Phenolic content and antioxidant activity of wholegrain breads from modern and old wheat (*Triticum aestivum* L.) cultivars and ancestors enriched with wheat sprout powder

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Abstract

In this study, we compared nutritional characteristics of breads from wholegrain flours of three modern cultivars, four old cultivars and one landrace population of common wheat (*Triticum aestivum*), one Khorasan (*Triticum turgidum* var *turanicum*) accession and one einkorn (*Triticum monococcum*) cultivar. One bread from refined flour was also included. All flours were enriched or not with 5% (w:w) of wheat sprout powder (WSP) to obtain enriched breads (EB) or control breads (CB), respectively. Total phenolics and flavonoids, reducing power, radical scavenging and thiobarbituric acid reactive substances production inhibition were measured on bread aqueous extracts. CB from old cultivars were generally higher in phenolic content and antioxidant activity than CB from modern cultivars. All EB showed increased phenolic content and antioxidant activity compared to CB, but the increase varied with the source flour, despite WSP supplementation was the same for all breads. In particular, the increase in phenolic content was much relevant for EB of modern cultivars.

Introduction

Bread is a basic food in human diet. In developed countries bread is mainly made with refined flour, which is low in fibre, bioactive compounds and antioxidants (Dziki et al., 2014), because these compounds are mainly present in the bran. Moreover, modern wheat cultivars have been obtained with breeding programs principally aimed at increasing yield, while neglecting bioactive compounds (Leoncini et al., 2012). Today, there is a growing interest towards foods high in bioactive compounds for their benefits in human health such as free radical scavenging properties or anticancer activity (Dziki et al., 2014). In this regard, old wheat cultivars are more and more reconsidered (Ghiselli et al., 2016). Leoncini et al. (2012) found that grains of Verna and Gentil Rosso were higher in phenolic compounds and antioxidant activity compared to grains of a modern cultivar and Migliorini et al. (2016), reported that breads from old wheat varieties (e.g., Verna, Gentil Rosso) were particularly appreciated by consumers. There is also an interest towards ancient *Triticum* species. It is the case of the Khorasan (*Triticum turgidum* var *turanicum*) Jakubz, which is rich in carotenoids like lutein and zeaxanthine, both involved in eye health (Abdel-Aal and Rabalski, 2013). Similarly, einkorn (*Triticum monococcum* L.) is rich in antioxidant compounds like bound phenolics (Benincasa et al., 2015), carotenoids, tococols, alkylresorcinols and phytosterols (Hidalgo and Brandolini, 2014). For these old cultivars, milling procedures often include the bran in the flour, with well known health benefits (Dziki et al., 2014), and single-cultivar products represent a sort of brand with a relevant niche-market. Actually, single-cultivar products are expected to have standard traits, although the environment (i.e., the location and season weather) may strongly affect the outcome. In fact, when comparing flours and their products, source grains should come from the same environment and cultivation condition. Moreover, to meet consumers’ expectation for this kind of niche products, the grains should better be obtained from crops grown organically.

An alternative means to improve the nutritional value of bread is represented by the supplementation of flours with plants extracts and plant parts (Dziki et al., 2014), including sprout powder (Gawlik-Dziki et al., 2014, 2017; Świeca et al., 2017). Sprouts, i.e., the young seedlings of many species are a new trend of healthy food. In particular, sprouts from *Triticum* species have been found to be high in bioactive compounds (e.g., phytochemicals, phospholipids, reducing glycosides, and low molecular weight peptides) (Calzuola et al., 2004;ucci et al., 2013; Benincasa et al., 2015; Falcinelli et al., 2017; Stagnari et al., 2017).
2017) having antioxidant, antimutagenic, and anti-ageing properties (Falcioni et al., 2002; Calzuola et al., 2004; Amici et al., 2008; Lucci et al., 2013). Sprouts are generally produced homemade or for ready-to-eat market, but can also be dried and ground to a fine powder to be used as supplements. The use of wheat sprout powder (WSP) to enrich wheat flours has been proposed recently (Žilić et al., 2014; Gawlik-Dziki et al., 2017; Świeca et al., 2017). In particular, Gawlik-Dziki et al. (2017) replaced a refined wheat flour with 5, 10, 15, 20% (w/w) of WSP and observed an increase of phenolics in breads, although they warned that the supplementation of WSP at high concentration may negatively influence the baking properties (Abderrahim et al., 2012). The WSP supplementation may be expected to have different nutritional effects on breads depending on the source flour, i.e., refined vs wholegrain and modern vs old varieties.

Thus, the aim of this study was to investigate the effect of WSP supplementation on the nutritional and sensory characteristics of breads obtained from wholegrain flours of modern and old cultivars of common wheat and ancestors, grown organically in the same field trial.

Materials and methods

Provenance of grains used for breads

Ten wholegrain flours from single-cultivar grains were compared (Table 1): Bologna, Blasco, Bolero (modern *Triticum aestivum* cultivars), Abbondanza, Gentil Rosso, San Pastore, Verna (old *T. aestivum* cultivars), Biancola (*T. aestivum* landrace population), Khorasan (*Triticum turgidum var. turanicum*), and Einkorn (*Triticum monococcum*, cv. Monlis). A Bologna refined flour (Bologna-REF) was also included as control. The seeds of most varieties were provided by the Associazione Italiana Agricoltura Biologica (AIAB). Seeds of the cultivar Monlis were provided by Prometeo s.r.l. (Canavaccio, Pesaro Urbino, Italy). All varieties were cultivated in the year 2014-2015 in an organic field trial located in S. Andrea di Agliano-PG-Italy (43°02’N, 12°24’E, 127 m a.s.l.) under the control of the Department of Agricultural, Food and Environmental Sciences of University of Perugia. The field experimental design was a randomised block with four replicates. Each experimental unit (plot) consisted of 8 rows 6 m long. Fertilisation was carried out with manure (30 kg N/ha) applied at tillering. Each cultivar was harvested when it reached complete maturity.

Production of wheat sprout powder and breads

The sprouts used for producing the WSP were supplied by NPP s.r.l., a spin-off company of the University of Perugia (Italy). Sprouts were obtained from grains of the cultivar Bologna following the method reported by Calzuola et al. (2004). Grains were sown on sterilised soft agar (0.8-1%) in plastic boxes for food and placed in a growth chamber at 20°C. Sprouts were collected 5 days after sowing, with the shoots about 5 cm long, they were dehydrated under a gentle and continuous flow of dehumidified air at 30°C for 48 h and powdered. The WSP was stored under vacuum at room temperature and used to enrich breads.

The control bread (CB) dough of each *Triticum* treatment was prepared by mixing 500 g of flour with 10 g of salt, 10 g of sugar, 5 g of yeast and 300 mL of water. The enriched bread (EB) dough was prepared with the same recipe but replacing 25 g of flour with 25 g of WSP. Each dough was kept at 4°C for 12 h and baked in oven at 200°C. After 3 h, breads were cut into slices and a representative subsample of 50 g was dried at room temperature for 48 h and finely ground for aqueous extraction and chemical analyses, which were performed in triplicate.

Aqueous extraction

Aqueous extractions were achieved following the method of Calzuola et al. (2004). The WSP and ground CB and EB were mixed with distilled water (1:5 w/v) and homogenised five times by a mixer, alternating 30 s of homogenisation and 30 s pause to prevent heating. Extracts were then centrifuged at 7000 rpm for 30 min at 4°C and the supernatants were stored at −20°C in small aliquots. Before the chemical analysis, aliquots were de-frozen and centrifuged at 10,000 rpm for 10 min at room temperature.

Total phenolic and flavonoid contents

The measurement of total phenolic content (TPC) was performed with phosphomolybdic-phosphotungstic acid reagent, according to the method of Singleton and Rossi (1965). 20 µL of extracts were mixed with 50 µL of Folin-Ciocalteu reagent, 100 µL of 20% Na₂CO₃ (w/v), 830 µL of deionised water and then incubated for 30 min at room temperature. For each sample, a blank control was prepared replacing the Folin-Ciocalteu reagent with the same volume of water. Absorbance was read at 760 nm by a spectrophotometer (Varian Cary 100 Scan) and values were evaluated as the difference between samples and their own blank control. The measured values were compared by using a calibration curve obtained with different concentrations of gallic acid as standard, and expressed as milligrams of gallic acid equivalents per grams of powder (mg GAE g⁻¹). The measurement of the flavonoid content

Table 1. Details on the *Triticum* cultivars and accessions used in this study.

<table>
<thead>
<tr>
<th>Species</th>
<th>Ploidy</th>
<th>Variety/accession</th>
<th>Modern/old</th>
<th>Year of release</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Triticum aestivum</em> L.</td>
<td>Hexaploid</td>
<td>Bologna</td>
<td>Modern</td>
<td>2002</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Blasco</td>
<td>Modern</td>
<td>2002</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bolero</td>
<td>Modern</td>
<td>1990</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Abbondanza</td>
<td>Old</td>
<td>1930</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gentil Rosso</td>
<td>Old</td>
<td>1911</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Biancola</td>
<td>Landrace</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>San Pastore</td>
<td>Old</td>
<td>1929</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Verna</td>
<td>Old</td>
<td>1953</td>
</tr>
<tr>
<td><em>Triticum turgidum var. turanicum</em> Jakube</td>
<td>Tetraploid</td>
<td>Khorasan</td>
<td>Ancestor</td>
<td>-</td>
</tr>
<tr>
<td><em>Triticum monococcum</em> L.</td>
<td>Diploid</td>
<td>Monlis</td>
<td>Ancestor</td>
<td>-</td>
</tr>
</tbody>
</table>

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(FC) was performed by the colorimetric (aluminium chloride) method (Chang \textit{et al.}, 2002). Each extract (50 µL) was mixed with 450 µL of distilled water and 30 µL of 5% sodium nitrite (NaNO₂) and incubated at room temperature for 5 min. Aluminium chloride (AlCl₃) 1% (300 µL) was added, and the mix was incubated for 6 min at room temperature. Sodium hydroxide (NaOH) 1 M (200 µL) was finally added and the absorbance of all samples was read at 510 nm wavelength. The values were computed by utilising the average of the dose-responses obtained with quercetin as standard, and expressed as milligrams of quercetin equivalents per grams of powder (mg QE g⁻¹).

**Reducing power**

The reducing power (RP) of extracts was measured using potassium ferricyanide as reagent, following the method of Yen and Chen (1995). An aliquot of 250 µL of extract was mixed with an equal volume of 0.2 M phosphate buffer, pH 6.6 and 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min. Then, an equal volume of 1% trichloroacetic acid was added and the mixture was centrifuged at 6000 rpm at room temperature for 10 min. The upper layer of solution was mixed with distilled water and 0.1% FeCl₃, at a ratio of 1:1:2 and the absorbance was measured at 700 nm. The RP was expressed as µmol of reduced potassium ferricyanide, which correspond to a half of optical density (700 nm), according to Calzuola \textit{et al.} (2004).

**Superoxide radical scavenging assay (nitrotetrazolium blue chloride assay)**

The measurement of superoxide radical scavenging (RS) activity was carried out according to Kirby and Schmidt (1997). An aliquot of 25 µL of 15 mM Na₂EDTA in buffer (50 mM KH₂PO₄/KOH, pH 7.4), 63 µL of 0.6 mM nitrotetrazolium blue chloride (NBT) in buffer, 38 µL of 3 mM hypoxanthine in 50 mM KOH, 62.5 µL of extract, and 137.5 µL of buffer were mixed in 96-well microplates (Falcon). In the blank, the extract was replaced by an equal volume of water. The reaction was started by adding 25 µL of xanthine oxidase solution in buffer (1 unit in 10 mL of buffer) to the mixture. The reaction mixture was incubated at 25°C and the absorbance at 570 nm was determined every 1 min in the first 5 min and then every 5 min until 60 min using a plate reader (Labsystems Multiskan MS). The RS activity was measured as inhibition of NBT reduction. Superoxide can reduce NBT to formazan, which is able to extract all hydrophilic antioxidants, vitamins and peptides included (Calzuola \textit{et al.}, 2004), which may react with the Folin-Ciocalteu reagent (Singleton \textit{et al.}, 2004), which may react with the Folin-Ciocalteu reagent (Singleton \textit{et al.}, 1999). Actually, water extraction is considered biologically meaningful, since human feeding usually implies water extracts. Moreover, water is non-toxic and gives advantages for certification and safety (Wong \textit{et al.}, 2005). The FC values of our breads were much lower than in WSP, except for the TPC of Gentil Rosso, which reached 2 mg g⁻¹. Among CB, old \textit{Triticum} cultivars and accessions had TPC values generally higher than modern cultivars: besides Gentil Rosso, also Abbondanza, Khorasan and Biancola showed TPC over 1 mg GAE g⁻¹ of bread.

Inhibition % (I %) of oxygen superoxide =100-(A x 100)       (1)

where A is the absorbance of the sample and A₀ is the absorbance of control.

**Inhibition of thiobarbituric acid reactive substances production**

The capacity of extracts to inhibit the production of thiobarbituric acid reactive substances (TBARS) from degradation of sodium benzoate was evaluated following the method developed by Koracevic \textit{et al.} (2001). Such inhibition was calculated as the difference of activity between the sample (S₁) and its blank control (S₀). A standardised solution of Fe-EDTA complex was prepared mixing equal volumes of 2 mM EDTA and 2 mM FeCl₃ and incubating it for 1 h at room temperature. An aliquot of 100 µL of extract was incubated at 37°C for 1 h in the presence of 400 µL of 0.1 M sodium phosphate buffer pH 7.4, 500 µL of 10 mM sodium benzoate, 200 µL of Fe-EDTA complex, 200 µL of 10 mM H₂O₂. In the reference control, the extract was replaced by an equal volume of the same sodium phosphate buffer. Acetic acid 20% (1 mL) was added prior incubation to the S₁ samples. After incubation, 1 mL of 20% acetic acid was added to samples S₁, and 1 mL of 0.8% thiobarbituric acid in 50 mM NaOH was added to all samples. Samples were then incubated at 100°C for 10 min and then cooled in ice, and the absorbance was read at 532 nm wavelength. S₀ readings were detracted from S₁ readings and resulting values (A) were expressed as percentage of inhibition of TBARS production as compared to the reference control (A₀).

Inhibition % (I %) of TBARS production =100-(A x 100)       (2)

Statistical analysis

All chemical analyses were carried out in triplicate for one sample of each bread. Data were subjected to analysis of variance and means were compared by using Fisher’s LSD.
substantially in line with the FC level of our CB although none included old wheat cultivars and accessions, most used organic extractions, some detected only single flavonoid compounds like quercetin and rutin (Lin et al., 2009), and some used a different standard (i.e., catechin) (Iraki et al., 2015). A significant difference was recorded between the refined and wholegrain CB of Bologna, confirming results obtained for other cultivars by Yu et al. (2013).

WSP-enriched breads (EB) showed a general increase of both TPC (Figure 1A) and FC (Figure 1B) compared to CB, with few exceptions for FC (Gentil Rosso and Biancola, where there was no change, and Einkorn, where there was a slight decrease). The expected general increase of phenolic compounds in EB confirms evidences from Gawlik-Dziki et al. (2017), who also supplemented wheat flour with 5 to 20% (w:w) of WSP, and from Gawlik-Dziki et al. (2014) who replaced wheat flour with 1% to 5% of broccoli sprout powder. In both works, these authors found that the increase of phenolic compounds was not proportional to the ratio of WSP supplementation and that replacing flour with 5% (w:w) of sprout powder represented a good compromise to increase phenolic content without worsening baking and sensory characteristics of breads. Changes in absolute values of TPC and FC from CB to EB varied with the treatment, despite WSP supplementation was the same for all breads. In particular, the increase of TPC was much relevant for EB of modern cultivars. The increase of both TPC and FC values from CB to EB was much relevant for Bologna refined, which reached values comparable or even higher than CB from most cultivars. Thus, the effect of WSP supplementation appears not strictly additive. Several factors may come into play to explain this, in particular the interaction between the phenolic compounds of the WSP, the phenols present in the flour, the other components of the flour and the thermal treatment. Turkmen et al. (2005) demonstrated that phenols in plant tissues may decrease or even increase after a thermal treatment, depending on the matrix. In fact, the thermal treatment may cause: i) the release of antioxidants by destruction of cell and subcellulars walls; ii) the production of new radical scavenging antioxidants from thermal chemical reactions; iii) the inactivation of oxidative enzymes suppressing antioxidants; and iv) the formation of novel antioxidant compounds (i.e., Maillard reaction products) (Jiménez-Monreal et al., 2009). For these reasons, it has been reported that the effects of thermal processing depend on polyphenols concentration and their chemical structure, oxidation level, localisation in the cell, interaction with other food components and type of thermal process applied (van Boekel et al., 2010). Similar comments can be addressed for the increase of FC in EB. The higher increase of TPC in EB from modern cultivars and even from Bologna-REF is relevant, because it makes WSP supplementation a powerful tool to improve nutritional quality of wheat cultivars which are more agronomically suitable and productive and of white breads which are traditionally widespread and available in the market.

Antioxidant activity of breads

In CB, either the reducing power (RP) (Figure 2) or the radical scavenging (RS) (Figure 3) or the TBARS production inhibition (Figure 4) were generally higher in old/ancient Triticum cultivars/accessions than in modern cultivars, with Gentil Rosso and Abbondanza showing the highest values for all the three assays. Khorasan and Biancola showed high values for RP and TBARS but not for RS. Bologna-REF did not differ much from Bologna. The high values of RP, RS and TBARS of breads from old cultivars is in line with data reported by Leoncini et al. (2012) for the grains, and the lack of difference between the refined and
wholegrain bread of Bologna is in line with results by Yu et al. (2013), who tested five different wheat genotypes. Of course, the thermal treatment caused by baking will have caused changes in antioxidants, but there is no reference to make a comparison. However, based on results from Yu et al. (2013), baking is expected to reduce antioxidant activity by 20-40% depending on wheat cultivars. The literature on breads reports antioxidant activities mainly evaluated with FRAP, DPPH and ABTS methods, which generally test same antioxidant properties and give comparable results (Thaipong et al., 2006). Differently, our three methods analyse different antioxidant properties, i.e. the ability of a sample to reduce Fe3+ (RP) (Yen and Chen, 1995), or to scavenge the superoxide (O2–) (RS) (Kirby and Schmidt, 1997) or the hydroxyl radical (OH–) (TBARS) (Koracevic et al., 2001). Thus, our three tests give a broad picture of the bread antioxidant activity. The substantial agreement of the three tests for CB indicates that all the antioxidant pool of source grains contribute to the overall antioxidant activity of breads. In particular only the RP of CB was correlated with the phenolic compounds (R2=0.83 for TPC and 0.67 for FC), which means that other antioxidant compounds are involved in the scavenging of superoxide (RS) and hydroxyl radical (TBARS).

All EB showed increased antioxidant activities compared to CB. In particular, the RP was much higher in EB of Gentil Rosso, Abbondanza, Biancola and Khorasan than in the other cultivars (Figure 2), while, unexpectedly, TBARS production inhibition of EB from these four cultivars showed lower values than EB from other several cultivars, including the modern ones (Figure 4). The RS was not much different among EB treatments (Figure 3). In general, the highest relative increases of antioxidant activities were recorded in those treatments having the lowest values for CB, in particular the modern cultivars. The different trends of the three tests for the EB indicates the substantial effect of WSP supplementation in enriching breads with a different pool of antioxidant molecules. Calzuola et al. (2004) reported several low-molecular weight compounds in wheat sprouts having antioxidant activity, like polyphenols, peptides, reducing glycosides, and found that many of them are resistant to heating. The non-additive effect of WSP supplementation for the different breads has to be interpreted in light of the above said interaction between the WSP components and the thermal treatment of baking. The most relevant result of this work is that the increase in bread antioxidant activity due to the addition of WSP was higher for breads having low antioxidant activity. Therefore, WSP supplementation may represent a valuable means to improve the nutritional value of breads even in case of low quality source grains.

**Conclusions**

Results indicate that, in many cases, breads from old cultivars and accessions had higher phenolic content and antioxidant activity than breads from modern cultivars, but differences were not always relevant. All wheat-sprout-powder enriched breads showed higher phenolic content and antioxidant activity compared to control breads, but the increase varied with the source flour, despite the wheat sprout powder supplementation was the same for all breads. In particular, the increase of total phenolic content was much relevant for enriched breads of modern cultivars. Overall, enriching breads with 5% of wheat sprout powder may represent a valuable means to increase the nutritional value of breads and their economic value in the healthy food market.

**References**


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