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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 lower 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 in basal condition and after 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 a hormetic effect, eliciting an adaptive response that protects the organism against both oxidative stress and inflammation.

1. 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 (Lutsey, Jacobs, Kori, Mayer-Davis, Shea, Steffen, et al., 2007), hypertension (Wang, Gaziano, Liu, Manson, Buring and Sesso, 2007), metabolic syndrome and type 2 diabetes (Kochar, Djousse, & Gaziano, 2007), and different types of cancer (Haas, Machado, Anton, Silva, & de Francisco, 2009). 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 (Slavin, Jacobs, & Marquart, 2001).

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-

Abbreviations: AACC, American Association of Cereal Chemist; ABTS, 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid); AOPP, advanced oxidation protein product; ARE, antioxidant-responsive element; b.w., body weight; CAP, canonical analysis of principal components; CD, celiac disease; DOX, doxorubicin; FID, free induction decay; GPx, glutathione peroxidase; GSH, glutathione; IBS, irritable bowel syndrome; KP, Kamut® khorasan pasta; MDA, malondialdehyde; NDC, non-digestible carbohydrates; NF-κB, nuclear factor κ-light-chain-enhancer of activated B cells; NMR, nuclear magnetic resonance; Nrf2, nuclear factor erythroid 2-related factor 2; n.s., not significant; ORAC, oxygen radical absorbance capacity; ROM, reactive oxygen molecule; SD, standard deviation; TAA, total antioxidant activity; TE, trolox equivalent; TrxR, thioredoxin reductase; TSP, trimethylsilyl propionate; WG, whole grain; WP, wheat pasta; WS, wheat sensitivity.

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related composition (Wijngaard & Arendt, 2006). 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 (Angioloni & Collar, 2011).

In two recent studies (Benedetti, Primiterra, Tagliamonte, Carnevali, Gianotti, Bordoni, et al., 2012; Gianotti, Danesi, Verardo, Serrazanetti, Valli, Russo, et al., 2011), 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 are 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, Rizzello, Pinto, and Gobbetti (2012) 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 celiac patients at the early stage of the gluten-free diet (Calasso, Vincentini, Valitutti, Felli, Gobbetti and Di Cagno, 2012). Rats were fed modern whole wheat pasta (WP) or ancient whole wheat Kamut® pasta (KP) for 7 weeks, and then half of the 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 (Danesi, Malaguti, Di Nunzio, Maranesi, Biagi and Bordoni, 2006). 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 (Benedetti et al., 2012; Gianotti et al., 2011) 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.

2. Material and methods

2.1. 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.

2.2. Pasta preparation and composition

According to the Italian guidelines of organic pasta processing, the process parameters accounted for a long (more than 6 h) 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 min. 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 contents of the cooked pasta were evaluated according to the standard AACC methods (Koontz, Phillips, Wunderlich, Exler, Holden, Gebhardt, et al., 2005); soluble and insoluble dietary fiber content was estimated according to the method described by Asp et al. (Asp, Johansson, Hallmer, & Siljestrom, 1983). The cooked pasta content of carotenoids, folic acid, vitamin E, Se, and total polyphenols was estimated as described previously (Gianotti et al., 2011).

2.3. 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 h of fasting, rats were anesthetized 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).

2.4. 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×) 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 (Marsh, 1992; Patel, Bradly, McIntire, Giusto, & S., 2009). In the lymph nodes and in the

spleen the morphology and diameter of lymphatic follicles were evaluated.

2.5. 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 min 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 more, the supernatants were combined and their pH was finally adjusted to 7.4 ± 0.02 (Jacobs, Deltimple, van Velzen, van Dorsten, Bingham, Vaughan, et al., 2008). 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 signal overlap in crowded regions, all free induction decays (FIDs) 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 weights and water and fiber contents. 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 (Ihaka & Gentleman, 1996) 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 analyses, 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.

2.6. Analyses in plasma

2.6.1. Total antioxidant activity (TAA)

TAA was measured in plasma using the method of Re, Pellegrini, Proteggente, Pannala, Yang and Rice-Evans (1999), 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.

2.6.2. 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. (2006). 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.

2.6.3. Plasma glucose estimation

Plasma glucose level was determined by the glucose oxidase enzymatic method (Barham & Trinder, 1972). 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.

2.7. Analyses in liver

2.7.1. Liver glutathione peroxidase (GPx) activity

One hundred milligrams of liver was 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 prescribed by the manufacturer (Cayman Chemical Co., Ann Arbor, MI, USA) (Flohe & Gunzler, 1984). Results were adjusted for the protein content in the sample and expressed as units per milligram of protein.

2.7.2. Liver thioredoxin reductase (TrxR) activity

One hundred milligrams of liver was 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 prescribed by the manufacturer (Sigma-Aldrich) (Holmgren & Bjornstedt, 1995). Results were adjusted for the protein content in the sample and expressed as units per milligram of protein.

2.7.3. Intracellular glutathione (GSH) level

One hundred milligrams of liver was 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 prescribed by the manufacturer (Fernández-Checa & Kaplowitz, 1990). Results were adjusted for protein content in the sample and expressed as nanomoles of GSH per milligram of protein.

2.7.4. Liver α-tocopherol and β-carotene content

One hundred milligrams of tissue was 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 (Aebischer, Schierle, & Schuep, 1999). Results for α-tocopherol and β-carotene were expressed as micrograms per gram of tissue.

2.7.5. Liver malondialdehyde (MDA) level

One hundred milligrams of tissue was homogenized in 1 mL of cold buffer (0.25 M Tris, 0.2 M sucrose, 5 mM 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 (Agarwal & Chase, 2002). Results were adjusted for protein content in the sample and expressed as nanomoles of MDA per milligram of protein.

2.7.6. Liver advanced oxidation protein product

One hundred milligrams of tissue was 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 (Witko-Sarsat, Friedlander, Capeillere-Blandin, Nguyen-Khoa, Nguyen, Zingraff, et al., 1996). Results were adjusted for protein content in the sample and expressed as nanomoles per milligram of protein.

2.7.7. Protein concentration

The protein concentration in all samples was determined according to the method of Bradford (1976).

Table 1
Composition of the experimental pasta.

	Wheat pasta (WP)	Kamut® pasta (KP)
Energy (kcal/100 g)	174 ± 12	190 ± 14
Energy (kJ/100 g)	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 acid)	20.1 ± 0.94	23.4 ± 1.0 *

The composition of the experimental pasta was determined as reported in Subsection 2.2. Statistical analysis was by Student's *t* test.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

2.8. 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 (Bauer, 1972). Canonical analysis of principal components (CAP) (Anderson & Willis, 2003) 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 Student's *t* test and the one-way ANOVA using Tukey's as post-test.

3. 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 pastas: 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).

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

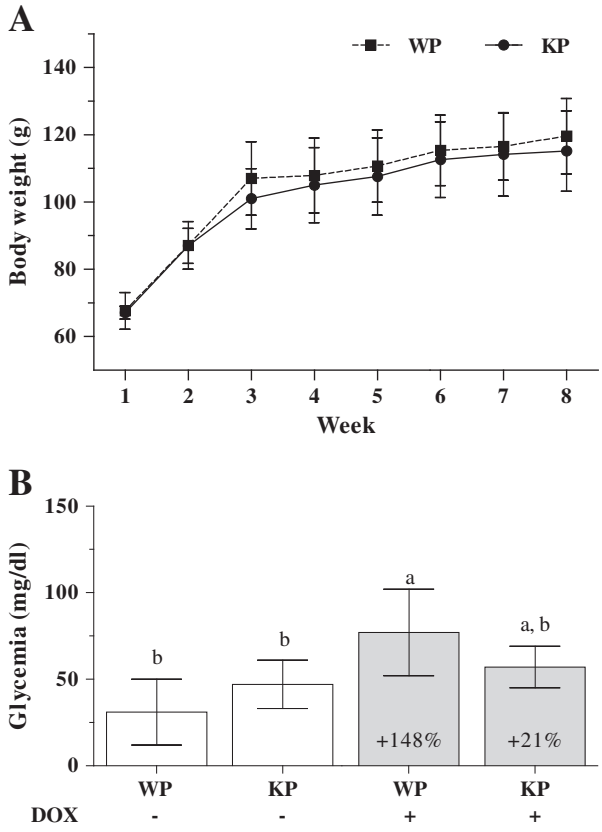

Fig. 1. Body weight gain (panel A) and glycemia (panel B) in rats fed the two experimental diets. Body weight was measured once a week during the dietary treatment. Data are means ± SD of 12 rats in each group. Glycemia was measured in basal (white bars) and stressed (gray bars) conditions as described in Subsection 2.6.3, and is reported as mean ± SD of 6 rats in each group. Statistical analysis was performed by 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.

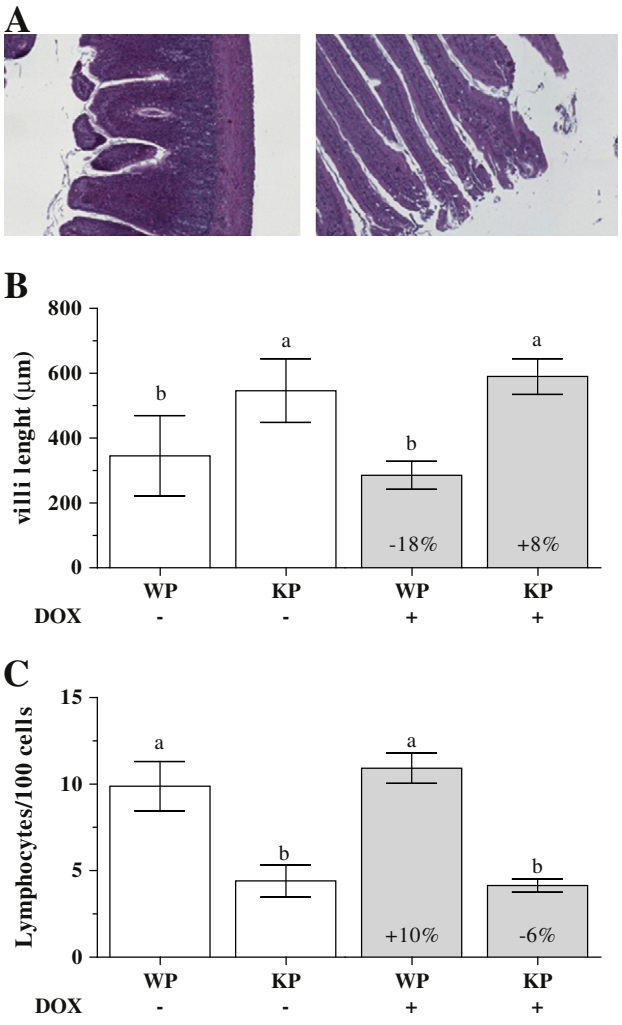

Fig. 2. Histological evaluation of the duodenum in rats fed the different experimental diets. In panel A, the different morphology of duodenum in WP (left side) and KP (right side) is reported. Villi length (panel B) was measured in basal (white bars) and stressed (gray bars) conditions as reported in Subsection 2.4. Lymphocyte infiltration of the mucosa (panel C) was evaluated in basal (white bars) and stressed (gray 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 õstatistical significance (at least $p < 0.05$). In stressed condition percent variations of the corresponding basal values are reported within the corresponding bar.

observed between the two dietary groups (Fig. 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 (Fig. 1B).

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 (Fig. 2A), confirmed by the measurement of the villi length (Fig. 2B). Furthermore, a higher lymphocyte infiltration was observed in WP animals (Fig. 2C).

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

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

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

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

Liver MDA and AOPP levels were also comparable in the two experimental groups in basal conditions (Fig. 7A and B, 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.

A typical ^{1}H NMR spectrum obtained on the fecal masses analyzed during the present investigation is represented in Fig. 8, together with the assignments of the main peaks, obtained through comparisons with the literature (Jacobs et al., 2008) and with the addition of pure compounds to the samples.

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 Fig. 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).

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 Fig. 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

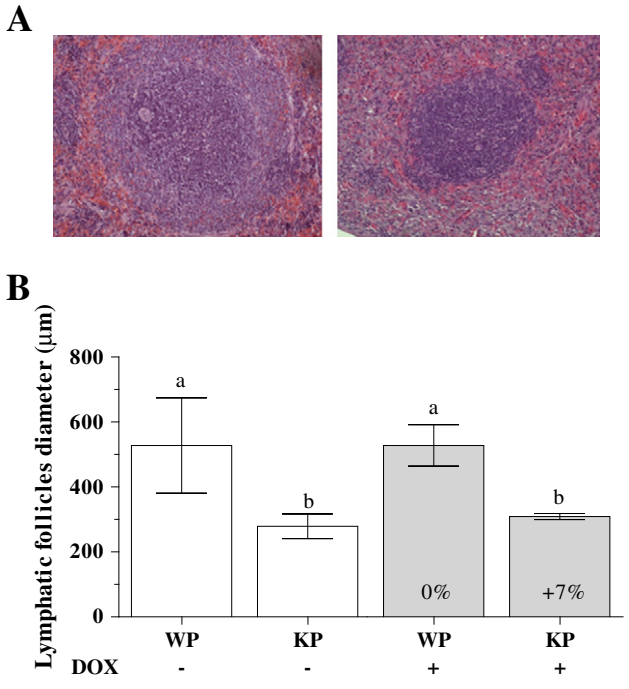

Fig. 3. Histological evaluation of the spleen and lymph nodes in rats fed the different experimental diets. In panel A, the different morphology of the spleen lymphatic follicles in WP (left side) and KP (right side) is reported. In these images the different thickness of the mantle around the follicles, 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 (gray bars) conditions as reported in Subsection 2.4 (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.

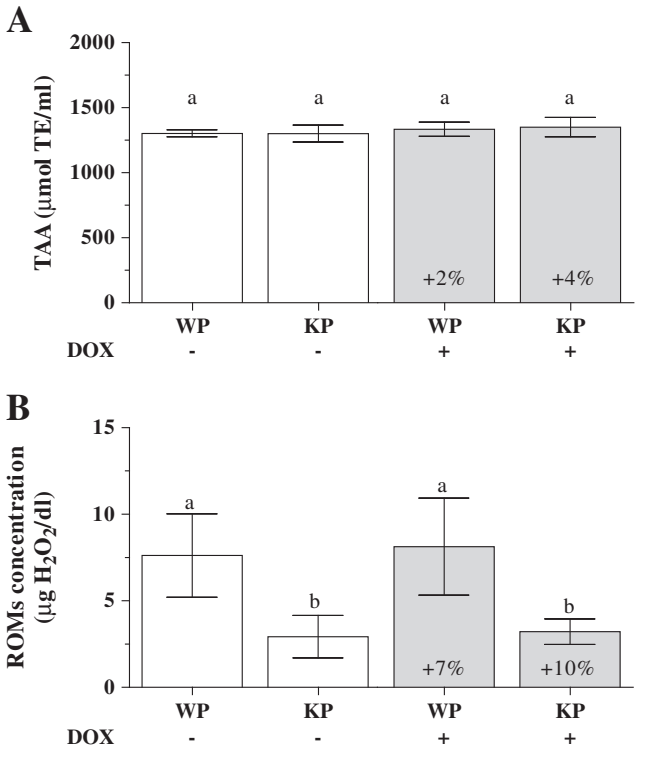

Fig. 4. Plasma TAA and ROM concentrations in rats fed the different experimental diets. The plasma TAA (panel A) and ROM concentrations (panel B) were measured in basal (white bars) and stressed (gray bars) conditions as reported in Subsections 2.6.1 and 2.6.2. Data are means ± SD of 6 rats in each group. 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.

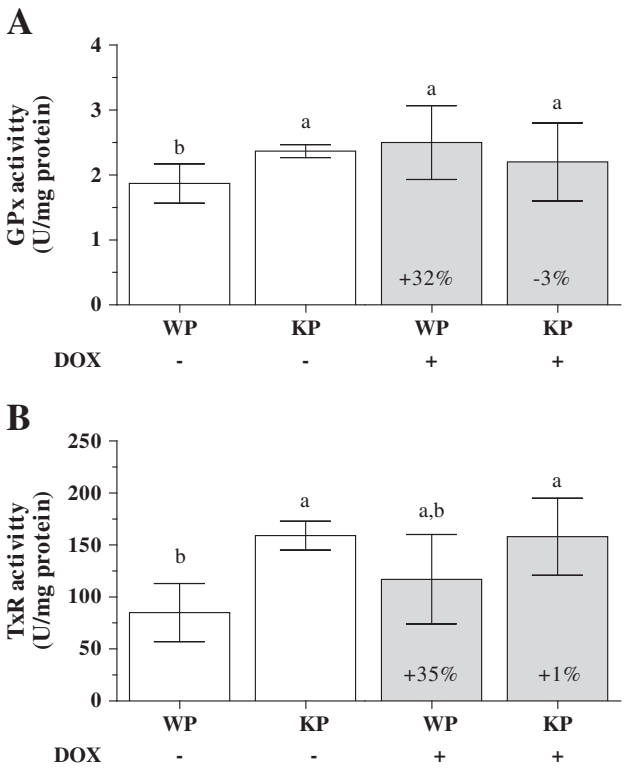

Fig. 5. Liver GPx (A) and TrxR (B) activities in rats fed the different experimental diets. The antioxidant enzyme activity in liver of rats fed the experimental diets was measured in basal (white bars) and stressed (gray bars) conditions as reported in Subsections 2.7.1 and 2.7.2. Data are means ± SD 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.

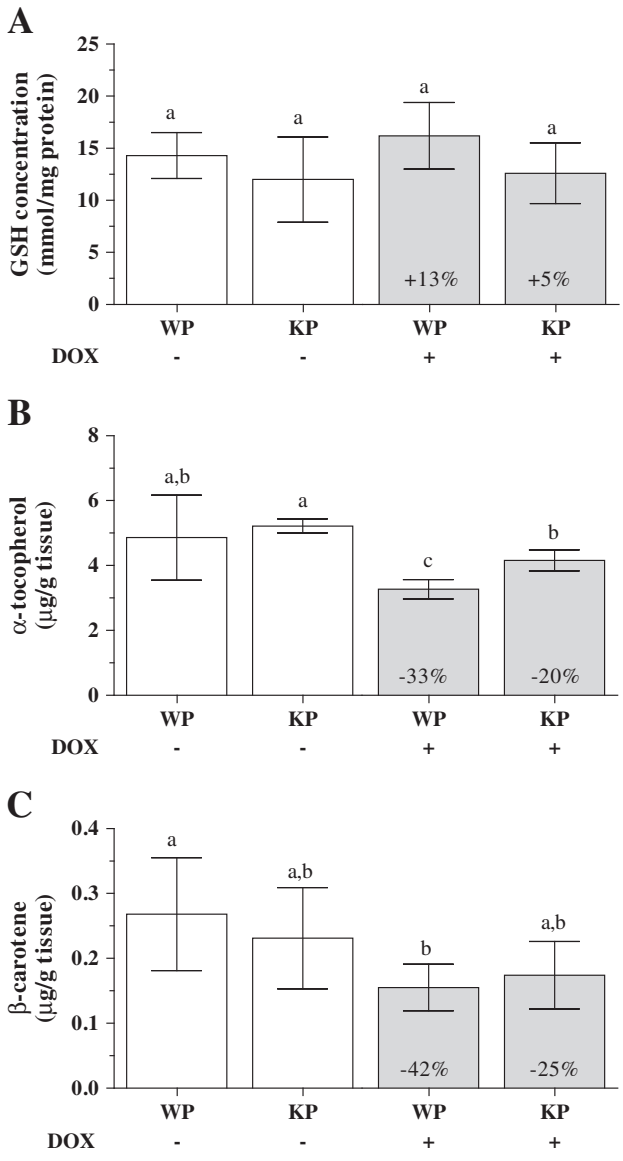

Fig. 6. Liver GSH, α-tocopherol, and β-carotene concentrations in rats fed the different experimental diets. Liver GSH (A), α-tocopherol (B), and β-carotene (C) concentrations measured in basal (white bars) and stressed (gray bars) conditions as reported in Subsections 2.7.3 and 2.7.4. Data are means ± 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.

type; and 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. Fig. 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.

4. Discussion

Celiac disease (CD) prevalence is estimated to be near to 1:100 in Western countries (Green & Cellier, 2007). 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 (Lundin & Alaedini, 2012). Recently, Carroccio, Mansueto, Iacono, Soresi, D'Alcamo, Cavataio, et al. (2012) 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 (Shepherd, Parker, Muir, & Gibson, 2008).

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 and Gregorini (2012) 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

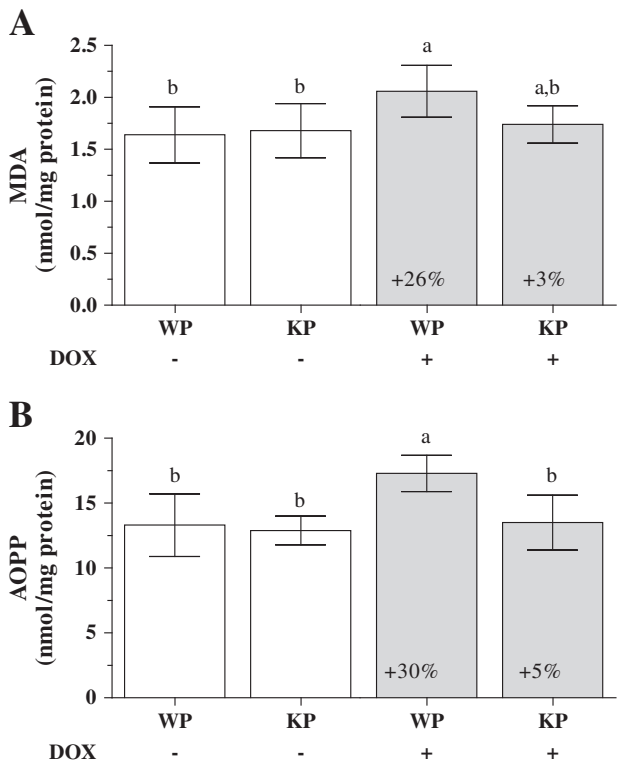

Fig. 7. Liver MDA and AOPP levels in rats fed the different experimental diets. Liver MDA (A) and AOPP (B) levels were measured in basal (white bars) and stressed (gray bars) conditions as reported in Subsections 2.7.5 and 2.7.6. Data are means ± SD of 6 rats in each group. Statistical analysis was performed 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.

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 (Lee & Johnson, 2004). 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 (Vauzour, 2012).

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 h 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 (Skulas-Ray, Kris-Etherton, Teeter, Chen, Vanden Heuvel and West, 2011).

Notably, the induction of GPx, also related to the higher selenium content in Kamut® pasta, has been reported to also inhibit inflammation (Brigelius-Flohe & Kipp, 2012).

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 do 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 (Pimentel & Chang, 2011), and host–microbe dialog 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 (Shanahan, 2011). WG cereals provide non-digestible carbohydrates (NDC) that can be fermented by the gut microbiota and act as a prebiotic; existing studies assess the effects of wheat-derived NDC on parameters related to gut bacterial metabolism and/or in obesity and glucose homeostasis (Neyrinck & Delzenne, 2010), and the primacy of

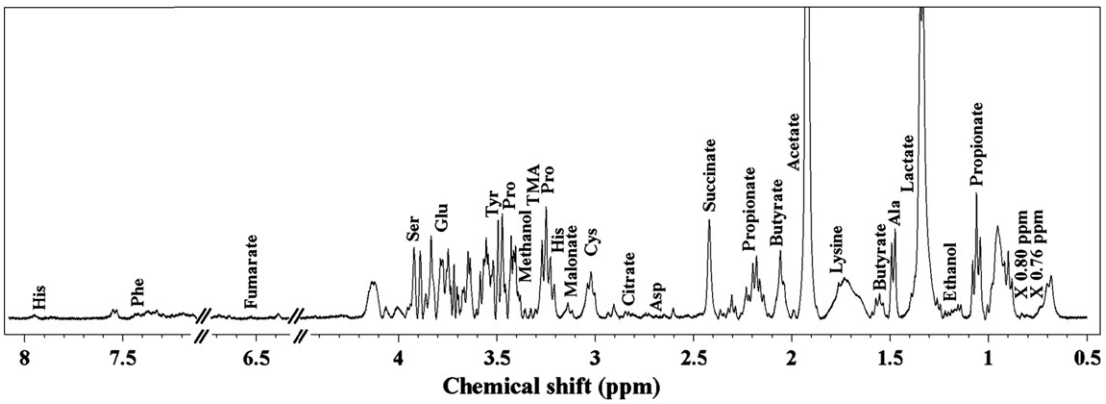

Fig. 8. A typical ^{1}H NMR spectrum obtained during the present investigation. The suggested assignments are based on the literature and/or by adding pure standard compounds.

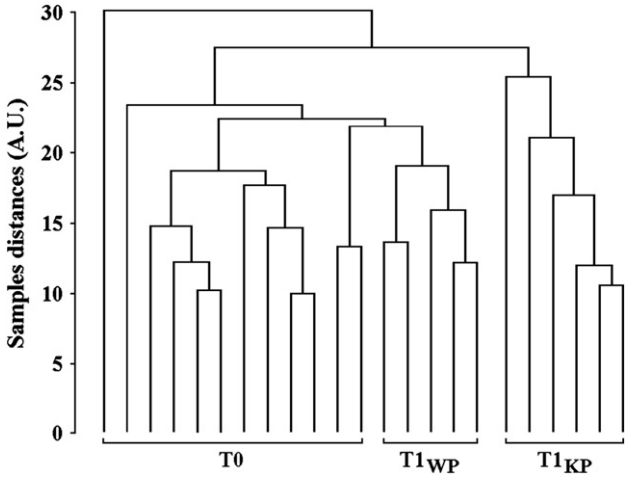

Fig. 9. Dendrogram obtained by cluster analysis based on Euclidean distance, using gut metabolites before (T0) and after the dietary intervention ($T1_{WP}$ and $T1_{KP}$ respectively).

environmental or lifestyle factors linked to changes in the gut microbiota in the development of inflammatory bowel disease, is increasingly evident (Shanahan, 2012). 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 (Macfarlane & Macfarlane, 2003).

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 amounts of acetate and propionate (Maslowski, Vieira, Ng, Kranich, Sierro, Yu, et al., 2009).

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 (2012), 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 seem to play a master role, indicating substantial differences between whole-grain durum and whole-grain Kamut® wheat. It is important to point up 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 a hormetic effect, eliciting an adaptive response that protects the organism against both oxidative stress and inflammation (Calabrese, Cornelius, Dinkova-Kostova, Iavicoli, Di Paola, Koverech, et al., 2012). This underlines the complexity of the nteraction 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 (Capozzi & Bordoni, 2013).

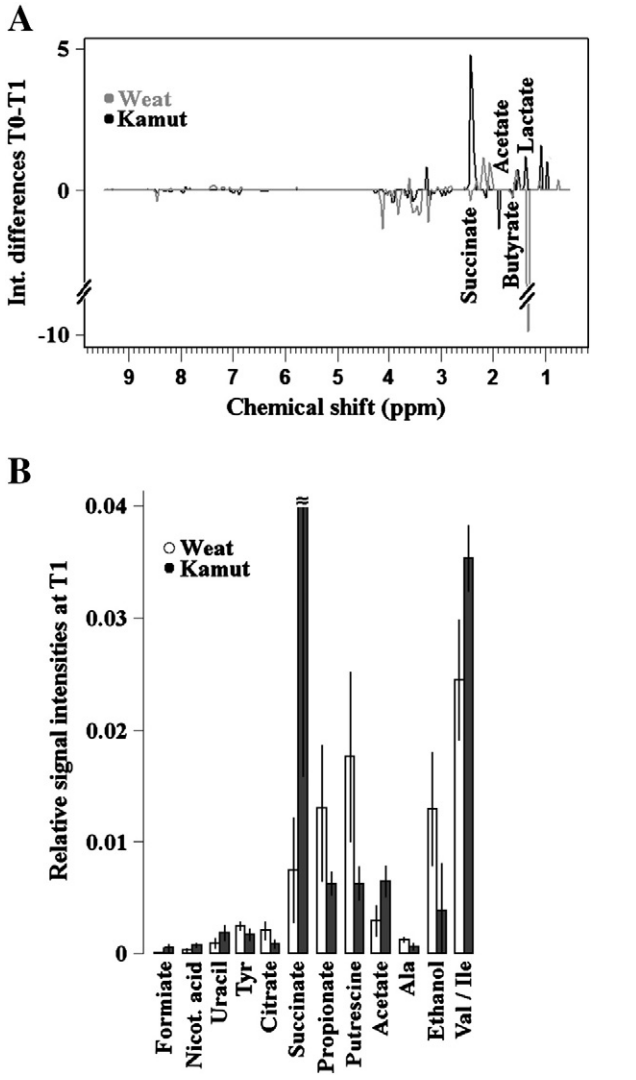

Fig. 10. A) Differences between the ^1H NMR spectra registered at T0 and T1 on the WP (light gray) and KP (dark gray) samples. B) Relative signal intensities registered at T1 on WP (white) and WP (dark gray) samples.

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