Barley and Emmer improve redox status and modulate some immune parameters in rats

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Abstract
Barley and Emmer contain many antioxidant compounds, but their biological effects in vivo, particularly at the level of redox status and immune functions have not been investigated. The objective of this study is to evaluate the physiological responses of some immunological and redox parameters to dietary barley and emmer flours in a...
rat model. Experimental diets, one rich in barley and the other in emmer flour (53%) have been fed for 6 weeks to growing male rats. After the experimental period total polyphenols content, total antioxidant capacity and protein carbonyl concentration were measured in plasma. Lymphocytes were isolated from mesenteric lymphnodes and cellular proliferation and T-cell subpopulations were analyzed. The barley and emmer flours showed elevated phenolic contents, induced elevated plasmatic antioxidant capacity and low oxidative status. The proliferative responses and the CD4+/CD8+ ratios of mesenteric lymphocytes of rats fed with barley and with emmer were also elevated without significative differences between them.

Parole chiave: orzo, farro, linfociti mesenterici, stato redox, ratti.
Key words: barley, emmer, mesenteric lymphocytes, redox status, rats.

Introduction
Minor cereals, such as emmer and barley, which have been very much utilized in the past and currently are underutilized, have now attracted the food industry and the consumers because of their organoleptic and nutritional characteristics, not only for the rediscovery of more traditional and natural foods, but also for the desire of healthy and equilibrated diets. Barley is rich in soluble dietary fiber such as beta glucan which has been associated with advantageous physiological effects: it reduces plasmatic cholesterol, lowers the postprandial blood glucose and insulin responses and selects favourable intestinal microflora (Bourdon, Yokoyama, Davis et al., 1999; Dongowski, Huth, Gebhardt, & Flamme, 2002). Diets containing barley significantly reduce lipids in mildly hypercholesterolemic men and women (Behall, Scholfiend, & Hallfrisch, 2004). The barley kernel contains many antioxidant compounds, mostly phenolic acids and vitamin E, that may improve the antioxidant status and related physiological functions (Zdunczyk, Flis, Zielinski et al., 2006). Among these functions immune activity and its regulation has been scarcely investigated. A recent study in mice showed that fermented barley extracts suppress the development of atopic dermatitis like skin lesions probably by inhibiting the production of inflammatory cytokines such as TNF gamma or IL-17 (Iguchi, Kawata, Watanabe, Mazumder, & Tanabe, 2009).

Emmer (Triticum dicoccum) is one of the three hulled wheat species cultivated mainly in regions of central Italy and known in Italy with the term of “Farro”.

The other two species are spelt (Triticum spelta), largely cultivated in Germany, Belgium, Austria, Slovenia and north Italy and einkorn (Triticum monococcum) cultivated in small mountain areas of Italy and France (Coda, Nionelli, Rizzello et al., 2009).

Emmer is rich in resistant starch, minerals and some vitamins (niacine and panthotenic acid) and it has been suggested that it should be included in the category of functional foods (Bonafaccia, Galli, Francischi et al., 2000). Studies on functional physiological properties of emmer and Barley, particularly at the level of the immune system are, however, lacking.

Furthermore most studies on the physiological effects of whole cereals have been carried out using single components isolated from other cereals and fed to animals or human subjects, but very limited data are available with complete diets rich in whole cereals (Slavin, 2003).

Supplementing the diet with cereals fraction rich in polyphenols has been shown to improve various leukocyte parameters and the antioxidant status in mice, so indicating possible health benefits for human population (Alvarez, Alvarado, Puerto, et al., 2006).

In a previous similar experiment we have shown that a diet containing whole wheat rich in dietary fiber and antioxidant compounds, given to rats for 6 weeks, enhance the immune responses of mesenteric lymphocytes in comparison with a diet containing refined wheat which has lost
theses compounds after the refining process (Molinari, Manzi, Ricci, et al., 2009).

The aim of this study is to verify whether emmer and barley flours, when consumed at constantly high levels in equilibrated diets, may have beneficial effects on the immune system, probably through the regulation of redox status, utilizing for this experiment two groups of rats fed for six weeks with one of two diets containing 53% of barley and of Emmer respectively.

**Materials and Methods**

**Animals and Diets**

Two groups of ten growing male Sprague Dowley rats, weighing about 100 grams have been fed for 6 weeks with experimental diets. The composition of the experimental diets is reported in Table 1. Weight and food consumption of rats were determined twice a week. The care and use of rats were approved by the Animal Care and Ethics Committee of Tuscia University.

**Preparation of flours**

Whole barley (var. Scarlett) and Whole emmer (var. Molise) were milled in a hammer mill mod 16/BV Beccaria s.r.l. (Scarnafigi, Cn, Italy), through 2 steps with 0.5 mm sieve, and working capacity: 30 kg/hr.

**Analysis of flours**

The samples of whole grain flours were analyzed using standard methods (ICC, 1995) for moisture, crude proteins, and ash. Soluble dietary fiber (SDF) and insoluble dietary fiber (IDF) were quantified by the enzymatic gravimetric procedures of Prosky (Prosky, Asp, Schweizer, Devries, & Furda, 1988), and methods 993.19 and 991.42 (AOAC, 1997). Total dietary fiber (TDF) was calculated as SDF+IDF.

**Determination of total phenolic content (TPC) of flours.**

The total phenolic content of flours was determined according to the method described by Shaidi & Nazck (1995). 1 g of flour was extracted with 80% aqueous methanol (1/10 g/ml), shaking for 2 hours at room temperature, and centrifuging at 12000g at 4°C. A 0.25 ml aliquot of the methanol extract was mixed with 0.25 ml of Folin-Ciocalteau reagent (previously diluted with water, 1:1 v/v), 0.5 ml of saturated sodium carbonate solution, and 4 ml of water. The mixture was allowed to stand at room temperature for 25 min, and then it was centrifuged at 2100 g for 10 min. Supernatant absorbance was measured at 725 nm using a spectrophotometer. The results have been expressed as mg of ferulic acid equivalents/g dry matter.

**Preparation of plasma**

At the end of experimental period blood was drawn into syringes from heart of each rats, placed in plastic tube containing heparin and centrifuged at 1500 g for 15 min at 4°C. After centrifugation plasma was collected in aliquots and immediately frozen for analyses of total polypehols content, total antioxidant capacity and protein carbonyls content.

**Analysis of plasma total phenols.**

Plasma total phenols were measured by the method of Serafini (Serafini, Maiani, & Ferro-Luzzi, 1998). Briefly, for total phenols, 500 μL of plasma were acidified and, following extraction of phenols complexed with alcoholic sodium hydroxide, proteins were precipitated using 0.75 M metaphosphoric acid and reextracted with a solution of acetone/water (1:1). Phenol content was measured by the Folin-Ciocalteau method using ferulic

| Table I. Composition of the experimental diets (g/100g) |
|---------------|-----------|--------|-----------|-------------|-------------|--------|-----------|
| Flour* | Sucrose | Casein | Corn oil | Salt** Mixture | Vitamin** Mixture | Coline | Methionine |
| 53    | 17      | 14     | 10       | 4           | 1           | 0.6    | 0.4       |

*Barley or Emmer; **Formulated according to AIN-93, 1993.
acids as standards and the results were expressed as μg ferulic acid equivalents/ml.

Determination of total antioxidant capacity (TAC) of plasma, by Ferric Reducing Antioxidant Parameter (FRAP)

FRAP assay was carried out by measuring the ability of plasma samples to reduce the colourless ferric-2,4,6-tripyridyl-s-triazine complex (TPTZ - Fe3+) to its ferrous colored form (TPTZ-Fe2+) according to the method of Benzie, & Strain (1996). 800 μl of FRAP reagent prepared daily (acetate buffer 0.3 M pH 3.6, 10 mM TPTZ in 40 mM HCl and 20 mM FeCl3 6H2O) were mixed with 50 μl of plasma diluted 1:2 with acetate buffer. The reagents were mixed and the absorbance at 593 nm recorded after 30 min incubation at 37°C. FRAP values were obtained using a standard curve with various amounts of 2mM FeSO4 and results expressed as Fe2+ equivalents/ml plasma.

Analysis of carbonyl content of plasma

The carbonyl content of plasma was determined according to the method of Reznich, & Packer (1994). For this measure 2 ml of plasma were divided in two aliquots of 1 ml each: one was marked as "test" and the other as "control". 4 ml of 10 mM 2,4-dinitrophenylhydrazine (DNPH) prepared in 2.5 mM HCl was added to the test sample and 4 ml of 2,5 M HCl alone was added to the control sample. The tubes were left in the dark for 1 h at room temperature and mixed by vortex every 15 min. 5 ml of 20% trichloroacetic acid solution were then added to both tubes for a 10 min incubation on ice, after which the tubes were centrifuged at 3000 g for 5 min at 4°C. The supernatant fluid was discarded and another wash performed by using 4 ml 10% trichloroacetic acid. Finally the precipitates were washed three times with 4 ml of ethanol/ethyl acetate mixture to remove unreacted DNPH and lipid remnants. The final protein precipitate was dissolved 1:1 in 2 ml of 6 M guanidine hydrochloride and incubated at 37°C for 10 min. The carbonyl content was calculated from peak absorption (370 nm) using an absorption coefficient of 22,000 M -1 cm-1. Each test sample was read against the control sample. The protein carbonyl content was expressed as nmole/ mg protein. Total protein content was determined on the HCl blank pellets using Bovine Serum Albumin (BSA) in guanidine hydrochloride as standard and reading the absorbance at 280 nm.

Preparation of lymphocytes from mesenteric lymphnodes

Lymphocytes were isolated from mesenteric lymphnodes of each rat, suspended in 10 ml of phosphate buffer saline (PBS), layered onto 5 ml of Histopaque-1083 and centrifuged at 720 g for 40 min. The lymphocytes were removed from the PBS-Histopaque interface and washed in PBS, centrifuging for 5 min at 720 g. The lymphocytes were counted in a Neubauer cell counter and diluted in RPMI 1640 culture medium with 5% antibiotics (penicillin, streptomycin) and 5% L-glutamine.

Analysis of lymphocytes proliferation

Lymphocytes proliferation was assayed by measuring [H3] - thymidine incorporation, after stimulation with the T-cell mitogen concanavalin A, (ConA). Lymphocytes were seeded at 3×105 cells/well in 96-well plates in complete RPMI 1640 culture medium with and without ConA (concentration range: 0-5 μg/ml) and incubated at 37°C in 5% CO2 for 24 and 48 hours. [H3]- thymidine (0.1 μCi) was added to each sample 18 h before harvesting. After incubation with ConA, the proliferative response was determined by radiochemical counting of each culture well. The cells were harvested on glass filter discs with an automated cell harvest. The filter discs were dried, transferred to scintillation vials and counted in a liquid scintillation counter. All assays were performed in triplicate. Lymphocytes proliferation was expressed as stimulation index (cpm of stimulated/cpm of unstimulated cells).

T-cell subpopulations analysis

The CD4+ and CD8+ T-cells were assayed by cytometry analysis. 1 x 106 mesenteric lymphocytes were fixed with 200 μl of 4% paraformaldehyde for 20 min at 4°C. After washing with 1 ml of PBS,
T-cells were stained with 50 μl of monoclonal fluorescein-labeled mouse anti-CD4+ at concentration of 1 μg/ml, and incubated for 40 min at 4°C. After incubation and washing to remove no-binding antibodies, lymphocytes were stained with 50 μl of monoclonal phycoerythrin labeled anti-CD8+ at concentration of 1 μg/ml and incubated for further 40 min. At the end, after another washing with 1 ml of PBS, samples were analyzed with flow cytometer (Becton-Dickinson, San José, CA).

**Statistical analysis**

The experimental data were reported as mean ± standard deviation of values. The results were subjected to two-way ANOVA test and the significance of mean differences were determined by t-test. Differences were considered significative when the P values were <0.05.

**Results**

**Behavior and growth of rats**

All diets were accepted by the rats and the animals remained healthy during the experimental period. No significant differences in body weight gain between the two experimental groups were observed (data not shown).

**Chemical composition of flours**

The flours have been characterized for their content in humidity, ash, proteins, fats, insoluble, soluble and total fiber (Table II).

**Total phenolic content of flours**

Barley has a significantly higher value than emmer (Table III).

**Plasma total polyphenols, total antioxidant capacity and carbonyl content**

The values of plasmatic polyphenols concentration do not statistically differ between the two cereals, independently of the values found in flours. However the FRAP values in plasma of barley fed rats are statistically higher than the emmer fed rats values. The carbonyl plasma concentration is sensibly lower in the 2 minor cereals groups of rats (Table IV) if compared with the values found in our previous paper (Molinari, Manzi, Ricci et al., 2009) on whole wheat groups (2.5 nmol/mg prot.).

<table>
<thead>
<tr>
<th>Samples</th>
<th>Moisture</th>
<th>Ash</th>
<th>Proteins</th>
<th>Fats</th>
<th>Insoluble fiber</th>
<th>Soluble fiber</th>
<th>Total fiber</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole Barley</td>
<td>10.4</td>
<td>1.7</td>
<td>11.1</td>
<td>2.8</td>
<td>9.2</td>
<td>3.6</td>
<td>12.8</td>
</tr>
<tr>
<td>Whole Emmer</td>
<td>9.9</td>
<td>1.4</td>
<td>13.7</td>
<td>2.3</td>
<td>7</td>
<td>3.3</td>
<td>10.3</td>
</tr>
</tbody>
</table>

Table III. Total phenolic content of Emmer and Barley flours (mg ferulic acid equivalents/g of dry matter)

<table>
<thead>
<tr>
<th>Whole Emmer</th>
<th>Whole Barley</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.76±0.08</td>
<td>1.5±0.4*</td>
</tr>
</tbody>
</table>

*Values are expressed as mean ± standard deviation.

<table>
<thead>
<tr>
<th>Diet</th>
<th>POLYPHENOLS (μg ferulic acid equivalents/ml)</th>
<th>FRAP (μM Fe2+equivalents)</th>
<th>CARBONYLS (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole Emmer</td>
<td>13.0 ± 2.0</td>
<td>656 ± 16*</td>
<td>0.35 ± 0.2</td>
</tr>
<tr>
<td>Whole Barley</td>
<td>10.6 ± 2.0</td>
<td>732 ± 38*</td>
<td>0.40 ± 0.2</td>
</tr>
</tbody>
</table>

*Values are expressed as mean ± standard deviation. P < 0.05.
Lymphocytes proliferation and T cell population

In Figures 1 and 2 the proliferative responses and the ratios of CD4+ and CD8+ T-cell isolated from mesenteric lymphonodes of rats fed for 6 weeks with the experimental diets are reported respectively. The results show that emmer and barley induce elevated responses, if compared with the results obtained in our previous study carried out with rats fed with refined and whole wheat diets (Molinari, Manzi, Ricci et al., 2009).

Discussion

The whole barley and emmer flours have an elevated content of polyphenols, with the maximum value for barley. The plasmatic level of polyphenols did not follow their in vitro content, probably because of various exogenous and endogenous factors which may interfere with the rate of absorption, transport and metabolism. So, for example, it has been shown that bound phytochemicals, which may be the major contributors to the antioxidant activity, are often not included in the analysis of phytochemicals, giving therefore an underestimate of total phytochemical values (Adom, & Lui, 2002). However the total antioxidant capacity of plasma showed a good correspondence with the in vitro concentrations of polyphenols, with barley showing the maximum value. This result can be explained by the presence of other antioxidant compounds not detectable with the Folin-Ciocalteau method, such as tocopherols and carotenoids which may affect the biological activity (Zdunczyk, Flis, Zielinski, 2006).

Figure 1. Proliferative responses of mesenteric lymphocytes of rats fed with the diets rich in Barley and Emmer flours

![Figure 1](image1.png)

*Stimulation index = cpm of mitogen stimulated /cpm of unstimulated cells.*

Figure 2. Ratio of T-cell sub populations CD4+/CD8+ of mesenteric lymphocytes of rats fed with the diets rich in Barley or Emmer flours

![Figure 2](image2.png)
Also the oxidative response in vivo (carbonyl concentration) is strongly reduced in minor cereals. The improvement in plasma redox status of rats fed with diets rich in barley and emmer may be responsible for the improvement of immune functions. For a correct function of immune system it is important to maintain a balance between ROS generation, which plays a key part of normal immune system and antioxidant functions within the immune cells (Knight, 2000).

Our results on the immune parameters of mesenteric lymphocytes showed that barley and emmer could modulate the biological responses. If we compare the present results with the results obtained in the previous experiment performed in identical experimental conditions (excepted for the dietary cereal which were whole and refined wheat) we may make the following considerations:

1) Barley and emmer flours enhance the stimulation index and the CD4+/CD8+ ratios of rat lymphocytes of about 30% with respect to refined wheat.

2) Barley and emmer flours have immunostimulating effects similar to those observed with whole wheat.

We cannot say if this effect is due to the antioxidant activity or also to the content of dietary fiber, since it has been shown that also dietary fiber may influence the immune functions. Diets supplemented with fermentable dietary fiber or isolated fructo-oligosaccarydes or β-glucan, may have immune-stimulating effects, such as the increase of immunoglobulin A secretion and the ratio CD4/CD8 ratio (Shley, & Field, 2002).

In conclusion, the present results indicated positive effects on the immune system of barley and emmer when consumed at constant by high content in the diet, and it may be important for practical nutrition, to include these two minor cereals in protective and healthy diets. Further work is needed in order better to understand factors and mechanisms involved in the physiological responses of the immune system to dietary barley and emmer.

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References
ALVARADO C., ALVAREZ P., JIMENEZ L., DE LA FUENTE M. (2006), Oxidative stress in leukocytes from young prematurely aging mice is reversed by supplementation with biscuits rich in antioxidant, Developmental and Comparative Immunology, 30(12), 1168-1180.


