats here projected gave an overall impression about the entity of the differences between their fecal metabolomes. A comparison between such distances is eased by the dendrogram depicted in Figure 9. The samples collected after 7 weeks formed two compact groups according to the research line, both separated from the samples collected at T0. This indicated an evolution of the metabolome of the rats, with differences ascribable to the two diets. In particular, the metabolic changes due to WP diet were less marked than the changes characterizing the gut metabolome of KP fed rats (Euclidean distance between the beginning and the end of intervention was 12.4% higher in KP compared to WP samples).

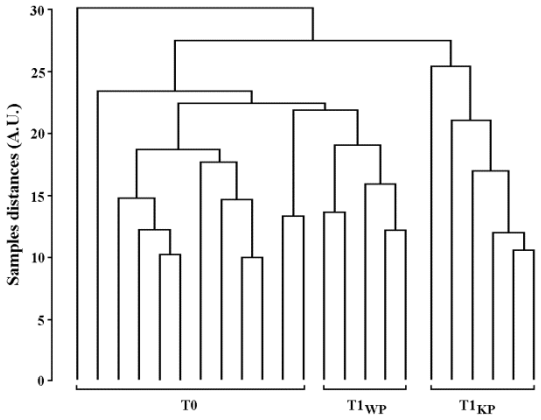

Figure 9. Dendrogram obtained by cluster analysis based on Euclidean distance, using gut metabolites before (T0) and after the dietary intervention ($T1_{WP}$ and $T1_{KP}$ respectively).

To identify the substances mainly responsible for such changes, the spectra from each stool sample collected at the beginning and in the end of the experiment were compared point by point. Both WP and KP diets led to significant differences in around the 15% of the points constituting each spectrum. As evidenced in Figure 10A the changes of the fecal metabolite composition observed comparing T0 and T1 were modulated by two concurrent criteria: a part of the statistically significant changes, such as the increase of butyrate concentration, was observed in both groups of rats and was thus not correlated to the cereal type; other changes, such as a dramatic decrease of lactate concentration in WP rats and a great succinate increase in KP ones, were specific for the two cereals. The overall effect of the two diets on the gut metabolome could be highlighted by comparing the spectra from the different animals at the end of the experimental period. Figure 10B shows the relative concentration of the molecules characterized by statistically significant differences between the samples pertaining to the two experimental groups. The concentration of succinate appeared as strikingly higher for KP samples, while WP samples were characterized by higher concentrations of ethanol, propionate and putrescine.

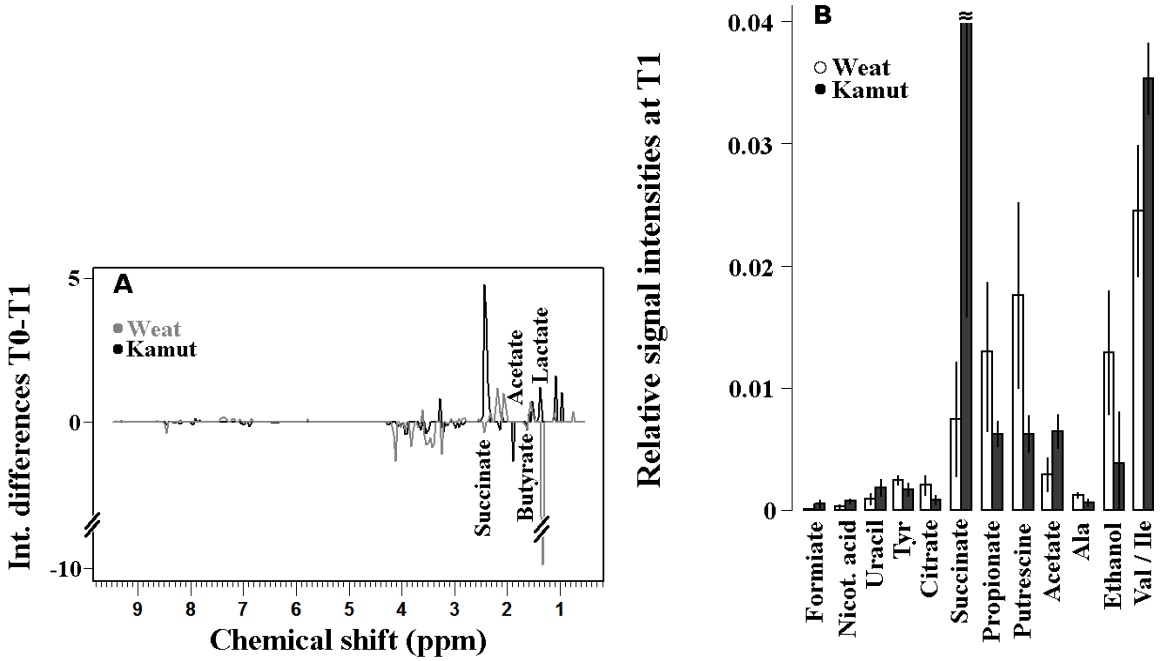

Figure 10. A) Differences between the ^{1}H-NMR spectra registered at T0 and T1 on the WP (light grey) and KP (dark grey) samples. B) Relative signal intensities registered at T1 on WP (white) and WP (dark grey) samples.

DISCUSSION

Celiac disease (CD) prevalence is estimated to be near to 1:100 in Western countries [30]. However, a much higher percentage (about 15-20%) of the general population than this 1% consider themselves to be suffering from wheat sensitivity (WS). Some of these wheat-reactive patients often present symptoms similar to CD but have negative CD serology and histopathology, and are therefore considered to be "simply" suffering from irritable bowel syndrome (IBS). The term "non-celiac gluten sensitivity" suggested for describing individuals who complain symptoms in response to ingestion of wheat without histologic or serologic evidence of celiac disease or wheat allergy, is a misnomer since a role for gluten proteins as the sole trigger of the associated symptoms remains to be established [31]. Recently, Carroccio *et al.* [32] demonstrated the existence of non-celiac WS as a defined clinical condition. In this study wheat, not gluten, was used for the challenges, so the Authors did not exclude the possibility that other components of wheat could be responsible for the resulting observations: i.e., fructans and poorly absorbed carbohydrates can induce symptoms by themselves [33].

Ancient wheat, not subjected to recent major genetic improvements in agronomic and processing characteristics, has been speculated to be better suited to be introduced into the diets of people suffering from non-celiac WS, although scientific or clinical evidences are lacking. Colomba *et al.* [34] pointed out that ancient wheat (Graziella Ra and Kamut® wheat) have greater amounts of both total and α-gliadin than modern ones (Cappelli, Grazia, Flaminio, and Svevo), thus challenging the "low-immunogenicity" hypothesis. In that work, a large series of α-gliadin epitope variants, mainly consisting of one or two amino acid substitutions were detected in all the accessions (including ancient ones); although their T-cell stimulatory capacity would need to be further investigated, the role of other wheat components than gluten in the triggering of non-gluten WS must also be carefully considered.

In the present study, the histological evaluation of the duodenum and spleen of rats fed modern durum pasta for 7 weeks clearly evidenced an inflammatory picture that could resemble non-gluten WS. On the contrary, rats fed ancient Kamut® pasta showed normal histological characteristics. At present it is not possible to clearly state if, and which specific durum components were responsible for the inflammatory reaction, or if and which specific Kamut® grain components had an anti-inflammatory action, or if a unique synergy of compounds was responsible. The hypothesis of the presence of anti-inflammatory agents is supported by the higher content of specific antioxidant components in the Kamut® pasta, whose role can be related not only to the prevention of oxidative stress but also to an anti-

inflammatory action. It is documented that phenolic compounds have antioxidant capabilities *in vitro*, but low bioavailability and low tissue concentrations make it unlikely that they act directly as antioxidants *in vivo*. Recent findings have suggested that in lower amounts, typical of those attained in the diet, phenolics may activate one or more adaptive cellular stress response pathways. Specific examples of such pathways include the Nrf-2/ARE pathway, and the NF-κB pathway. The nuclear factor erythroid 2-related factor 2 (Nrf2) is the transcription factor that binds to the antioxidant-responsive element (ARE) with high affinity and plays a central role in the upregulation of genes implicated in the modulation of the cellular redox status and the protection of the cell from oxidative insult [35]. The transcription factor NF-κB is a master regulator of inflammation, and numerous phenolics have been shown to inhibit NF-κB in different cell types [36].

In the present study the antioxidant protective effect of Kamut® wheat-based food was clearly detected, and at least in part accountable to the higher activity of liver antioxidant enzymes such as GPx and TxR.

Since rats were sacrificed after 12 hours of fasting, it is conceivable that this had completely abolished the differences in TAC due to a direct scavenging activity of absorbed compounds. In fact, it has been evidenced that plasma TAC (as total ORAC) increases 30 min after an antioxidant rich-meal, coming then back to basal value in further 30 min [37].

Notably, the induction of GPx, also related to the higher selenium content in Kamut® pasta, has been reported to also inhibit inflammation [38].

Regarding antioxidant protection, results reported in the present study confirmed those previously obtained feeding rats durum and Kamut® bread, thus indicating that different types of processing does not affect Kamut® grain protective effect. In both studies, Kamut® pasta fed rats evidenced a lower oxidative status in basal condition and a better response to the exogenous oxidative stress. In addition, data herein reported clearly evidenced the inflammatory role of modern durum wheat pasta itself. Although this inflammatory effect was surely exacerbated by feeding rats pasta only, it is worth noting that no signs of inflammation were detected in rats fed only Kamut® pasta.

Kamut® anti-inflammatory effects could have been mediated at least in part by modifications induced in the gut microflora. Over the last few years, growing evidence has supported a link between inflammatory bowel diseases and alterations in intestinal bacterial composition [39], and host-microbe dialogue has been showed to be involved not only in the maintenance of mucosal homeostasis but also in the pathogenesis of inflammatory disorders of the gut [40]. WG cereals provide non-digestible carbohydrates (NDC) that can be fermented by the gut

microbiota and act as prebiotic; existing studies assess the effects of wheat-derived NDC on parameters related to gut bacterial metabolism and/or in obesity and glucose homeostasis [41], and the primacy of environmental or lifestyle factors linked to changes in the gut microbiota in the development of inflammatory bowel disease, is increasingly evident [42]. In our study, some modifications occurred in the fecal metabolite profiling of rats fed both experimental diets; it is conceivable that diets uniquely based on whole grain pasta probably "per se" significantly modified the initial gut metabolome, regardless of the type of cereal eaten. The whole grain pasta could have cause an increase of fermentative activity of bacteria from the Firmicutes phylum which are known to produce high amounts of butyrate, the major energy source for colonocytes [43]. In addition, the metabonomic approach allowed to clearly distinguish between WP and KP rats, strongly supporting the development of a very different microbiota in the two groups. As example, the higher acetate concentration in WP feces could be accounted to a selective prebiotic effect on the Bacteroidetes phylum, which is known to produce large amount of acetate and propionate [44].

In conclusion, herein presented results confirm the antioxidant protection by Kamut® grain-based foods, and further evidence their anti-inflammatory role. As recently reviewed by Lefevre and Jonnalagadda [45], epidemiological studies support for an association between diets high in whole grains and the reduction of subclinical inflammation, but interventional studies do not demonstrate a clear effect of increased whole-grain consumption on markers of inflammation. Issues related to insufficient length of intervention, extent of dietary control, and population selection, may underlie these discrepant findings; in the light of our results the types of whole grains seems to play a master role, indicating substantial differences between whole-grain durum and whole-grain Kamut® wheat. It is important to point out that results herein reported were obtained using foods typical of the human diet; in fact pasta was administered after cooking to simulate real life conditions. This approach increases the predictive potential of the rat model.

Further studies should prioritize investigations on the mechanisms involved in the observed effects, evaluating the role of the different Kamut® wheat components in both the host and microbiota. The challenge ahead is to proceed cautiously until rigorous randomized controlled clinical trials have been undertaken to determine whether Kamut® grain and other ancient wheat could have wide spread efficacy in individuals affected by non-gluten WS. Based on available data, it is conceivable that Kamut® components can act through an hormetic effect, eliciting an adaptive response that protects the organism against both oxidative stress and inflammation [46]. This underline the complexity of the interaction between food and

humans, and the need of a new comprehensive approach to food and nutrition, since the overall vision of the food-human interaction can be achieved only by merging results coming from different scientific fields, using a foodomic approach [46].

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In this study Veronica Valli performed the analyses in plasma, contributed to the statistical analysis and to the critical interpretation of the results, and participated in the writing and editing of the manuscript.

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Anti- oxidative and anti- inflammatory effects of whole grain durum wheat and Kamut® cookies in HepG2 cells

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ABSTRACT

Consumption of whole grain based products is associated with a reduction of the risk of developing many diseases. This protective activity is related to different phytochemicals, whose concentration has been reported higher in ancient crops and/or minor cereals (e.g. Kamut®, barley, spelt, rye, etc). The present study was aimed to assess antioxidant and anti-inflammatory properties of cookies made with whole grain durum wheat or Kamut® khorasan flour (from Italy and USA), and fermented using *S. cerevisiae* or *S. cerevisiae* plus lactic acid bacteria. Cookies were *in vitro* digested and ultrafiltered in order to supplement HepG2 cells with a mix of compounds whose size was compatible with the intestinal absorption.

Results herein reported highlight the overall healthy effect of Kamut® and whole grains. All supplementation evidenced antioxidant and anti-inflammatory effects, that were more evident in cell supplemented with the digested Kamut® cookies.

INTRODUCTION

Cookies are a popular foodstuff, consumed by a wide range of populations, due to their varied taste, long shelf-life and relatively low cost [1]. Because of the increased demand for healthy and functional products, attempts are being made to improve cookies nutritive value and functionality by modifying their composition [1-4].

Whole grains (WG) are both concentrated sources of dietary fiber and rich in health-beneficial phytochemicals including trace minerals and phenolic compounds. The concentration of WG bioactive components has been reported as higher in ancient crops and/or minor cereals (e.g. Kamut®, barley, spelt, rye, etc), thus increasing the interest on the use of ancient grains because of their better health-related composition [5]. Epidemiological studies confirm that

high whole grain intake protects against cancer, cardiovascular disease, diabetes, and obesity [6]. In previous studies we proved that the ancient grain Kamut® khorasan is effective in reducing both oxidative damage and inflammatory status in rats [7-9].

This work was aimed to assess and compare the anti-inflammatory and anti-oxidant properties of cookies made with whole grain durum wheat or Kamut® khorasan flour.

Since the level of phytochemicals in vegetables is strongly affected by agronomic and environmental factors that could therefore have a deep impact on the protective activity of edible plants [10], Kamut® khorasan flours from two different geographical area, Italy and USA, were used.

Furthermore cookies were fermented using *S. cerevisiae* or *S. cerevisiae* and lactic acid bacteria (LAB). LAB are considered to have several beneficial properties, such as antimicrobial activity, ability to modulate immune response, anti-tumorigenic activity and antioxidant activity [11, 12]. It has been also shown that fermentation by yeast and LAB can influence the food nutritional quality for example improving the properties of the dietary fiber complex and increasing the uptake of minerals, vitamins and phytochemicals [13, 14].

Experiments evaluating the protective effects of whole grain wheat and Kamut® as ingredients of cookies were performed using cell cultures. Cell cultures are usually good experimental model, but do not allow to monitor and evaluate all modification elapsing in the tested products during digestion. Digestion is a very important process, defined as the enzymatic breakdown of organic macromolecules into their components. This dynamic physiological event is mandatory for obtaining molecules available for absorption, and it is influenced by both gastrointestinal condition and physical and chemical characteristics of the food matrix [15]. The overall nutritional value of foods relies on bioavailability as well as concentration of nutrients and other bioactive components. In order to get closer to the *in vivo* situation, cookies were *in vitro* digested and the mix of the more bioavailable compounds resulting from digestion was used for cell supplementation. *In vitro* total antioxidant capacity (TAC) and total phenolic content (TPC) of not-digested and digested cookies were also verified.

HepG2 cells, a human hepatoma cell line considered a good model to study *in vitro* cytotoxic agents [16], were chosen as model system given that the liver is the organ mainly involved in xenobiotic metabolism [17].

Protection from the oxidative damage induced by cell exposure to hydrogen peroxide 0.4 mM was assessed measuring cell viability, reduced glutathione (GSH) and reactive oxygen species (ROS) intracellular content, cytosolic TAC and thiobarbituric acid reactive substances (TBARS) content in the media.

To evaluate the potential anti-inflammatory effect of the different digested cookies, the level of a pro-inflammatory (IL-8) and an anti-inflammatory (IL-10) cytokine was estimated in the media in both basal condition and after cell treatment with LPS, known to be a strong stimulator of inflammatory response [18].

In this study we supplemented cells with a food which is present in the human diet, and not with flours or cereal-derived compounds. Although this protocol does not allow to define which components are the protective ones, it must not be considered a limitation of the study since our aim was to evaluate and compare the possible protective effect of real foods, and our approach allowed to consider the synergism between the different antioxidant molecules and the importance of the food matrix, well recognized factors of the overall antioxidant effectiveness [19] that are ignored in studies evaluating the effect of pure compounds.

MATERIALS AND METHODS

Chemicals: Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin and Dulbecco's phosphate-buffered saline (DPBS) were from Lonza (Milan, Italy). Ethanol, and 1-propanol were from Carlo Erba (Milan, Italy)

All other chemicals were from Sigma-Aldrich (Milan, Italy). All chemicals and solvents were of the highest analytical grade.

Ingredients for cookies recipe: butter, eggs, sugar and salt were purchased at local markets. Kamut® flours were a kind gift of Kamut® Enterprise of Europe, while wheat flour was purchased at a local market.

Methods

Cookies preparation

Six different types of cookies were tested:

1) Cookies made with **Italian whole-grain durum wheat** fermented using ***S. Cerevisiae*** (**WS_{IT}**);

2) Cookies made with **Italian Kamut® wheat** fermented using ***S. Cerevisiae*** (**KS_{IT}**);

3) Cookies made with **USA Kamut® wheat** fermented using ***S. Cerevisiae*** (**KS_{US}**);

4) Cookies made with **Italian whole-grain durum wheat** fermented using ***S. Cerevisiae*** and **LAB** (**WL_{IT}**);

5) Cookies made with **Italian Kamut® wheat** fermented using ***S. Cerevisiae*** and **LAB** (**KL_{IT}**);

6) Cookies made with **USA Kamut® wheat** fermented using ***S. Cerevisiae*** and **LAB** (**KL_{US}**);

The LAB used were: *Lb. Plantarum, Lb. Sanfranciscensis, Lb. Brevis.*

Cookies were made according to the same recipe (Table 1) and cooked in oven at the same temperature (175°C) for the same time (10 minutes). Each kind of cookie was prepared twice in two different days, and the two preparations were mixed in order to consider the variability that can derive from these preliminary phases.

Ingredients	**WL_{IT}, KL_{IT}, KL_{US}**	**WS_{IT}, KS_{IT}, KS_{US}**
Flour (g)	140	140
Butter (g)	56,5	56,5
Eggs (g)	44	44
Sugar (g)	24,25	24,25
Salt (g)	3	3
Mix of LAB (mL)	15	-
***S. Cerevisiae* (mL)**	5	20

Table 1. Summary of cookies recipe, modified by Drewnowski *et al.* [20].

In vitro digestion

Cookies were digested *in vitro* according to Bordoni *et al.* [21] with slight modifications. Briefly, the *in vitro* digestion was simulated inside a 100 ml flask kept at 37°C by means of a water bath on a magnetic stirrer equipped with a heating plate. Chemical composition of the digestive fluid, pH and residence time periods were adjusted in series to simulate the physiological conditions of mouth, stomach and small intestine. A buffer solution (120 mM NaCl, 5mM KCl, 6 mM $CaCl_2$ - pH 6.9) was added in proper volumes at every step. For 1 g of biscuit dry matter, 2: 4: 4 ml of such buffer was added to resemble saliva, gastric juice and duodenal juice respectively. The absolute volumes of the digestive juices were, for practical reasons, scaled to the content of food used. Mastication and oral digestion were simulated adding buffer solution with 90 U ml^{-1} α-amylase and grounding the biscuits with mortar and pestle for 5 minutes. Then, buffer solution was added and the pH was decreased to 2.0 by drop-wise addition of 37% HCl. Gastric digestion was started with the addition of pepsin to a final concentration of 3 mg ml^{-1}. After a 60 minutes incubation, buffer solution was added and pH increased to 5 with 1.5 M $NaHCO_3$ to stop peptic digestion. Duodenal digestion started with the addition of pancreatin (0.4 mg ml^{-1} final concentration) and bile (2.4 mg ml^{-1} final concentration). The pH was adjusted to 6.5 with 1.5 M $NaHCO_3$ and the digestion was followed for another 180 minutes.

Digestion was performed three times for each kind of biscuits and the resulting final digested solutions were mixed and frozen, allowing us to supplement cells with the same solution in each experiment.

Prior to supplementation, the mix was centrifuged at 4,000g for 5 minutes and again at 21,000g for 20 minutes. The supernatant was filtered with 0.2 µm membranes (TQ - *talis quails*- digested sample), and an aliquot was sequentially ultrafiltered with Amicon Ultra at 3 kDa of molecular weight cut-off (3K digested sample), allowing the separation of compounds which size is small enough to allow absorption through the intestinal mucosa. The 3K digested samples, containing molecules with molar mass <3 kDa, were used for cell supplementation.

Cookies extraction

Cookies extraction was performed according to Danesi *et al.* [22], with some modifications. A precisely weighed amount of the sample (5 g) was extracted with 20 mL ethanol/water (70:30 v/v) acidified with 0.1% HCl, or with the buffer solution used for digestion. After a 20 min incubation at 40°C, samples were centrifuged at 3000g for 5 min and the supernatant collected. The extraction was repeated with 10 mL of acidified ethanol/water or buffer, and the supernatants were combined. Two extraction replicates were performed for each sample.

***In vitro* total antioxidant capacity (TAC) using ABTS assay**

TAC was measured using the method of Re *et al.* [23], 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) ($ABTS^{\bullet+}$). The decolorization of $ABTS^{\bullet+}$ 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. Samples subjected to this analysis were both the digested fractions (TQ and <3 kDa) and the extracted cookies.

Determination of Total Phenolic Content (TPC)

TPC was determined using Folin-Ciocalteau's method [24], adapted to a 96-well plate assay according to Dicko *et al.* [25] with slight modifications. Briefly, 45 µL of water were first pipetted into each well. Then, 5 µL of extract 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 to have a final volume of 200 µL. The absorbance was measured after 60 min at 750 nm with a Tecan Infinite M200 microplate reader (Tecan, Männedorf, Switzerland). A blank measure, for which the sample was replaced by water, was subtracted from the absorbances. Gallic acid prepared in ethanol/water (70:30 v/v) was used

as standard for calibration and results were expressed as mg gallic acid equivalent (GAE)/g of cookie.

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 of 95% air and 5% CO2 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 6-well or 12-well plates at the concentration of $1 \cdot 10^6$ cells/mL. Cell counting was carried out using the TC20™ Automated Cell Counter (Bio-Rad Laboratories; Hercules, CA, USA).

After 24 hours (75-80% confluence) cells were incubated with serum-free DMEM containing the different <3K digested mixtures at 50 µL/mL medium concentration. Control cells (C) and unsupplemented cells (US) received a corresponding amount of sterile water.

For experiments aimed at investigating the antioxidant activity, 24 h after supplementation cells were washed twice with warm DPBS and exposed for 1 h to 0.4 mM H_2O_2 in Earle's balanced salt solution (EBSS) (116 mM NaCl, 5.4 mM KCl, 0.8 mM NaH2PO4, 26 mM NaHCO3, 2.38 mM CaCl2, 0.39 mM MgSO4); C cells received EBSS without H_2O_2. After 1 h EBSS was removed, centrifuged at 400g for 3 min and used for the thiobarbituric acid reactive substances (TBARS) assay.

For experiments aimed at investigating the anti-inflammatory activity, 6 h after supplementation cells were washed twice with warm DPBS and exposed for 18 h to 100 ng/mL LPS in RPMI-1640 without phenol red and added of 100 U/mL penicillin, 100 µg/mL streptomycin and 200 mM glutamine. C cells received RPMI without LPS. After 18 h medium was removed and maintained at -20°C until cytokines quantification.

Cell viability

Cell viability was measured using the 3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay [26]. The test is based on the capacity of mitochondrial dehydrogenase in viable cells to convert MTT reagent to a soluble blue formazan dye. Briefly, after two washing with DPBS, 1 mL of MTT reagent diluted in RPMI-1640 medium without phenol red (final concentration 0.5 mg/mL) was added to each well, and the cell cultures were incubated for 1 h at 37 °C. Medium was completely removed, 1-propanol added to dissolve formazan product, and after 20 min shaking absorbance formazan production measured determined spectrophotometrically at 560 nm against a propanol blank.

Thiobarbituric acid reactive substances (TBARS) concentration

TBARS, the end-products of lipid peroxidation, were assayed in EBSS as reported [27]. 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) [28]. TBARS level was expressed as relative fluorescence units (RFU) and normalized for milligrams of proteins in each well.

Measurement of intracellular ROS concentration

To evaluate the ability of digested mixture to reduce ROS levels produced by cells, the conversion of DCFH-DA to DCF was monitored spectrofluorometrically according to Valli *et al.* [29]. Briefly, 30 min before oxidative stress, DCFH-DA, dissolved in absolute ethanol, was added to cells to a final concentration of 0.02 mM. 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 of value in non-stressed US cells.

Cytosolic TAC

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. Cytosolic TAC was measured on the supernatant using the method of Re *et al.* [23], as described above. Results were normalized for protein content in the sample.

Thiolic content

Cells were lysed with 700 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.

The method, that determine the total thiol content considered as GSH, was based on the reaction of GSH with DTNB that produces the TNB chromophore with a maximal absorbance at 415 nm [30]. One hundred µl of the supernatant were incubated with 50 µL of DPBS and 50 µL of reagent buffer (160 mM sodium phosphate pH 8.0, 4 mM EDTA, 4% SDS and 500 µM DTNB) for 30 min. The concentration of GS-TNB proportional to the concentration of GSH was measured spectrophotometrically and the obtained results were compared to the concentration-response curve of standard GSH solutions, normalized for protein content in the sample, and expressed as nmol of thiols calculated as GSH/mg protein.

Evaluation of the cytokines secretion in the cell media

The level of the pro-inflammatory (IL-8) and the anti-inflammatory (IL-10) cytokine was estimated in the media in both basal condition and after cell treatment with LPS using the Multi-Analyte ELISArrey Kit (Quiagen; Hilden, Germania) using a quantitative sandwich immunoassay. Results were expressed as Optical Density and normalized to cell protein content; proteins were extracted with RIPA buffer following the manufacturer's instructions with slight modifications. Briefly, cells were washed with cold DPBS, scraped and centrifuged at 500g for 5 min. The pellet was resuspended in 100 µL RIPA buffer and added of a protease and phosphatase inhibitors mixture (Protease Inhibitor Cocktail, Sigma). After a 5 min incubation, cell lysates were centrifuged again at 14,000g for 15 min and the supernatant collected.

Protein content

Protein content was determined according to Bradford [31] using bovine serum albumin as standard.

Statistical analysis

Data on cookies antioxidant capacity and phenolic content are reported as means ± SD (n=3) whereas data obtained in cell cultures are reported as means ± SD of at least 6 samples derived from 3 independent cell cultures.

Statistical analysis was performed using one-way ANOVA, followed by Tukey's post-test or Dunnet's post-test as appropriate.

RESULTS

In vitro TAC and TPC of digested and not digested cookies

Total antioxidant capacity (TAC) and total phenolic content (TPC) of not-digested (Figure 1) and digested (Figure 2) cookies were assessed. Not digested cookies were extracted with both an ethanol/water (70:30 v/v) solution acidified with 0.1% HCl, and with the same buffer used for the *in vitro* digestion.

In non-digested samples, the use of the acidified ethanol-water solution allowed a better extraction of phenolics in all samples, that consequently showed an higher TAC. This was particularly evident for the KL_{US} cookies. A significant correlation was observed between TAC and TPC (Pearson correlation coefficient: $r^2=0.86$; $p<0.001$).

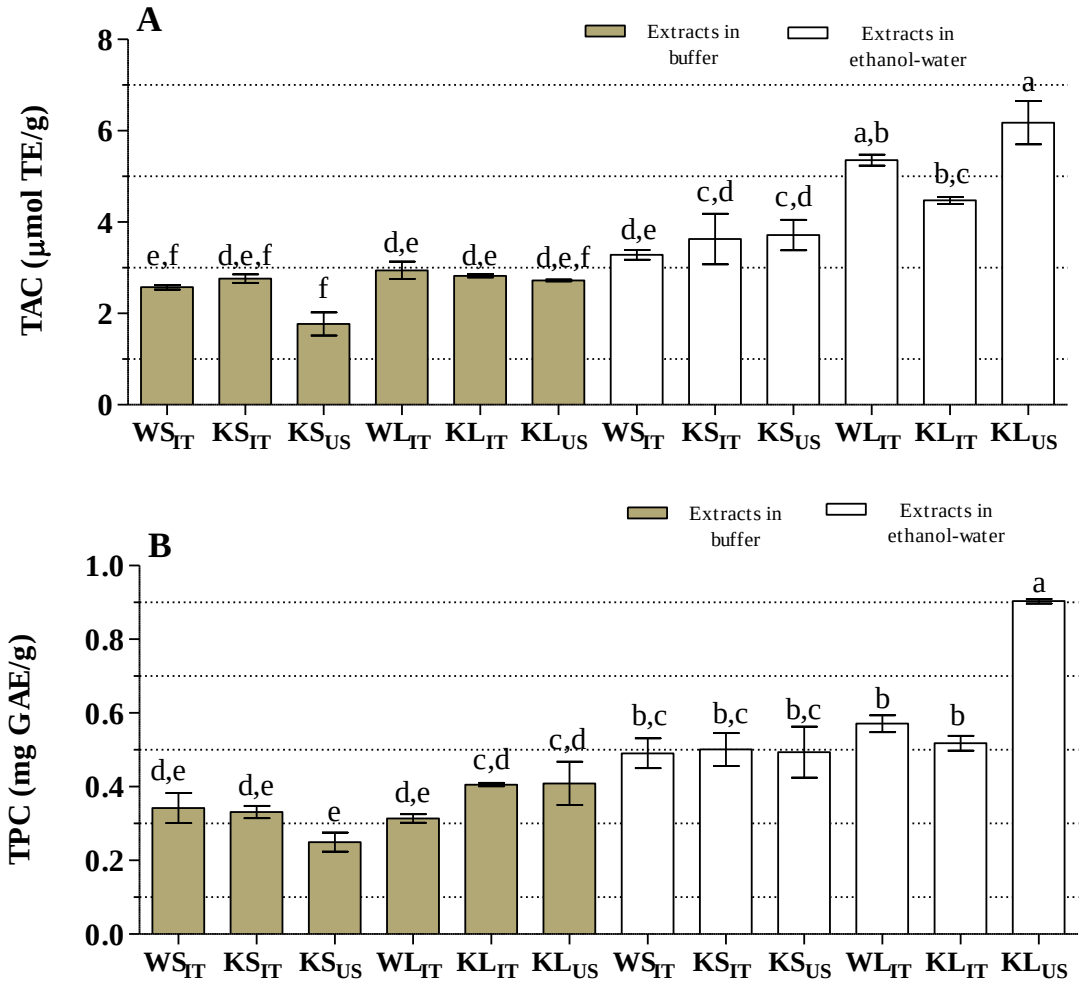

Figure 1. Total antioxidant capacity (TAC) (panel A) and total phenolic content (TPC) (panel B) of cookies extracts.

Data are means ± SD. Statistical analysis was by one-way ANOVA (A $p<0.001$; B $p<0.001$) with Tukey's post-hoc test. Different letters indicate significant differences (at least $p<0.05$).

Both TAC and TPC hugely increased in the digested samples compared to the not digested counterparts, the TAC of not digested cookies being about 10 times lower than the corresponding digested counterparts. Similarly, the TPC of digested cookies was considerably higher than that one of cookies extracts.

In the digested samples the major contribution of total TAC and TPC was ascribable to the <3kDa fraction, containing compounds with dimension compatible to the intestinal absorption. KS_{US} and KL_{US} cookies evidenced the highest TAC, and KL_{US} cookies the highest TPC. A significant correlation was observed between TAC and TPC (Pearson correlation coefficient: $r^2=0.83$; $p<0.001$).

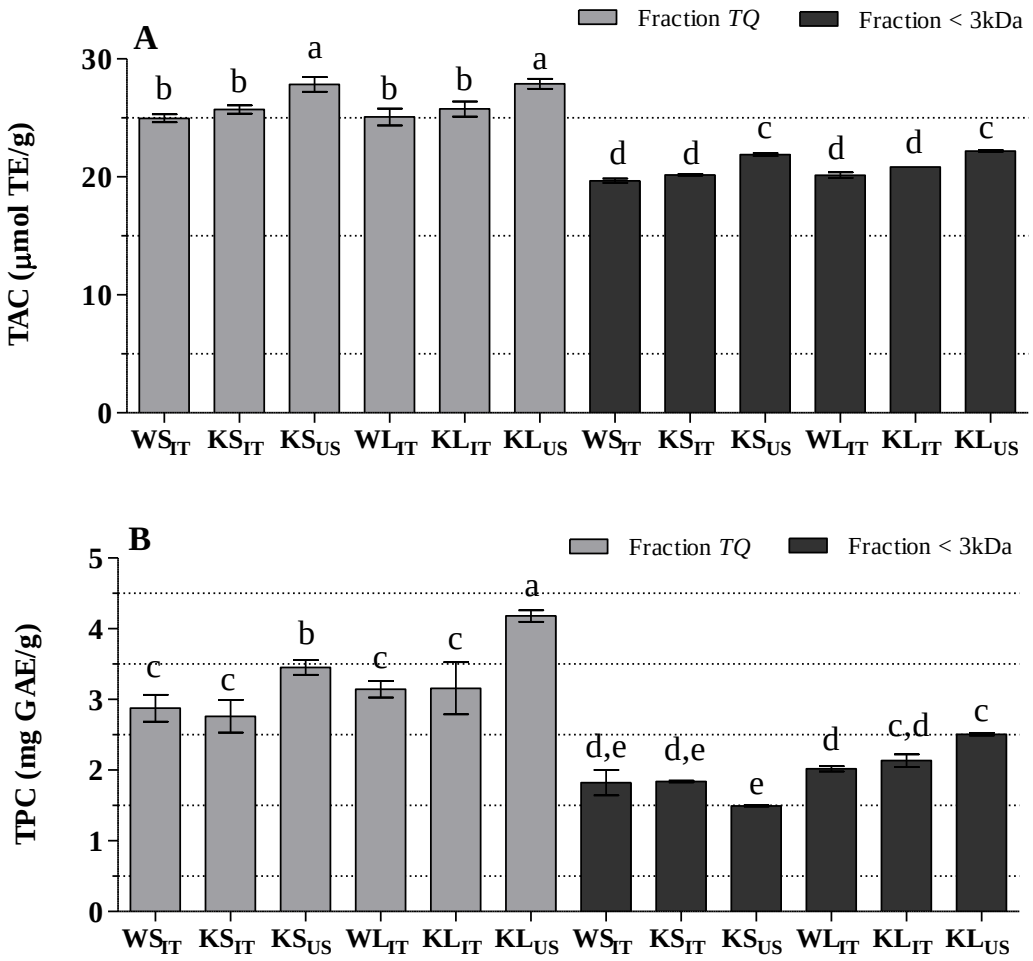

Figure 2. Total antioxidant capacity (TAC) (panel A) and total phenolic content (TPC) (panel B) of digested fractions.

Data are means ± SD. Statistical analysis was by one-way ANOVA (A $p<0.001$; B $p<0.001$) with Tukey's post-hoc test. Different letters indicate significant differences (at least $p<0.05$).

Results on biological system

The <3kDa samples were then used for HepG2 cell supplementation, since this fraction is the one resembling more closely the compounds that are adsorbed after intake and digestion of cookies.

To set the suitable concentration for cell supplementation preliminary experiments were performed to assess a possible cytotoxicity due to the supplemented compounds. The 50 µL/mL concentration didn't cause any cytotoxic effect in basal condition as assessed by MTT assay (data not shown) and was therefore use for further experiments.

1. Protection against the oxidative damage

Compared to control cells, the exposure to H_2O_2 caused a reduction in cell viability in all cells but those supplemented with US Kamut® cookies (Figure 3). The decrease in cell viability was more evident in unsupplemented (US) cells than in supplemented ones ($p<0.001$ in all cases by the Dunnett's post-test)

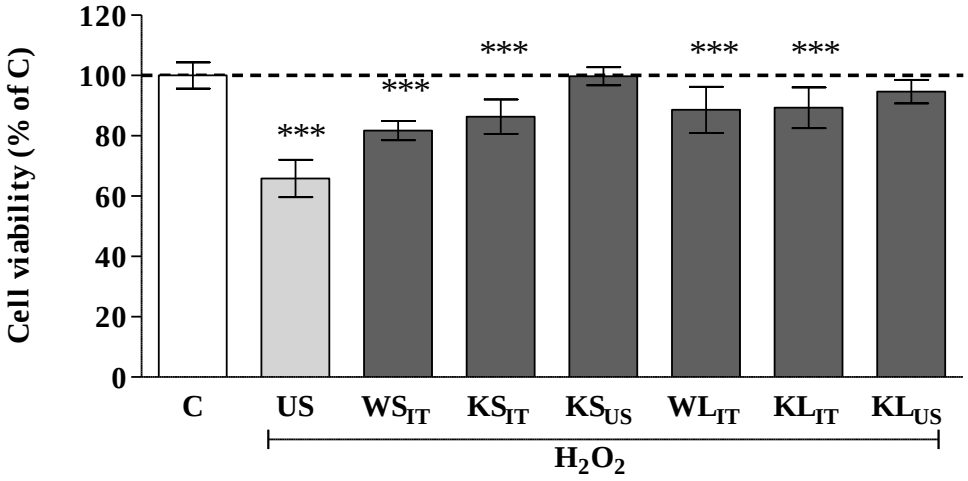

Figure 3. Cell viability in control and supplemented cells after the oxidative damage.
Data are means ± SD. Statistical analysis was by the one-way ANOVA ($p<0.001$) with Dunnett's post-hoc test compared to C cells: *** $p<0.001$.

Cell exposure to H_2O_2 greatly increased TBARS concentration in the medium, and the increase was higher in unsupplemented cells than in supplemented ones ($p<0.001$ in all cases). TBARs production was different among supplemented cells, and the lower increase was observed in cells supplemented with US Kamut® cookies ($p<0.001$ by the one way ANOVA) (Figure 4A).

Upon the oxidative stress, ROS concentration significantly increased in unsupplemented cells, while it was unchanged or even lower in all the supplemented conditions with respect to C (Figure 4B).

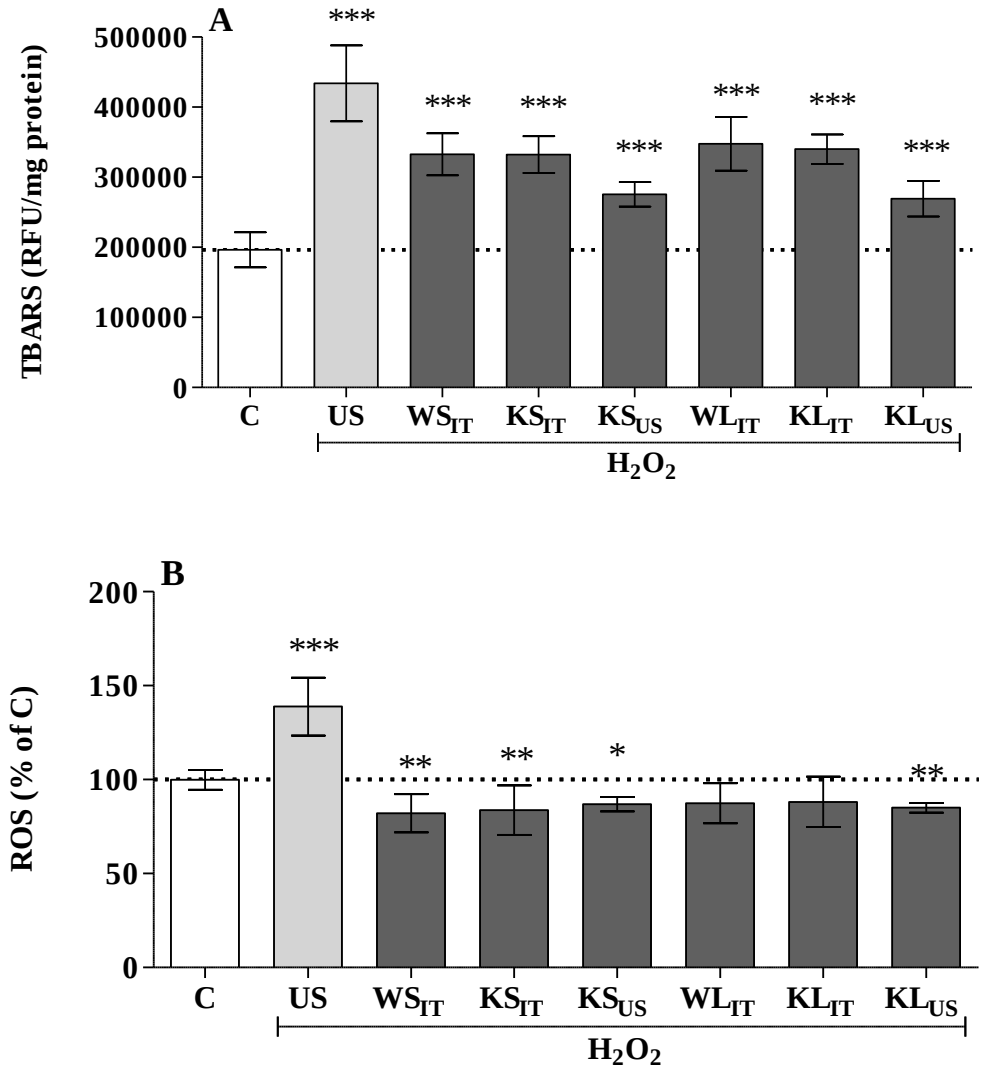

Figure 4. TBARS (panel A) and ROS (panel B) concentration in control and supplemented cells after the oxidative damage.

Data are means ± SD. Statistical analysis was by the one-way ANOVA ($p<0.001$) with Dunnett's post-hoc test (*$p<0.05$; **$p<0.01$; ***$p<0.001$).

In oxidative condition the total antioxidant capacity (TAC) significantly decreased in unsupplemented cells only; no differences were detected among stressed, supplemented cells (Figure 5A).

Total thiol intracellular content was not modified by the oxidative stress in unsupplemented cells; on the contrary it was increased in supplemented ones regardless the type of supplementation (Figure 5B).

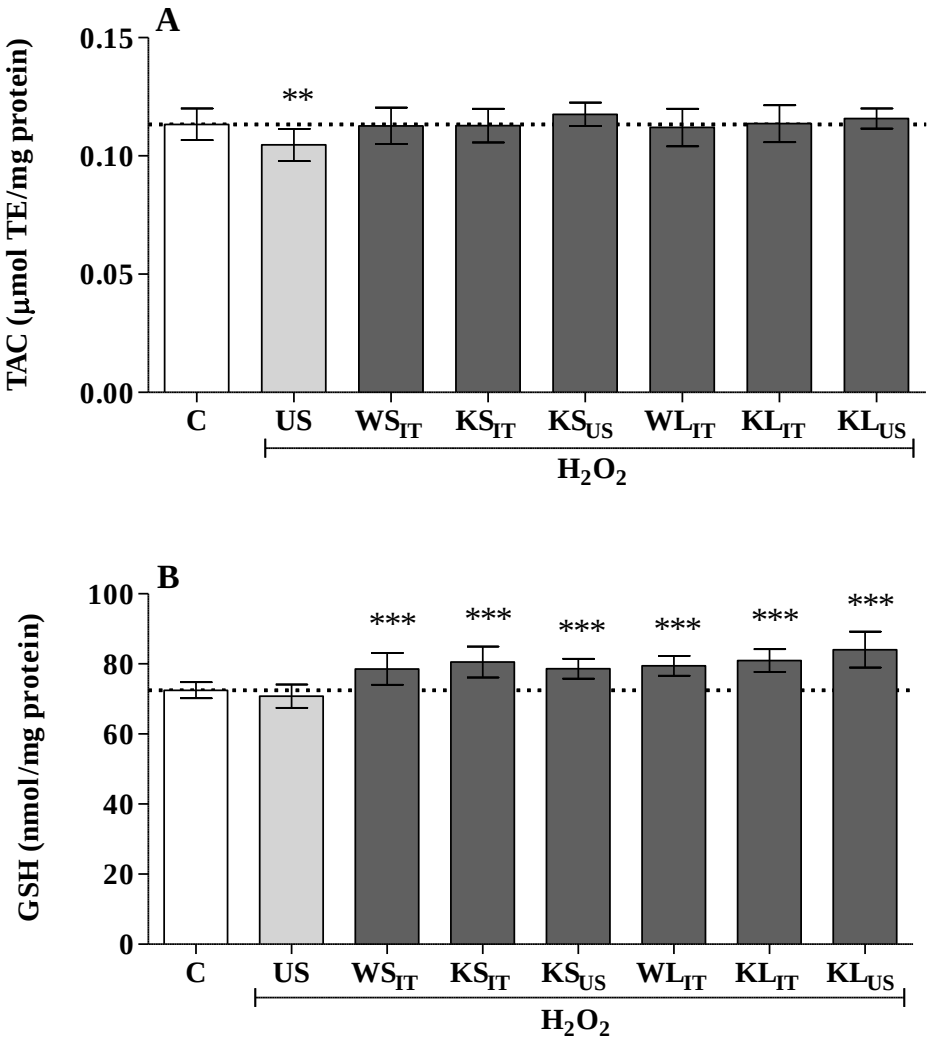

Figure 5. Cytosolic TAC (panel A) and GSH (panel B) content in control and supplemented cells after the oxidative damage.

Data are means ± SD. Statistical analysis was by the one-way ANOVA (panel A: $p<0.001$; panel B: $p<0.001$) with Dunnett's post-hoc test (** $p<0.01$; ***$p<0.001$).

2. Anti-inflammatory activity

Under basal condition (no LPS stimulation) all supplemented cells but WS_{IT} evidenced a significant decrease in pro-inflammatory IL-8 production, and the lower IL-8 secretion was detected in the medium of the cells supplemented with Kamut® cookies prepared using lactic fermentation (Figure 6A).

In a complementary way the secretion of the anti-inflammatory IL-10 increased in all supplemented cells and the lower increase was detected in WS_{IT} supplemented cells. (Figure 6B).

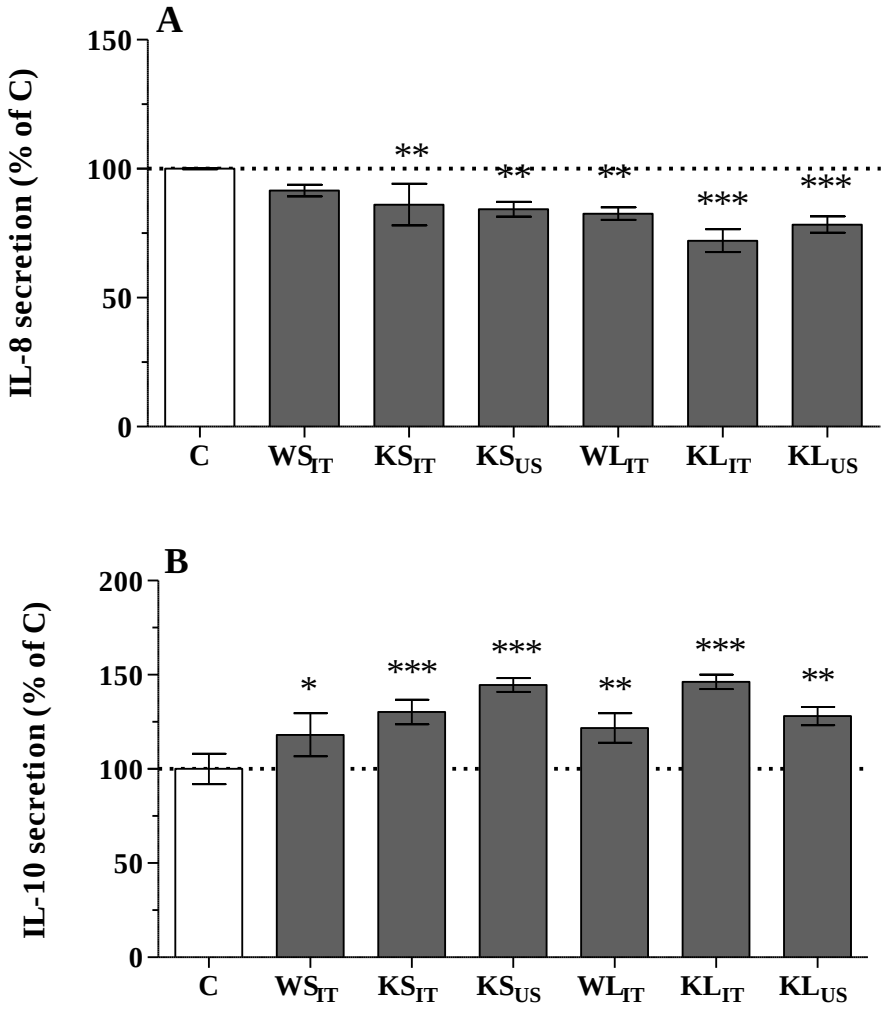

Figure 6. IL8 (panel A) and IL-10 (panel B) secretion in basal conditions.
Data are means ± SD. Statistical analysis was by one-way ANOVA ($p<0.001$) with Dunnett's post-test: * $p<0.05$; **$p<0.01$; ***$p<0.001$.

Upon LPS exposure, unsupplemented cells (US) showed a reduction in cell viability that was not observed in supplemented cells, regardless the type of supplementation (data not shown).
In US cells LPS exposure caused a significant increase of IL-8 secretion compared to basal condition (C) (Figure 7A). All supplementations protected against the pro-inflammatory effect of LPS, K_{US} supplemented cells showing the lower IL-8 secretion compared to C.
After a certain time the physiological response of cells to an inflammatory stimulus is also the production of anti-inflammatory cytokines. In fact, after 18 h exposure to LPS, IL-10 secretion was higher in both unsupplemented and supplemented cells but the one receiving the wheat cookies prepared with lactic fermentation. In stressed condition Kamut® cookies

supplemented cells evidenced a higher IL-10 secretion than unsupplemented ones (at least $p<0.05$ by Dunnet multiple comparison test) (Figure 7B).

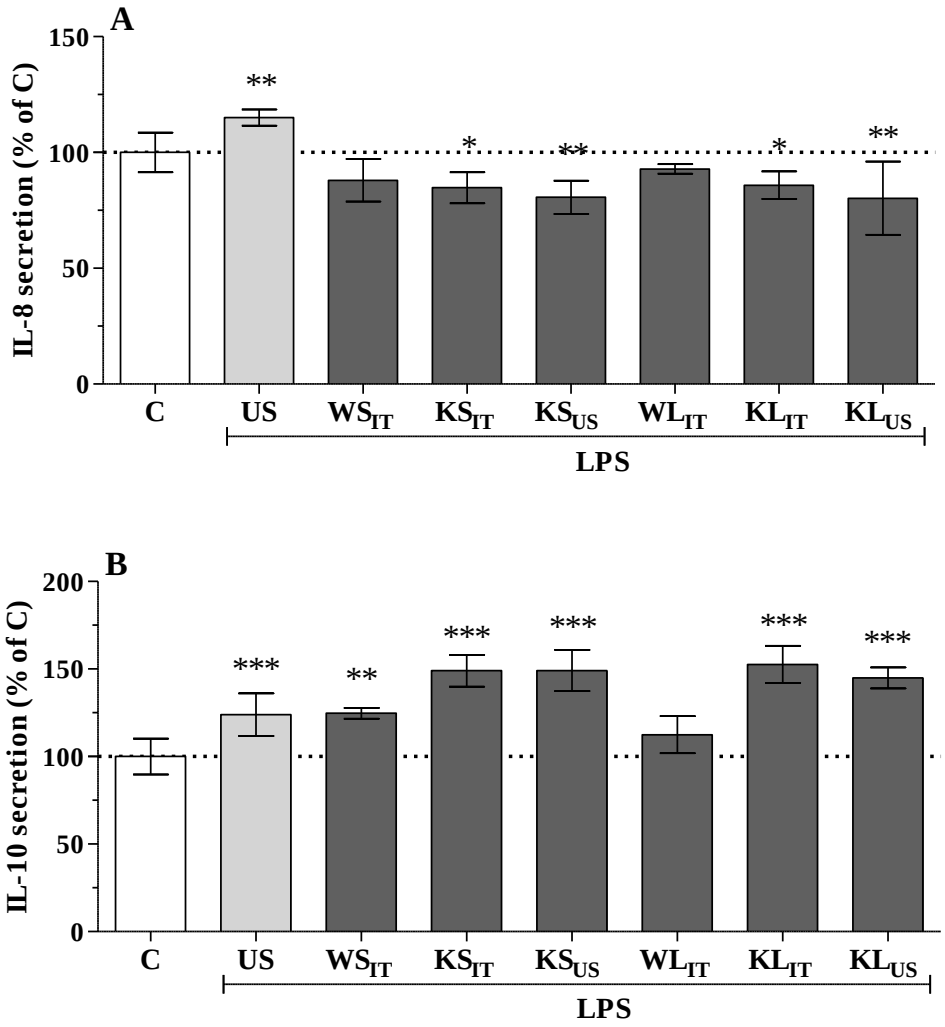

Figure 7. IL-8 (panel A) and IL-10 panel (B) secretion after the pro-inflammatory stimulus.

Data are means ± SD. Statistical analysis was by one-way ANOVA ($p<0.001$ both in panel A and in panel B) with Dunnett's post-test: * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

DISCUSSION

All tests used to evaluate the protective effect of the different supplementations against the induced oxidative stress confirmed the effectiveness of all the digested foods in reducing the damages due to hydrogen peroxide exposure. Although all digested cookies appeared protective, the ones made with US Kamut® better preserved cells from the increase in TBARs and the decrease in cell viability.

Since several of the phytochemicals in whole grain have been reported to exert not only antioxidant, but also anti-inflammatory effects [32], cytokines secretion after cells supplementation with the different digested cookies was also evaluated.

IL-8 and IL-10 were chosen as markers of the cell inflammatory status because HepG2 cells are capable of producing IL-8 and IL-10 in response to specific cytokines stimulation. Moreover, IL-10 is a prototypical regulatory cytokine, exerting several immunomodulatory effects and cereals have shown to stimulate its production in monocytes [33], whereas IL-8, a cytokine with multiple roles, is a pro-inflammatory molecule also inducing cytotoxic effects [34]. Serum IL-8 levels are markedly elevated in patients with alcoholic hepatitis [35].

The decrease in IL-8 and the increase in IL-10 secretion observed in supplemented cells in basal condition suggest a lower inflammatory status. This effect was more evident after LPS stimulation, that evoked a lower IL-8 and a higher IL-10 production in supplemented cells than unsupplemented ones. The anti-inflammatory effect was more evident in cell supplemented with the digested Kamut® cookies than the digested wheat ones, independent of either the Kamut® origin (IT or US) or the type of fermentation used.

The anti-inflammatory effect observed in cultured HepG2 cells is in agreement with previous *in vivo* results. In fact, feeding rats with Kamut® wheat pasta or durum wheat pasta we observed histological modifications in the lymph nodes and spleen, and morphological alterations of the intestinal villi that are suggestive of an anti-inflammatory effect of Kamut® components. These *in vivo* results are reported in this PhD thesis (pp. 47-72). Furthermore Sofi *et al.* [36] have recently evidenced in humans that a replacement diet with Kamut® products is effective in reducing some markers of inflammation.

Previous studies in rats [7, 8] and humans [36] showed a greater protection from oxidative stress by Kamut®-based foods than whole-grain durum wheat-based foods. In this studies we also observed a higher protective activity by US Kamut® cookies than IT Kamut® and wheat ones.

Whole grain cereal antioxidant properties are mainly ascribed to their phenolic content; ancient grain varieties were shown to present unique health-beneficial phytochemicals in their phenolic profile [37], and this can explain the differences observed between wheat and US Kamut®. The observed higher activity of US Kamut® could therefore be related to a different phenolic profile, however a different selenium content could have also contributed to the higher effectiveness of the cereal grown in the US.

The essential trace mineral, selenium, is of fundamental importance to human health. It is needed for the proper functioning of the immune system and it is a constituent of

selenoenzymes with antioxidant functions [38]. The concentration of selenium in plant foods is strongly correlated with its concentration in soils [39] and selenium level in US soils is generally higher than in European ones [40].

Further studies are in progress to evaluate the phenolic profile and selenium content in the cookies used in this study.

It is important to consider that all cookies were prepared using whole grain flours. The higher bioactive content of whole grain cereals is well known [6, 41], and this could have reduced differences in the antioxidant and anti-inflammatory potential among the studied cereals. Other molecules than phenolics could contribute to the overall effect of whole grain products; we supplemented cells with the digested cookies and not with a single class of cookies-derived compounds in order to evaluate the possible synergistic effect among all different components.

Results in cultured cells may diverge from those in whole organisms since the most of dietary (poly)phenolics appear in the circulatory system not as the parent compounds, but as phase II metabolites produced by intestinal and liver cells [42]. Although in this study the use of *in vitro* digestion and human hepatic cell culture, that possess phase II enzymes and can therefore metabolize phenolics, reduced in part the distance to the physiological situation in animals, the existing differences must be considered before drawing conclusions.

In any case, considering that most studies regarding the healthy properties of cereals are based on unprocessed or partially processed cereals rather than on cereal food products, and differences in bioactives solubility and/or bioavailability within the digestive tract are not taken into account, our results represent a first step for the evaluation of different cereals and processing and highlight the potential health effects of whole grains and particularly of the ancient variety Kamut® khorasan.

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Chapter 4

Modulation of adipocyte differentiation and proadipogenic gene expression by sulforaphane, genistein, and docosahexaenoic acid - A first step to counteract obesity

V. Valli, K. Heilmann, C. Gerhäuser, A. Bordoni - DHA, Genisteina e Sulforafano: bioattivi anti-obesità - Riunione Nazionale SINU "Comprendere e applicare i LARN" - Florence, 21-22 October 2013 (ISBN: 978-88-97843-09-2).

Excessive caloric intake, related to high fat and high simple sugars foods consumption, is one of the main cause of obesity. For this reason, confectionery products are always classified in a negative way from a nutritional point of view. However, theoretically, compounds able to influence adipogenesis could potentially be used in the formulation of sweet products and contribute to treat or to prevent obesity. In this chapter, the ability of three bioactives in inhibiting adipocytes differentiation is investigated. Further studies aimed at successfully incorporating the bioactive compounds in a confectionery product and at testing the final sweet product on humans would be interesting.

ABSTRACT

Obesity is characterized by excess body fat accumulation due to an increase in size and number of differentiated mature adipocytes. Adipocyte differentiation is regulated by genetic and environmental factors, and its inhibition could represent a strategy for obesity prevention and treatment. The current study had two aims: i. to evaluate changes in the expression of adipogenic markers (*C/EBPα*, *PPARγ* variant1 and variant 2; and *GLUT 4*) in 3T3-L1 murine pre-adipocytes at four stages of the differentiation process; and ii. to investigate the anti-obesity effectiveness of sulforaphane, genistein, and docosahexaenoic acid by evaluating both lipid accumulation and the modulation of *C/EBPα*, *PPARγ*, and *GLUT 4* mRNA expression in mature adipocytes. The bioactive compounds were shown to suppress adipocytes differentiation, decreasing the expression of the adipogenic markers and lipid accumulation to the levels of pre-adipocytes. These results set the stage for further studies considering natural food constituents as important tools in preventing or treating obesity.

INTRODUCTION

Obesity is the main dysfunctions of adipose tissue, and associates with premature death and the development of chronic diseases as cardiovascular diseases, type 2 diabetes, hypertension, certain cancers and inflammation [1]. Environment, lifestyle and genetic susceptibility certainly contribute to the increased risk of obesity, which is characterized by an excess accumulation of white adipose mass, resulting from both the increase in adipocyte cell size and the development of mature cells from undifferentiated precursors. Particularly, *de novo* generation of fat cells plays a key role in the development of obesity.

Obesity is considered as one of the most easy to recognize and the most difficult to treat medical conditions [2], and anti-obesity drugs lack physiology specificity and have side effects [3]. Discovering compounds able to regulate size, number and function of adipocytes and understanding their mechanisms of action could greatly contribute to obesity prevention and treatment; particularly, natural compounds could represent a potential novel strategy already exploited for preventing metabolic disorders [4].

Bioactive food compounds are essential and non-essential constituents naturally occurring in small quantities in foods that have been found to possess a large range of beneficial health effects [5, 6].

Certain bioactive compounds have been shown to have specific effects on biochemical and metabolic functions of adipocytes [7, 8]. In particular, they were shown to inhibit differentiation of pre-adipocytes, stimulate lipolysis, and induce apoptosis of existing adipocytes [9]; all of these actions contributing to a possible decrease of adipose tissue amount [10].

The aim of the current study was to investigate the anti-adipogenic ability of three bioactives, namely docosahexaenoic acid (DHA), genistein (GEN), and sulforaphane (SFN).

DHA (C22:6 n-3) is an n-3 polyunsaturated fatty acid (PUFA) abundant in fish. It is considered effective in the prevention of many chronic diseases, mainly cardiovascular diseases [11].

GEN (4,5,7-trihydroxyisoflavone), the most abundant isoflavone found in soybeans, has received particular attention for its structural similarity to estrogen that has high affinity to the estrogen receptor. It is a well-known antioxidant, chemopreventive and anti-inflammatory agent [12, 13].

SFN (sulforaphane), an isothiocyanate compound, is a phytochemical constituent of cruciferous vegetables such as broccoli sprouts, Brussels sprouts and cabbage. SFN is known to have antioxidant, immunomodulatory, anticancer and antidiabetic properties [14, 15].

To evidence the anti-adipogenic effect of the three bioactives the 3T3-L1 cell line, isolated from the disaggregated Swiss 3T3 mouse embryos, was chosen as model system; 3T3-L1 cells are the most frequently used adipocytes in literature and show many properties similar to those of normal adipocytes [16].

First, changes in the expression of three adipogenic markers were evaluated at various stages of the differentiation process. Indeed, it has been well documented that adipogenesis is finely controlled by key transcription factors such as peroxisome proliferator-activated receptor-γ (*PPARγ*) and CCAAT-enhancer binding protein-α (*C/EBPα*) that induce the expression of various genes determining the adipocytes phenotype and involved in insulin sensitivity, lipogenesis and lipolysis such as the glucose transporter *GLUT4* [17, 18].

Then pre-adipocytes were supplemented during and post-differentiation with DHA, GEN, and SFN, and both lipid accumulation and the mRNA expression of *PPARγ, C/EBPα,* and *GLUT4* were evaluated to evidence their potential inhibitory activity on adipogenesis.

Results herein reported suggest that the tested bioactives are able to reduce the expression of adipogenic markers to the levels of pre-adipocytes, and to inhibit adipogenesis. Although *in vitro* studies always need confirmation in vivo, our results could be useful to understand the process leading to the influence of food bioactives on de novo differentiation of adipocytes, contributing to the development of new strategies to prevent obesity.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM)/F12 Glutamax I was purchased from Invitrogen (Germany), Donor Bovine Serum (DBS) was from Gibco Life Technologies (Germany), Fetal Bovin Serum (FBS GOLD) was from PAA Laboratories (Austria) and TRIzol Reagent was from Ambion, Life Technologies (Germany). All other chemicals were purchased from Sigma (Germany). All chemicals were of the highest analytical grade.

Methods

Cell culture and differentiation

3T3-L1 mouse pre-adipocytes were obtained from American Type Culture Collection and maintained at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2; pre-adipocytes were sub-cultured every three days when 80% confluent or less into a new 175 cm^2 flask. Cells were cultured in DMEM/F12 Glutamax I added of D-glucose (3151 mg/L f.c.) (GM) and containing 10% DBS and 1% penicillin/streptomycin. Cells were seeded in 12-well plates or 25 cm^2 flask at a concentration of 50,000 cells/mL. Three days after seeding

cells were stimulated to differentiate with GM supplemented with 10% FBS, 1% penicillin/streptomycin, insulin (10 µg/mL), dexamethasone (1µM), isobutylmethylxanthine (0.2 mM), and rosiglitazone (10 µM) (differentiation medium). After further 3 days (differentiation) cells were then maintained in GM with FBS, PS, and insulin (10 µg/mL, post-differentiation medium) for another 5 days (post-differentiation); after this period approximately 90% of cells displayed the characteristic lipid-filled adipocyte phenotype.

Bioactive supplementation

In some experiments DHA, GEN and SFN (dissolved in DMSO) at were added at final concentrations of 10, 25 or 50 µM, respectively, to the differentiation or post-differentiation medium. Medium was changed every two days during post-differentiation.

Unsupplemented control cells (US) received a corresponding amount of DMSO as solvent control (< 0.5% final concentration).

The effect of the bioactives on adipogenesis was evaluated morphologically by staining accumulated lipids with Oil Red O [19]. Briefly, cells were fixed with 4% formalin in PBS for two hours, washed with water, rinsed with isopropanol 60% and stained with Oil Red O for 30 minutes at room temperature. After washing with distilled water for 3 times, the lipid droplets were quantified by dissolving Oil Red O in isopropanol 100% and measuring the optical density at 500 nm.

The lowest bioactive concentrations able to influence lipid accumulation were then used in gene expression experiments.

Gene expression analysis

To evaluate transcript levels of adipogenesis marker genes at different stages of adipocyte differentiation, cells were collected at 4 different steps of the differentiation protocol: 1 day after seeding (T1); 3 days after seeding when cells were post-confluent, before the beginning of differentiation (T2); at the end of the differentiation, before the addition of the post-differentiation medium (T3); at the end of post-differentiation (T4).

In following experiments, GEN at a concentration of 10 µM, SFN at 10 µM, and DHA at 25 µM were added to the differentiation and post-differentiation medium as described above. At the end of the post-differentiation period (T4), cells were collected, and total RNA was extracted with TRIzol Reagent following the manufacturer's protocol. Contaminating DNA was eliminated by DNase treatment (DNA-free Kit from Ambion, Life Technologies, Darmstadt, Germany). RNA quantity and quality, respectively, were assessed by spectophotometric analyses at 260/230 nm using a Nano-Drop ND-2000 spectrophotometer

(Thermo Fisher Scientific, Wilmington, DE, USA) and by the microfluidics-based Bioanalyzer platform (Agilent Technology).

First-strand cDNA was synthesized from 0.5 µg or 1 µg of DNase-treated total RNA using Superscript II reverse transcriptase (Invitrogen, Darmstadt, Germany) according to manufacturer's instructions.

Real-time qPCR was performed using the Universal Probe Library system (Roche, Mannheim, Germany) on a Roche Lightcycler 480 Real-time PCR system (Roche, Mannheim, Germany). The cycling program for analysis was 15 min at 95 °C followed by 45 cycles of 10 s at 95 °C, 20 s at 55 °C, and 10 s at 72 °C with the following primer pairs and respective mono color hydrolysis probes:

Gene	**Forward Primer**	**Reverse Primer**	**Probe number**
PPARγ transcript variant 1	gaaagacaacggacaaatcacc	gggggtgatatgtttgaacttg	7
PPARγ transcript variant 2	tgctgttatgggtgaaactctg	ctgtgtcaaccatggtaatttctt	2
C/EBPα	aaacaacgcaacgtggaga	gcggtcattgtcactggtc	67
GLUT4	gacggacactccatctgttg	gccacgatggagacatagc	5
β-actin	gtgggagagcaaggaagaga	cactcttggcccagtctacg	56
HPRT1	tcctcctcagaccgctttt	cctggttcatcatcgctaatc	95
TBP	cggtcgcgtcattttctc	gggttatcttcacacaccatga	107

Expression levels of target mRNAs were normalized to the three housekeeping genes *β-actin, HPRT1, TBP*.

Statistical analysis

Gene expression data were analyzed using DataAssist Software version 3.01 (Applied Biosystems; Foster City, CA, USA). Average fold change and standard deviation (SD) were obtained from 3 biological replicate samples per condition.

All data were analyzed by one-way ANOVA, using Dunnett post-hoc test in the case of comparisons with the untreated controls or Tukey post-hoc test for comparing differences among all the groups. Statistical analysis of the data was performed using the GraphPad Prism 5 software (San Diego, CA, USA).

RESULTS

1. Expression of adipogenesis marker genes at different stages of adipocyte differentiation

Differentiation requires the activation of numerous transcription factors which are in charge of the coordinated induction and silencing of more than 2000 genes related to the regulation of adipocyte in both morphology and physiology [20]. To characterize the differentiation process mRNA expression of the transcription factors *PPARγ* (variant 1 and variant 2) and *C/EBPα*, and the *GLUT4* gene was evaluated at 4 different stages of adipocyte differentiation. Expression of all the marker genes was very low and similar at T1 and T2. Three days after the addition of the differentiation medium (T3), expression of *PPARγ* (variant 1 and variant 2), *C/EBPα* and *GLUT4* started to increase significantly. A prominent increase in transcription was observed in mature adipocytes (T4) (Figure 1).

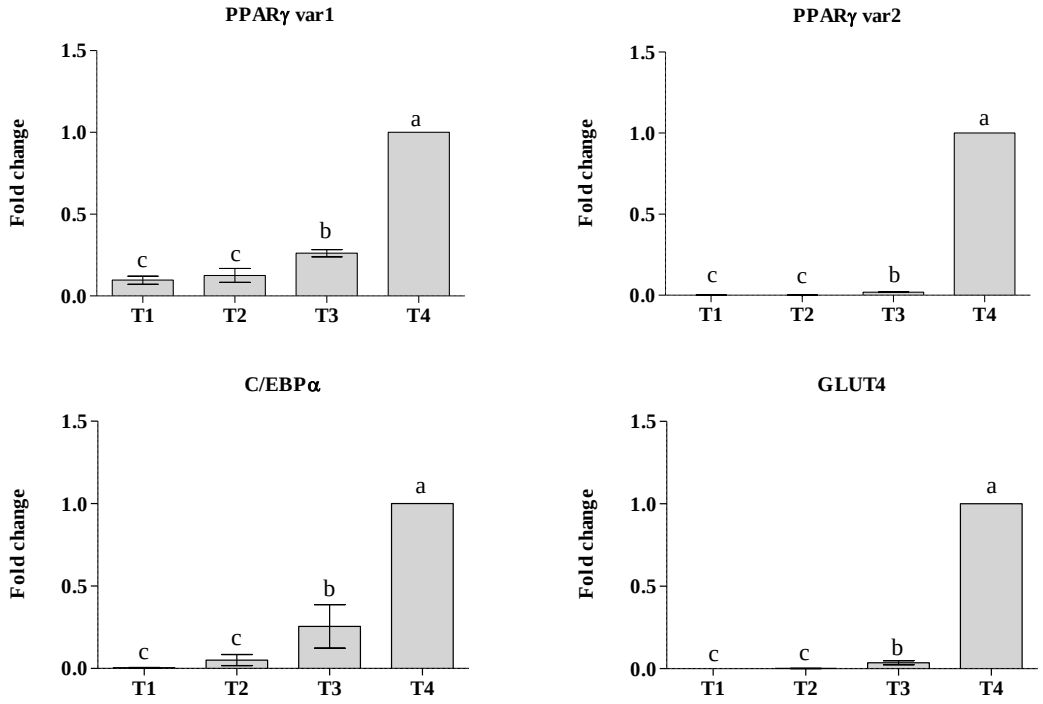

Figure 1: *PPARγ* var1, *PPARγ* var2, *C/EBPα* and *GLUT4* mRNA expression at 4 different stages of adipocyte differentiation.

T1: 1 day after seeding; T2: 3 days after seeding, before the beginning of differentiation; T3: end of the differentiation, before the addition of the post-differentiation medium: T4: end of post-differentiation (mature adipocytes).

Gene expression data are presented as the mean fold change of relative expression compared to mature cells (T4), normalized to 1. Statistical analysis was by one-way ANOVA ($p<0.001$) with Tukey as post-hoc test. Different letters indicate statistical significance (at least $p<0.05$).

During differentiation, pre-adipocytes took on the characteristics of mature adipocytes. At T2, non-differentiated cell showed typical fibroblastoid morphology, while at the end of the differentiation process (T4) cells had abundant intracytoplasmic lipid accumulation, showing typical white adipocyte morphology (Figure 2).

T2 **T4**

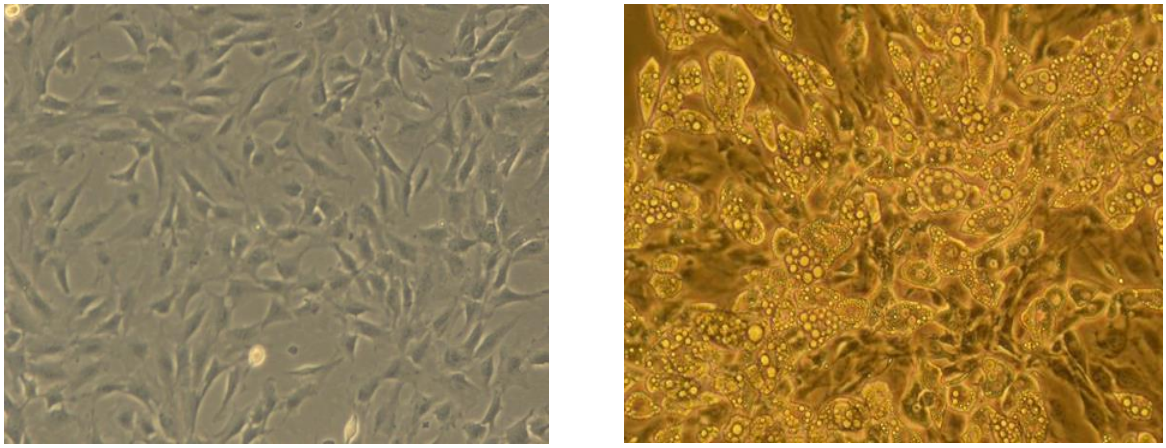

Figure 2. Morphological changes between pre-adipocytes (T2) and mature adipocytes (T4)

Two images showing different cells morphologies were captured at steps 2 and 4 (T2 and T4) using a Leica DMIL Microscope (Germany). Magnification 10X.

2. Inhibition of lipid accumulation

The anti-obesity potential of DHA, GEN, and SFN was first investigated evaluating the bioactive influence on pre-adipocyte differentiation into adipocytes. With this aim, lipid accumulation was detected by Oil-Red-O staining in 3T3-L1 cells treated at increasing concentrations (10 µM, 25 µM and 50 µM) of the test compounds during the differentiation and post-differentiation periods, as described above. All bioactives markedly reduced lipid droplet formation compared to vehicle controls. GEN and SFN appeared effective even at the lowest concentration used (10 µM), while a higher DHA concentration (25 µM) was required to reduce lipid accumulation (Figure 3).

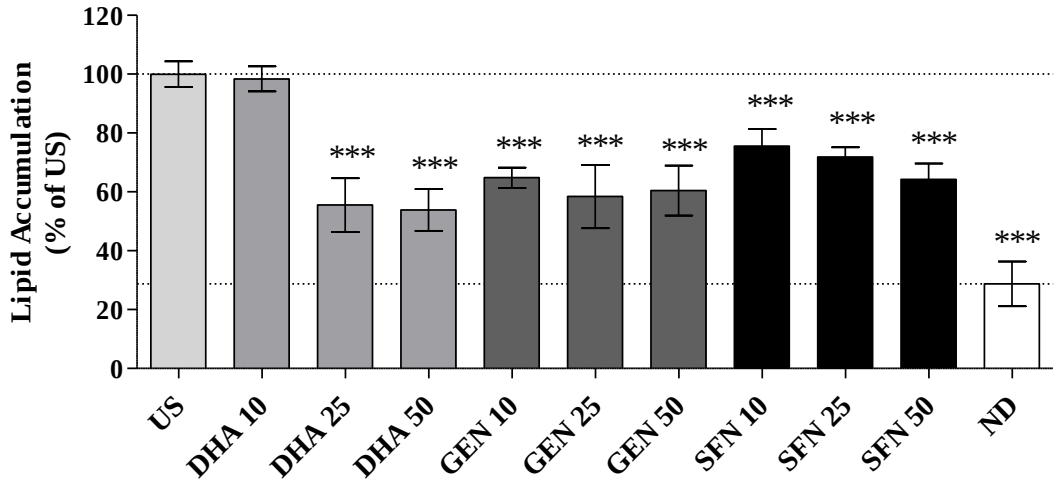

Figure 3. Lipid accumulation in supplemented and control cells

Data are means ± SD and are expressed as a percentage relative to unsupplemented cells (US), assigned as 100%. Statistical analysis was by one-way ANOVA ($p<0.001$) with Dunnett post-hoc test: ***<0.001 vs. unsupplemented (US) cells. ND: non differentiated cells, before the beginning of the differentiation process.

3. Effects of GEN, SFN and DHA on *PPARγ* var 1 and 2, *C/EBPα*, and *GLUT4* expression

To determine whether the bioactive-induced decrease in lipid accumulation was related to a reduction in mRNA levels of adipogenesis marker genes, cells were supplemented during differentiation and post-differentiation with the three bioactives at the lowest concentrations causing a significant decrease in lipid accumulation.

At T4, all bioactives significantly reduced transcript levels of *PPARγ* var 1 and var 2, *C/EBPα*, and *GLUT4*, the effect of GEN and SFN on *PPAR γ* and *GLUT4* expression being stronger than that of DHA (Figure 4).

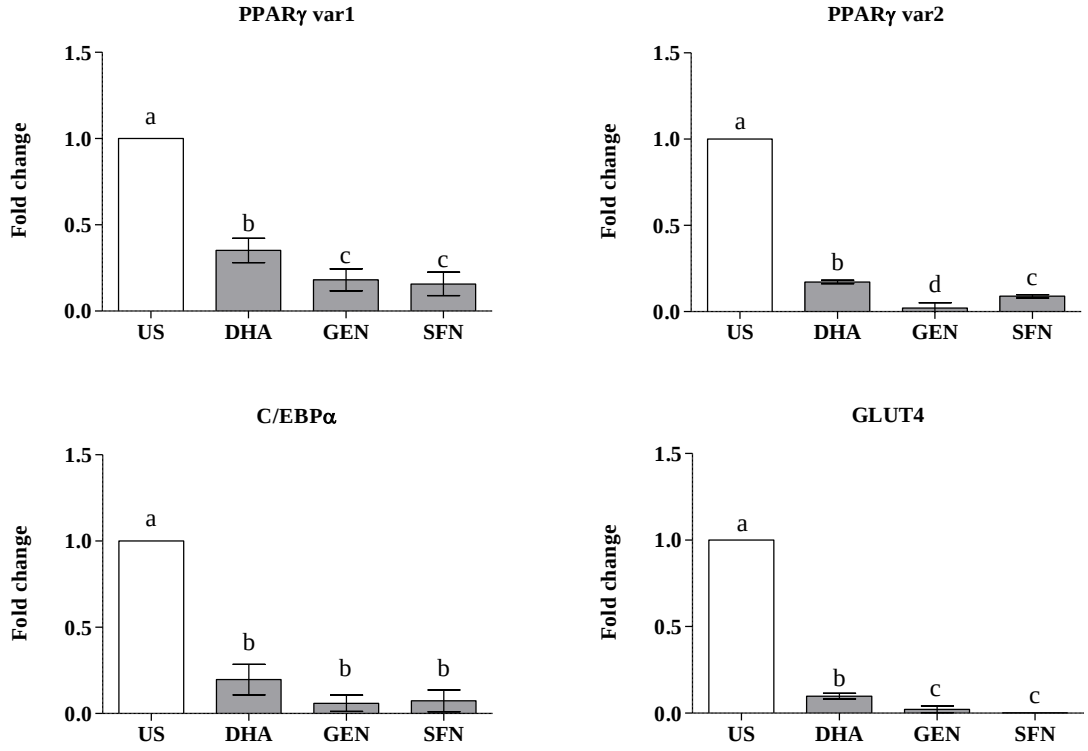

Figure 4. Effects of GEN, SFN and DHA on *PPARγ* var 1 and 2, *C/EBPα*, and *GLUT4* mRNA expression

Gene expression data are presented as the mean fold change of relative expression compared to unsupplemented (US) cells at T4, normalized to 1. Statistical analysis was by one-way ANOVA (values $p<0.001$ for every graph) with Tukey as post-hoc test. Different letters indicate statistical significance (at least $p<0.05$).

DISCUSSION

Adipose tissue has an important function in the energy balance of the body by regulation of lipid metabolism, glucose homeostasis, and adipokine secretion. Thus, its dysfunction is critical in developing metabolic diseases [21].

In general, obesity is related to the extent of adipocyte differentiation, intracellular lipid accumulation and lipolysis [22]. The process of adipocytes differentiation is ascribed to the activation of the expression of adipocytes specific genes; several transcriptional regulators, including C/EBP and PPARγ, play pivotal role in this process.

PPARγ as a master regulator is both necessary and sufficient for adipogenesis [23, 24]. *PPARγ* has two isoforms, *PPARγ1*, ubiquitously expressed, and *PPARγ2*, expression of which is restricted to adipose tissue. Both isoforms are strongly induced during pre-adipocytes

differentiation and are highly expressed in adipose tissue in animals. *PPARγ1* is induced at earlier time points than PPARγ2 and is maintained at higher levels than *PPARγ2* [25].

The *C/EBP* family includes the members *C/EBPα*, *C/EBPβ*, and C/*EBPδ*. All three are expressed sequentially during the differentiation of pre-adipocytes to adipocytes. *C/EBPβ* and *C/EBPδ* are expressed relatively early and have been shown to play a role in induction of *PPARγ*. Consistent with its late expression during fat cell differentiation, *C/EBPα* has been implicated in the maintenance of the terminally differentiated adipocyte phenotypes [20, 26, 27].

In adipocytes *C/EBPα* has been reported to be important for the expression of the gene encoding for GLUT-4, the major insulin-responsive glucose transporter in adipose tissue as well as in skeletal and cardiac muscles [28].

In this study, 3T3-L1 cells at four stages of differentiation were characterized by the expression levels of the marker genes *C/EBPα*, *PPARγ*, and *GLUT 4*.

Results obtained on gene expression were consistent with previous reports [29, 30], and are in agreement with results on lipid accumulation, confirming that differentiation of 3T3-L1 cells includes distinguishable multiple stages.

The second part of this study focused on the effect of GEN, DHA and SFN on adipocyte differentiation.

Firstly, it was shown that 3T3-L1 treatment with DHA, GEN, and SFN during differentiation and post-differentiation suppressed lipid accumulation, and the lowest concentration causing a significant decrease in lipid accumulation was chosen to treat 3T3-L1 cells for evaluating the influence of the three bioactives on the expression of adipogenic marker genes at the end of the differentiation process (T4).

mRNA expression levels of *PPARγ*, *C/EBPα*, and *GLUT4* were hugely decreased by cell treatment with 25μM DHA, 10μM GEN, and 10 μM SFN, respectively, during differentiation and post-differentiation, and GEN and SFN were more effective than DHA.

At T4 expression levels of *PPARγ*, *C/EBPα*, and *GLUT4* in treated cells was comparable to the expression level in unsupplemented cells at the first stages (T1, T2 or T3) of adipocytes differentiation, indicating that all tested compounds could have efficiently blocked adipocytes differentiation.

Most recent advances in food and nutrition sciences have highlighted the possibility of controlling body weight through food intake and in particular by incorporating functional ingredients [31]. Yun [32] arranged natural products having anti-obesity effects into five categories based on their distinct mechanisms: 1) decreased lipid absorption; 2) decreased

energy intake; 3) increased energy expenditure; 4) decreased pre-adipocytes differentiation and proliferation; 5) decreased lipogenesis and increased lypolisis. Different studies have already explored the action of the three bioactives on adipocyte life-stages, revealing that they act on different critical pathways. In particular our results are in accordance with literature studies on 3T3-L1 models showing that DHA [33, 34], SFN [22, 35], and GEN [36-39] may exert antiobesity effects by inhibiting differentiation to adipocytes, inducing apoptosis in post-confluent pre-adipocytes and promoting lipolysis.

Concluding, our results represent a first step for the evaluation of the antiadipogenic effects of natural bioactive molecules. DHA, GEN, and SFN were shown to exhibit anti-adipogenic activity mediated by the inhibition of the expression of PPARγ, C/EBPα and *GLUT4*. These transcription factors and gene are critical to the final stages of adipocyte differentiation and progression, and our results are therefore important for the characterization of the anti-adipogenesis mechanism of the considered bioactive compounds.

According to our results DHA, GEN and SFN could contribute to the prevention and treatment of obesity, and could potentially be used for the formulation of new functional food products devoted to a new dietetic strategy for overweight counteraction.

Authors are aware that further investigations are needed to verify if the anti-obesity effects evidenced *in vitro* do translate into *in vivo* actions, especially in humans. As well, in this study single compounds have been studied separately and not as part of whole foods, ignoring both the matrix effect and the eventual synergism between the selected compounds and others that could be present in the food, and this issue also deserves future attention.

Notwithstanding, our results emphasize the potential anti-obesity activity of DHA, GEN, and SFN, and considering the link between food consumption and obesity the study herein reported can be considered as a first step in the way to find natural anti-obesity strategies.

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FINAL CONSIDERATIONS

The over-consumption of unhealthy food, coupled with lives that are increasingly sedentary, is producing large numbers of people who are overweight and obese, primarily in high-income countries, but also in emerging middle-income countries.

Confectionery and sweet products, often consumed as snack foods outside the three main daily meals, are classified 'extra' foods because it is not necessary to consume them to obtain the essential nutrients that the body needs. They are energy dense, high in sodium, low in micronutrients and can contribute in large amounts to the excess of energy intake, which may have potential negative impacts on health, including excess of weight gain. Sometimes, also in metropolitan areas, small supermarkets, canteens, bars and vending machines don't have healthy choices for snacks or dessert. It can be true that healthy products cost more than unhealthy ones, however the economic costs of diet-related chronic diseases, would be dramatically reduced by healthy diets.

An increased emphasis on healthy lifestyles is an imperative for governments facing rising healthcare costs, particularly in developed economies that are battling childhood obesity.

Actually, in regards to children, key factors in the development of a child's food preferences and eating behaviors are food availability and accessibility as well as adverts for junk food on television and they will tend to choose these high fat and sugar snack foods, if made available, in preference to more nutritious options.

Many industries should therefore provide better nutrition in indulgent products like cookies, ice-cream, chocolates, cakes, candies. An increase in the potential health and functional benefits of such goods can be important for different categories of consumers, not only children, but also athletes, elderly, and stressed people.

The overall aim of this PhD project was to find possibilities for increasing confectionary products healthy value. We showed that both new ingredients and some ancient ones too can help in improving the nutritional aspects of sweet foods. The healthier alternatives do not have to replace the traditional products but can provide a choice to consumers whenever they want/need a bit more nutrition in their sweet moments. Indeed, sweets can be part of a balanced diet as long as people pay attention to portion sizes and choose healthier treats. Developing confectionery and sweet products with increased levels of bioactive compounds has the potential to maintain our health and wellbeing through our diet.

In particular we evidenced different approaches aimed at this nutritional and healthy improvement:

1) **Exploitation of by-products.** The sustainable addition of agro-industrial by-products as sugar molasses and wheat bran was shown to provide specific natural antioxidants as phenolic compounds.

2) **Rediscovery of ancient grain varieties.** The substitution of the modern durum wheat flour with the Kamut khorasan grain resulted highly positive increasing the antioxidant and anti-inflammatory potential of the final products

3) **Addition of single bioactive compounds with specific functions.** Addressing the global and rising problem of obesity, sulforaphane, genisteine, and docosahexaenoic acid exhibited anti-adipogenic characteristics that could be important in the formulation of new foods.

The common base of these approaches resulted the use of ingredients rich in food bioactives. Sources of these valuable molecules can really be the key for a positive impact on human health, mainly considering that our modern diets often contain low concentration or minimal intake of bioactives and that they can naturally occur in plant varieties that disappeared due to the lack of interest or in food industry wastes and residues that are not usually recovered.

Certainly, further multidisciplinary studies are needed to develop more deeply the healthy possibilities studied in this PhD thesis, but taking into account that food science is greatly growing developing new foods, designing processes, creating new packaging materials, improving sensory characteristics, our results can be useful for driving research and industry towards specific aims and specific procedures.

Finally, considering the enormous impact on what we eat on our health and the need people have to reach health and wellbeing also through nutrition, the global vision given by this PhD thesis may be important both for giving a new idea of sweet confectionary products that, healthy improved, could be also recommended within an optimal diet.

PhD Portfolio

COURSES

Compulsory courses organized by the Agricultural, Environmental and Food Science and Technology PhD school at the University of Bologna	Attendance hours
Philosophy of science and research methodology	10
Research financing and project design in agricultural sciences	20
Statistical methods in agriculture and data analysis	30
Setting up a research protocol	4
Writing a scientific paper	8
Work safety	10
Mathematical models in environmental, crop and food science	20
Bibliographic services to support research	4
Artificial neural networks: modeling, software packages and research applications	6
Issues of risk-benefit assessment in not heat-treated food	3
English course – Academic writing	24
Other courses	
NAMASTE Training Course Practical Aspects of Working with Industry (Organized within the activities of EU project NAMASTE by the Campden BRI at the University of Bologna)	4
Training School "Food Digestion and Human Health" (Organized by the COST Action FA1005 INFOGEST at Gdansk - Poland)	20

RESEARCH PERIODS ABROAD

Institution and Place	Tutor	Period
Karolinska Institutet (KI), Department of Medecine Stockholm, Sweden	Dr. P. Arner	01/02/2013-01/03/2013
German Cancer Research Center (DKFZ), Division Epigenomics and Cancer Risk Factors Heidelberg, Germany	Dr. C. Gerhäuser	03/03/2013-31/07/2013

PARTICIPATION AT WORKSHOPS AND CONGRESSES

Type of scientific event/Title	Place	Date
II International Conference FoodOmics: A science for nutrition, health and wellness in a post-genomic era	Cesena (Italy)	22-24 June 2011
Riunione Nazionale della Società Italiana di Nutrizione Umana (SINU): Nutrizione e Rischio Cardiovascolare	Naples (Italy)	12-13 October 2011
Workshop Fresh Fish: Tradizione e consumo, Produzione e ricerca, Commercializzazione e Ristorazione	Cesena (Italy)	02 December 2011
I International Conference on Food Digestion	Cesena (Italy)	19-21 March 2012
XVII Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology	Cesena (Italy)	19-21 September 2012
Traditional Food International 2012: Traditional food from culture, ecology and diversity, to human health and potential for exploitation	Cesena (Italy)	04-05 October 2012
XXXV Congresso Nazionale SINU: LARN - Revisione 2012	Bologna (Italy)	22-23 October 2012
IUNS 20th Interantional Congress of Nutrition	Granada (Spain)	15-20 September 2013
Riunione Nazionale SINU: Comprendere e applicare i LARN	Firenze (Italy)	21-22 October 2013
EFFoST Annual Meeting: Bio-based Technologies in the Context of European Food Innovation Systems	Bologna (Italy)	12-15 November 2013

AWARDS

Type of award	Work presented	Place/Date
PRIZE YOUNG RESEARCHER Supported by SINU	Melasso di canna e di barbabietola: possibili alternative funzionali allo zucchero raffinato	Bologna, 22/10/2013
STUDENT OF THE YEAR AWARD 2013 supported by Cargill and EFFoST	Anti-oxidative and anti-inflammatory effects of whole grain durum wheat and Kamut cookies in HepG2 cells	Bologna, 14/11/2013

LIST OF BUBLICATIONS

- A. Gianotti, F. Danesi, V. Verardo, D.I. Serrazanetti, **V. Valli**, A. Russo, Y. Riciputi, N. Tossani, M.F. Caboni, M.E. Guerzoni, A. Bordoni "Role of cereal type and processing in whole grain *in vivo* protection from oxidative stress" Frontiers in Bioscience 2011, 1(16): 1609-1618

- F. Danesi, F. Ferioli, M.F. Caboni, E. Boschetti, M. Di Nunzio, V. Verardo, **V. Valli**, A. Astolfi, A. Pession, A. Bordoni "Phytosterol supplementation reduces metabolic activity and slows cell growth in cultured rat cardiomyocytes" The British Journal of Nutrition 2011, 106: 540-548

- M. Di Nunzio, **V. Valli**, A. Bordoni "Pro- and anti-oxidant effects of polyunsaturated fatty acid supplementation in HepG2 cells" Prostaglandins, Leukotrienes and Essential Fatty Acids 2011, 85: 121-127

- A. Bordoni, G. Picone, E. Babini, M. Vignali, F. Danesi, V. Valli, M. Di Nunzio, L. Laghi, F. Capozzi "NMR comparison of *in vitro* digestion of Parmigiano Reggiano cheese aged 15 and 30 months" Magnetic Resonance in Chemistry 2011, 49: S61-70

- **V. Valli**, A. Gómez-Caravaca, M. Di Nunzio, F. Danesi, M. Caboni, A. Bordoni "Sugar Cane and Sugar Beet Molasses, Antioxidant-rich Alternatives to Refined Sugar" Journal of Agricultural and Food Chemistry 2012, 60: 12508-12515.

- A. Carnevali, A. Gianotti, S. Benedetti, M.C. Tagliamonte, M. Primiterra, L. Laghi, F. Danaesi, **V. Valli**, M. Ndaghijimana, F. Capozzi, F. Canestrari, A. Bordoni "Role of Kamut® brand khorasan wheat in the counteraction of non-celiac wheat sensitivity and oxidative damage" Food Research International 2014, *in press*, doi: 10.1016/j.foodres.2014.01.065

- F. Danesi, **V. Valli**, S. Elementi, L.F. D'Antuono "The agronomic techniques as determinants of the phenolic content and the biological antioxidant effect of palm-tree kale" Food and Nutrition Sciences 2014, 5: 1-7

- A. Bordoni, L. Laghi, E. Babini, M. Di Nunzio, G. Picone, A. Ciampa, **V. Valli**, F. Danesi, F. Capozzi "The foodomics approach for the evaluation of protein bio-accessibility in processed meat upon *in vitro* digestion" Electrophoresis 2014, *in press*, doi: 10.1002/elps.201300579

- L. Laghi, E. Babini, A. Bordoni, A. Ciampa, F. Danesi, M. Di Nunzio, G. Picone, **V. Valli**, F. Capozzi "Time Domain Measurements and High Resolution Spectroscopy are Powerful Nuclear Magnetic Resonance Approaches Suitable to Evaluate the *In Vitro* Digestion of Protein-rich Food Products" (2013) Magnetic Resonance in Food Science: Food for Thought (J. Van Duynhoven, H. Van As, P.S. Belton, G.A. Webb eds.) pp 201-212, The Royal Society of Chemistry, Cambridge, UK (ISBN: 978-1-84973-634-3)

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