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# Molecular features of fermented teff flour relate to its suitability for the production of enriched gluten-free bread

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ABSTRACT

The effects of fermentation of teff flour by a mixture of lactic acid bacteria and yeasts present in a gluten-free sourdough have been considered. Fermentation had a major impact on the physicochemical properties of teff starch and on its pasting behavior, and a somewhat more limited impact on teff proteins, leaving essentially intact protein components of possible relevance for formation of a protein network. Either fermented or non-fermented teff were added to a 25% level to a commercial corn-based gluten-free bread mix, containing chemical leavening agents. The bread enriched with fermented teff had improved physical properties and a lower staling rate with respect to a non-enriched control or to a bread enriched with non-fermented teff flour.



## 1. Introduction

Fermentation is one of the oldest and most economical biotechnological pre-treatments of grains for producing and preserving food. Fermentation also provides a "natural" option whenever there is a need to remove undesirable components, to enhance the nutritive value and flavour of the food, and to decrease the energy required for cooking and to increase the product safety (Wood, 2004). In the tradition of African and Asian countries, fermentation is a natural process that involves mixed cultures of yeasts and bacteria indigenously present on the substrate (Blandino, Al-Aseeri, Pandiella, Cantero, & Webb, 2003). These fermented foods originated as household products, but expanded to the cottage industry level as a consequence of increasing consumer demand (Steinkraus, 1997).

The effects of fermentation on cereal grains (such as millet, sorghum, teff, etc.) have been investigated quite extensively (Elkhalifa & El Tinay, 1994; Usha, Sripriya, & Chandra, 1996 a, b; Elkhalifa, Schiffler, & Bernhard, 2004; Yigzaw, Gorton, Solomon, & Akalu, 2004). Some of these crops are used after a biotechnological pre-treatment of grains or flours - usually fermentation or sprouting - in order to improve flavor, structure, and stability of baked goods (Guyot, 2010; Hugo, Rooney, & Taylor, 2003). However, most of these studies were mainly focused on the nutritional features of the fermented grains and on their use for preparing indigenous fermented foods and beverages.

Studies on non-conventional plant materials are a topic of growing popularity in cereal science, responding to the consumers' request for an increased range of cereal-based products with improved nutritional value. In this frame, given the absence of celiac-toxic sequences in its proteins (Taylor & Emmambux, 2008), teff is well suited as an ingredient for the production of gluten-free foods. Teff (*Eragrostis tef*) is a small tropical grain, originating from Ethiopia and typically used for the production of *injera*, a fermented wheat flatbread of local tradition (Bultosa & Taylor, 2004).

Because of the tiny dimensions of teff seeds, the whole meal flour is characterized by the presence of significant amounts of coating layers and sprout, resulting into high levels of insoluble polysaccharides. Teff presents a starch/protein organization morphologically similar to that of sorghum. As in sorghum, the major protein fractions in teff are globulins and prolamins, typically present as compact aggregates in protein bodies surrounding the starch granules. This peculiar structure calls for pre-treatment of flour from either sorghum or teff as almost mandatory to facilitate transformation into either the common foods consumed in the countries of origin (Elkhalifa & El Tinay, 1994; Elkhalifa et al., 2006; Hassan & El Tinay, 1995) or in foods closer in their appearance to those consumed in the Western world (Marengo et al., 2015). However, very little molecular-level information is available on starch-protein and protein-protein interactions in fermented teff. Reportedly, teff fermentation has a positive impact on nutritional properties such as the bio availability of some minerals (mainly iron, calcium, phosphorus and copper) and B1 vitamin (Bultosa & Taylor, 2004). Destruction of phytic acid has been implied in contributing to improve the bioavailability of iron and other metals of nutritional relevance from diets where fermented teff foods are staple components (Wood, 2004).

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Taking all of this into account, the main objectives of this study were: *i)* assessing the nature and extent of starch and protein modifications occurring during teff fermentation; *ii)* evaluating the possible use of fermented teff flour for producing teff-enriched gluten free bread; *iii*) combining the above information to understand the role played by individual macromolecules (and of fermentation-dependent modifications) in defining the properties of the enriched gluten-free bread.

## 2. Materials and methods

### *2.1. Teff flour*

Teff was purchased from Innovative Solutions Ltd. (Mayfield, UK). Whole grains were ground to flour (<0.5 mm) with a laboratory mill (IKA Universalmühle M20, Staufen, Germany), fitted with a water cooling jacket in order to avoid overheating during grinding. The resulting flour was fermented by using a gluten-free sourdough prepared as described by Marti et al. (2015) as the source of the required microorganisms. The gluten-free sourdough (500 g) was maintained in spring water (1000 mL) for 20 min at room temperature, and an aliquot of the watery phase (300 mL) was then added to teff flour (500 g). After a first fermentation step (24 h at 20 °C), fresh spring water (180 mL) and an additional amount of teff flour (300 g) were added to the fermented dough, and the resultant dough was fermented again for 3 h at 30 °C. This dough refreshment step was repeated daily for 8 d to give the fresh fermented teff, that was freeze-dried (alfa 2-4, Martin Christ Gefriertrocknungsanlagen GmbH, Germany) and ground to produce the dry fermented teff flour (particle size < 0.25 mm) used for further studies.

### *2.2. Bread samples*

Teff flours (as such or after fermentation) were added at 25% replacement levels to a gluten-free breadmaking blend of patented composition (Molino Quaglia S.p.A., Vighizzolo D'Este, Italy), containing corn starch, skimmed milk, sugar, guar gum, psyllium fiber, and corn maltodextrin. Blends were mixed with the amount of water suggested by the manufacturer of the gluten-free blend (ratio of solids: water = 1:0.8), with NaCl (1.5 g/100 g of blend), and with *Saccharomyces cerevisiae* (3 g/100 g of blend). Mechanical mixing was carried out for 12 min at room temperature in an automatic spiral mixer (Bomann, Clatronic s.r.l., Italy). Immediately after mixing, the dough (1500 g) was allowed to rest for 15 min at room temperature, divided into 300 g portions, molded into cylinder shapes, put in baking pans (8 × 15 × 5 cm) and allowed to rest for 45 min in a proofing chamber at 30 °C and 70% relative humidity. Baking was carried out for 60 min at 190 °C in an oven (Self Cooking Center®, Rational International AG, Landsberg, Germany), with steam injection (70% relative humidity) in the first instants of baking. Two hours after removal from the oven the samples were packaged in perforated orientated polypropylene film and stored under controlled conditions (20 °C, 60% RH) for 3 d. Bread prepared from 100% commercial gluten-free blend was used as a control. Bread-making trials were carried out in duplicate.

### *2.3. Chemical analysis of teff flour before and after fermentation*

Moisture, ash, starch, proteins and fat were determined according to the approved methods AACC 44–15, 08–12, 76–13, 46–12, and 30–10, respectively (AACC, 2001). The amount of total dietary fiber was determined according to the gravimetric enzymatic method of Prosky, Asp, Schweizer, DeVries, and Furda (1998). Sugar content was determined according to Zygmunt et al. (1982). Water activity ($a_w$) was measured by an electronic hygrometer (Aqua Lab, CX-2 – Decagon Devices, Pullman, WA), based on the determination of the dew point and calibrated with standard solutions of LiCl and NaCl (prepared by High-Purity Standards for Decagon Devices). Total titratable acidity was determined on 10 g of sample, homogenized with 90 mL of distilled water and was expressed as the volume (mL) of 0.1 M NaOH required for bringing the pH of the suspension to a value of 8.5 as determined on a Crison GPL22 pH meter (Crison Instruments, Alella, Barcelona, Spain). All measurements were performed in triplicate.

### *2.4. Microbiological analysis of teff flour before and after fermentation*

Ten grams of each sample were aseptically weighed and suspended into a sterile bag, mixed with 90 mL of sterile 0.85% tryptone/salt solution, and homogenized with a Stomacher Calworth 400 Circulator (PBI International, Milan, Italy) at 230 rpm for 1 min. Tenfold progressive dilutions were prepared for the following microbiological determinations: *i)* Total Bacterial Count (TBC), on Plate Count Agar (PCA, VWR GmbH, Darmstadt, Germany) and incubation at 30 °C for 48 h (ISO, 2003); *ii)* Total Lactic Acid Bacteria (LAB), on de Man Rogosa Sharpe agar (MRS; Merck, Darmstadt, Germany) and incubation under anaerobic conditions (gas pack) at 30 °C for 48 h (De Man, Rogosa, & Sharpe, 1960); *iii)* yeasts, by spread technique on Yeast Glucose Chloramphenicol (YGC, Merck, Darmstadt, Germany) and incubation at 30 °C for 48 h (ISO, 1992). All microbiological analyses were carried out in duplicate, and the results are expressed as Colony Forming Units (CFU) per gram sample.

### *2.5. Microstructural features*

Microscopy images were obtained by means of an Olympus BX50 microscope (Olympus, Tokyo, Japan), after staining with Toluidine Blue (O'brien, Feder, & McCully, 1964).

### *2.6. Protein solubility and thiol accessibility*

Protein solubility under native or denaturing conditions was determined by suspending 0.5 g of sample in 10 mL of 0.05 mol/L sodium phosphate buffer, pH 7.0, containing 0.1 mol/L NaCl, and 8 mol/L urea or 8 mol/L urea and 0.01 mol/L dithiothreitol (DTT) when indicated. Suspensions were stirred for 60 min at 25 °C, and centrifuged (10,000×*g* for 20 min, 20 °C). The amount of protein in the supernatant was determined by a dye-binding method (Bradford, 1976) using bovine serum albumin as a standard. Results are expressed as mg proteins (g sample)$^{-1}$. Accessible –SH groups were measured by suspending 0.5 g of sample in 10 mL of 0.05 mol/L sodium phosphate buffer, pH 6.8, containing 0.1 mol/L NaCl and 0.2 mmol/L 5,5′-dithiobis(2-nitrobenzoate) (DTNB; Ellman, 1959). After 15 min at 25 °C, insoluble material was removed by centrifugation (10,000×*g*, 20 min, 20 °C), and the absorbance at 412 nm of the supernatant was read against a DTNB blank (Barbiroli et al., 2013; Marengo et al., 2015). Total accessible thiols were measured according to the same protocol, but adding urea (8 mol/L) to the DTNB-containing buffer.

### 2.7. SDS-PAGE

The polypeptide profile of individual samples and of solubilized protein fractions was analyzed by SDS-PAGE in a 12% gel after denaturation in the absence/presence of 1% (v/v) 2-mercaptoethanol as indicated, using a MiniProtean Apparatus (BioRad, Richmond, VA) as described in previous reports (Barbiroli et al., 2013; Marengo et al., 2015). Gels were stained with Coomassie Blue (BioRad, Richmond, VA, USA). Sample volumes were adjusted to load 0.01 mg of protein per lane. Molecular weight markers were from Amersham Biosciences, Amersham, UK.

### 2.8. Starch properties

Starch susceptibility to alpha-amylase hydrolysis was determined according to the official enzyme-based method AACC 76–31, 2001. Pasting properties were measured in a Brabender Micro-Visco-AmyloGraph (Brabender OHG, Duisburg, Germany). Twelve grams of sample were dispersed in 100 mL of distilled water, scaling both sample and water weight on a 14% flour moisture basis. The pasting properties were evaluated at constant speed (250 rpm) with the following temperature profile (heating/cooling rate, 3.0 K/min): heating from 30 to 95 °C; holding at 95 °C for 20 min; cooling from 95 to 30 °C. The following indices were considered: pasting temperature (temperature at which the initial increase in viscosity occurs); peak viscosity (maximum paste viscosity achieved during the heating cycle), and setback (increase in viscosity during cooling, corresponding to the difference between the final viscosity and the viscosity reached after the first holding period). Measurements were performed at least in duplicate.

### 2.9. Bread characterization

A reflectance color meter (CR 210, Minolta Co., Osaka, Japan) was used to measure the lightness and saturation of the color intensity of bread crumb by utilizing the CIE-LAB-System uniform color space procedure. Values for L*, a*, and b* (as measures of lightness, redness-greenness, and yellowness–blueness, respectively) were recorded for each sample. Each measurement was replicated five times. The volume of five loaves was determined by a rapeseed displacement method, 2 h after baking. The weight of bread was recorded and the specific volume was determined through the volume/mass ratio and expressed in mL $g^{-1}$. The moisture of the crumb core was determined in triplicate using a single-stage drying process for 16 h at 105 °C. The crumb core water activity ($a_w$) was measured in triplicate.

Crumb texture was assessed using a testing machine (Z005, Zwick Roell, Ulm, Germany) equipped with 100 N load cell. To evaluate hardness, three central slices (1.5 cm thickness) of each loaf were compressed to 30% of their height, using a 30 mm diameter cylindrical aluminum probe and a test speed of 2 mm $s^{-1}$. Crumb hardness was measured (n = 6) after 0, 1, and 3 d and expressed as the load (N) at 30% strain.

### 2.10. Statistical analysis

Statistical analysis was performed using Statgraphics XV version 15.1.02 (StatPoint Inc., Warrenton, VA, USA). ANOVA test was performed, and samples were used as factor. When a factor effect was found significant ($p \leq 0.05$), significant differences among the respective means were determined using Fisher's LSD test.

## 3. Results and discussion

### 3.1. Microstructural features of fermented flour

Microscope images of teff flours (Fig. S1) show that, before fermentation, starch granules are inside the flour particles, so that flour main components (starch and protein) are not easily recognizable. The images in Fig. S1, taken after staining with the protein-specific dye Toluidine Blue, indicate the presence of proteins between individual starch granules, confirming previous findings (Bultosa, Hall, & Taylor, 2002; Hager, Wolter, Jacob, Zannini, & Arendt, 2012a; Elkhalifa et al., 2006). As expected, the proteolytic events occurring during fermentation have an impact on the structure of the protein matrix, allowing liberation of the starch granules.

### 3.2. Chemical and microbiological properties of fermented flour

The chemical characteristics of fermented and un-fermented teff flours are compared in Table 1. The chemical composition of the unfermented teff used in this study is similar to that found by other authors (Hager, Wolter, Jacob, Zannini, & Arendt, 2012a), and confirms the nutritional value of teff (Thompson, 2009). Fermentation of teff causes a decrease in starch content, probably due to the simultaneous action of endogenous amylases and of those produced by lactic acid bacteria (Baye, Mouquet-Rivier, Icard-Varnière, Rochette, & Guyoy, 2013). The content of proteins and fat remains almost unchanged after fermentation. Although the total amount of fiber remains unchanged, fermentation results in a 35% decrease of the insoluble components of the fiber. This is interesting from a nutritional standpoint, given the reported positive effects of the soluble fraction of fiber on human health and well-being (Slavin, 2005). As expected, the fermentation by microorganisms determined a decrease in the total sugar content, and in particular of sucrose, raffinose, and fructose, which were no longer detectable in the fermented teff flour.

Microbiological determinations (Table 2) gave a Total Bacteria Count (TBC) around 4 log CFU $g^{-1}$ in the unfermented sample. The bacterial species in unfermented teff flour were mostly aerobic spore-forming bacteria, whose growth is greatly limited by the low water activity ($a_w = 0.54$). The microbial composition drastically changed after fermentation, when the yeast population increased and Lactic Acid Bacteria (LAB) became the most important microbial population, constituting the virtual totality of the TBC. The lactic acid produced by LAB is responsible for the increase in acidity measured in fermented teff flour, as indicated by the significant pH de-

**Table 1**
Proximate analysis of teff flours (figures in percent, on a dry matter basis).

| | Unfermented | Fermented |
|---|---|---|
| Total Starch | 78.81 ± 0.43* | 72.66 ± 0.18* |
| Protein | 8.41 ± 0.29 | 9.03 ± 0.02 |
| Lipid | 3.32 ± 0.17 | 2.82 ± 0.08 |
| Total fiber | 8.0 ± 0.14 | 7.51 ± 0.17 |
| Soluble fiber | 1.15 ± 0.14* | 1.80 ± 0.14* |
| Insoluble fiber | 6.80 ± 0.01* | 5.72 ± 0.06* |
| Sugars | 1.77* | 0.15* |
| Glucose | 0.45 ± 0.07* | 0.15 ± 0.01 * |
| Sucrose | 0.91 ± 0.01 | n.d. |
| Raffinose | 0.20 ± 0.03 | n.d. |
| Fructose | 0.21 ± 0.01 | n.d. |

Means ± standard deviation (n = 3) followed by an asterisk (*) in any given row are statistically different ($p \leq 0.05$).
n.d., not detectable.

**Table 2**
Chemico-physical and microbial characteristics of teff samples.

| | Unfermented | Fermented |
|---|---|---|
| pH | 6.25 ± 0.18* | 4.41 ± 0.02* |
| Total titratable acidity (mL 0.1 M NaOH/10 g) | 4.53 ± 0.45* | 12.08 ± 0.55* |
| Moisture (g/100 g) | 12.5 ± 0.05* | 5.1 ± 0.03* |
| Total Bacteria Count (CFU $g^{-1}$) | 50,000 ± 3600* | 2,000,000 ± 126,000* |
| Lactic Acid Bacteria (CFU $g^{-1}$) | <100 | 2,400,000 ± 248,000 |
| Yeast (CFU $g^{-1}$) | 3000 ± 180* | 1000 ± 160* |

Means ± standard deviation (n = 3) followed by an asterisk (*) in any given row are statistically different ($p \leq 0.05$).

crease (from 6.25 to 4.1) and by the corresponding increase in titratable acidity (from 4.5 to 15) in fermented teff flour.

### 3.3. Organization of the protein network in fermented teff flour

Information on the nature of the inter-protein interactions in cereal- and pseudocereal-based materials can be provided by measuring protein solubility in different media (Barbiroli et al., 2013; Bonomi et al., 2012; Cabrera-Chávez et al., 2012; Iametti et al., 2006; Marengo et al., 2015). In particular, conditional solubility studies in the absence/presence of denaturants and of disulfide-breaking agents offer useful hints as for the role of hydrophobic interactions and of disulfide bonds in the stabilization of protein aggregates and protein networks (Bonomi et al., 2012; Marengo et al., 2015).

Fermentation-dependent changes in protein solubility are shown in Fig. 1A, and suggest that fermentation result in modest variation in the overall protein organization. The observed decrease in buffer- and urea-soluble proteins in the fermented flour are consistent with reports on fermented sorghum flour (Elkhalifa et al., 2006; Hugo et al., 2003; Marengo et al., 2015). The observation that proteins solubilized in the presence of urea and of a disulfide-breaking agent also decrease indicate that proteins are likely among the primary nutrients used for microbial growth also in fermented teff.

Cysteine thiols (–SH) and intra- or intermolecular disulfides (–S–S–) have a fundamental role in defining the technological properties of cereal flours, since their presence and location play a fundamental role in the stabilization of protein networks through formation of covalent bonds upon processing (Bonomi et al., 2012; Iametti et al., 2013). Evaluating the amount and accessibility of protein –SH groups has been shown to represent a useful predictive tool to evaluate cereal performance. This approach has been proven useful when trying to understand the molecular determinants of some physical traits of either cereal-based or gluten-free products enriched with non-cereal components (Bonomi et al., 2012; Cabrera-Chávez et al., 2012; Marengo et al., 2015; Marti et al., 2014a).

The accessibility of thiols in teff flours is shown in Fig. 1B. Apparently, all thiols in teff flour are readily accessible even in the absence of a denaturant. A decrease in reactive -SH groups was detected in the fermented samples, and suggests that LAB microflora involved in fermentation may have taken up and used for their own growth most of the cysteine-containing peptides released upon proteolysis, as observed in previous studies on fermented sorghum (Elkhalifa et al., 2006; Marengo et al., 2015).

Finally, the nature of the proteins involved in the events outlined above was investigated by SDS-PAGE analysis of the proteins solubilized in different media from the samples (Fig. 2). The SDS-PAGE pattern of proteins in untreated teff flour shows four main fractions with molecular mass around 96, 90, 66, and 58 kDa. The intensity of

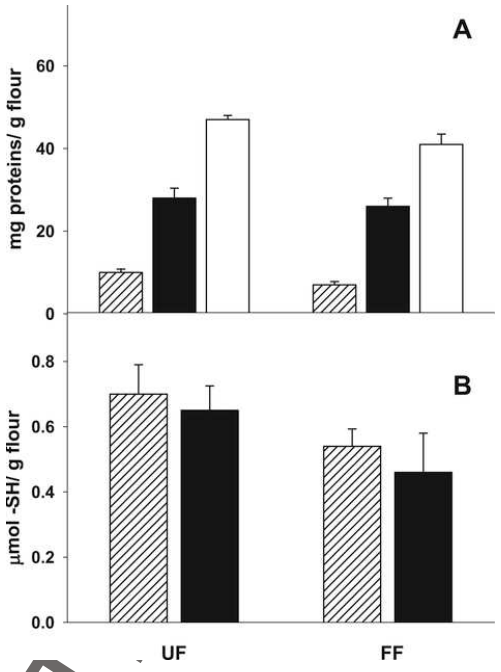


**Fig. 1.** A: Solubility of proteins from unfermented (UF) and fermented (FF) teff flour samples in different media. Aliquots of the two samples were suspended under stirring in 0.05 mol/L sodium phosphate, 0.1 mol/L NaCl, pH 7.0, in the presence/absence of 8 mol/L urea and 10 mmol/L DTT, as indicated. Shaded bars, buffer only; black bars, + urea; empty bars, + urea and DTT. After 60 min at 25 °C, the suspensions were centrifuged (10,000×*g*, 20 min, 20 °C) and the protein concentration in the supernatant was determined by the Bradford assay. Standard deviation is given for each sample (n = 3). B: Thiol content of proteins in unfermented (UF) and fermented (FF) teff flour samples. Thiols were assessed on flour samples suspended in 0.05 mol/L sodium phosphate, 0.1 mol/L NaCl, pH 6.8, in the presence/absence of 8 mol/L urea as indicated. Shaded bars, buffer only; black bars, + urea. The buffer contained 0.2 mmol/L DTNB. After 15 min at 25 °C, the samples were centrifuged (10,000×*g*, 20 min, 20 °C) and the absorbance of the supernatant was read at 412 nm. Results are expressed as micromol thiol/(g flour). Standard deviation is given for each sample (n = 3).

all these protein bands decreased in the fermented flour. However, the 66 kDa component appears more resistant to proteolysis than other proteins. The component at 52 kDa is preferentially degraded when present in a non-disulfide-linked form. Taking into account the extent of proteolysis of individual components (as indicated by the SDS-PAGE tracings) and the information on the aggregation state (derived from solubility measurements), we hypothesize that residual proteins in fermented teff are mainly responsible for the formation of inter-protein bonds in this matrix.

### 3.4. Starch properties of fermented flour

The effect of fermentation on starch properties was first assessed by measuring the amount of starch that appears to be rapidly susceptible to hydrolysis by alpha-amylase (Table 3). Fermentation significantly decreases the amount of susceptible starch, as observed in sorghum (Elkhalifa et al., 2006). This is mainly attributable to the action of microorganisms, that may preferentially take up this readily available starch fraction.

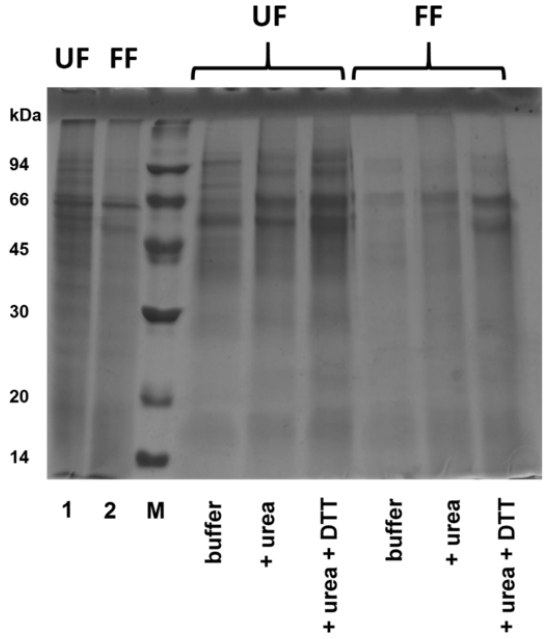


**Fig. 2.** SDS-PAGE patterns of proteins solubilized in different media from the two samples of teff flour. Samples were denatured in the presence of 2-mercaptoethanol, and diluted to allow loading the same amount of protein (0.01 mg) in each lane. Lane 1 and 2 refer to SDS-PAGE pattern obtained by treating teff flours with denaturing buffer. M: molecular weight markers.

**Table 3**
Effect of fermentation on properties of teff starch.

| | Unfermented | Fermented |
|---|---|---|
| Susceptibility to amylase (g released glucose/100 g starch) | 4.35 ± 0.43* | 1.66 ± 0.18* |
| Pasting temperature (°C) | 72.3 ± 0.3* | 76.1 ± 0.1* |
| Peak viscosity (BU) | 212 ± 2* | 246 ± 3* |
| Breakdown (BU) | 38 ± 2* | 70 ± 4* |
| Setback (BU) | 374.5 ± 0.5* | 365 ± 13* |

Means ± standard deviation (n = 2) followed by an asterisk (*) in any given row are statistically different ($p \leq 0.05$).

The pasting properties of teff flours are also compared in Table 3, and clearly indicate that they were vastly affected by fermentation. The viscoamylographic tracing of untreated teff flour is characterized by a low peak viscosity, a low loss of viscosity at high temperatures (breakdown), and a limited tendency to retrogradation (setback) compared to the pasting profiles of other cereals (Bultosa & Taylor, 2004). This trend could be related to the morphological characteristics of the starch, as small starch granules are characterized by a low ability to absorb water, to swell, and to show viscosity during the heating steps (Bultosa et al., 2002).

After fermentation, teff flour exhibited a higher onset gelatinization temperature compared to the untreated sample, suggesting a decreased ability of the starch to absorb water and swell. This could be related to the decreased accessibility of starch granules after fermentation. Fermentation also causes an increase in peak viscosity during heating, as observed for sorghum (Elkhalifa et al., 2006). The fermented teff suspension shows a higher value of breakdown during holding at 95 °C, compared to the untreated sample, exhibiting a great loss of viscosity as a result of the combination of thermal and mechanical stress. Finally, fermentation did not seem to affect the ability of teff starch to retrograde, as indicated by viscosity values after the cooling step.

### 3.5. Teff-enriched gluten-free bread

The characteristics of gluten-free breads enriched in either unfermented or fermented teff are reported in Table 4. The specific volume of bread significantly ($p \leq 0.05$) decreased when teff was added. Specific volume is one of the parameters used in the bakery industry to assess bread development. Values of about 4–5 mL $g^{-1}$ are typical of wheat breads - depending on the formulation and the method of baking - whereas values between 1.3 and 2.4 mL $g^{-1}$ are common in gluten-free bread (Hager et al., 2012b). Use of fermented teff led to a significant ($p \leq 0.05$) increase in specific volume compared to bread from unfermented teff flour, maybe due to microbial gas production that might have favored expansion of the dough (Wood, 2004). Changes in fiber solubility after the fermentation process should be also taken into consideration. Indeed, fermentation promoted a decrease in insoluble fiber (Table 1), that negatively affect the formation of a three dimensional protein network.

The central slice of gluten-free breads is shown in Fig. 3, that highlights important differences in porosity among the samples. Teff-enriched gluten-free breads exhibited a less dense structure than control, as already observed for wheat-based bread (Alaunyte, Stojceska, Plunkett, Ainsworth, & Derbyshire, 2012). Fermented teff-enriched bread shows a more open crumb structure with a lower number of cells, larger than those of bread containing unfermented teff. This latter - in turn - showed a more regular porosity. The mouth feel of bread is known to be strongly influenced by these cell characteristics, and a high presence of large cells has been associated with a decrease in crumb hardness (Marti et al., 2014b). Loaf volume is also considered to be a major determining factor of crumb firmness (Axford, Colwell, Cornford, & Elton, 1968).

The crumbs of gluten-free bread made with 25% of either unfermented or fermented teff had a more intense color than control. Addition of teff made the bread crumb darker (lower L* values), redder, and less yellow (Table 4). Using fermented flour significantly ($p \leq 0.05$) decreased the yellowness of the product, but gave no significant ($p > 0.05$) differences in luminosity and redness.

Changes in crumb hardness during storage are reported in Fig. 4. Due to their higher fiber content, initial crumb firmness was significantly ($p < 0.05$) higher in teff-enriched breads than in control, confirming previous studies (Hager et al., 2012b). Also, bread made from

**Table 4**
Bread-making performance.

| | Control bread | 25% Enriched bread | |
|---|---|---|---|
| | | Unfermented teff | Fermented teff |
| Crumb luminosity (L*) | 62.06 ± 0.47[b] | 43.96 ± 0.79[a] | 43.57 ± 0.74[a] |
| Crumb redness (a*) | −5.56 ± 0.36[a] | 8.88 ± 0.35[c] | 8.36 ± 0.25[b] |
| Crumb yellowness (b*) | 11.20 ± 0.32[c] | 9.22 ± 0.16[b] | 3.50 ± 0.37[a] |
| Crumb moisture (g/100 g) | 50.3 ± 0.32[b] | 48.5 ± 0.23[a] | 50.1 ± 1.81[b] |
| Crumb water activity ($a_w$) | 0.964 ± 0.007[a] | 0.972 ± 0.004[b] | 0.983 ± 0.006[c] |
| Unit weight (g) | 218.5 ± 2.5[b] | 230.9 ± 4.3[c] | 208.1 ± 10.9[a] |
| Unit volume (mL) | 288.0 ± 29.7[b] | 195.0 ± 19.1[a] | 269.0 ± 44.2[b] |
| Specific volume (mL $g^{-1}$) | 1.3 ± 0.14[b] | 0.8 ± 0.09[a] | 1.3 ± 0.16[b] |

Values marked the same letter in a column are not significantly different ($p \leq 0.05$; LSD).

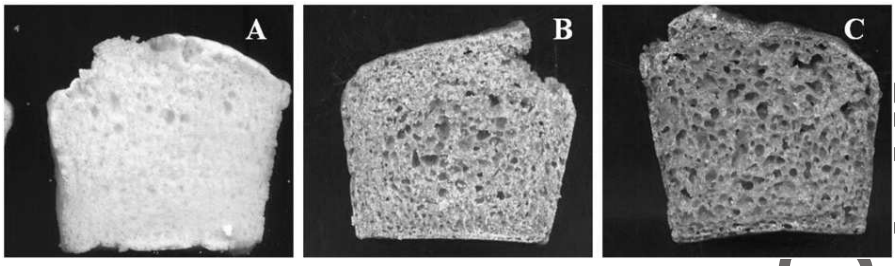


**Fig. 3.** Images of bread samples. Control bread (A); 25% unfermented teff-enriched bread (B); 25% fermented teff-enriched bread (C).

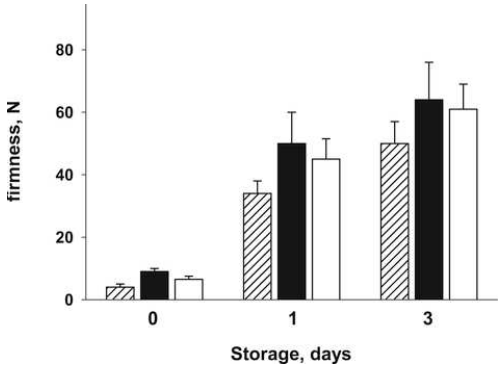


**Fig. 4.** Changes in crumb firmness of bread samples during storage for 3 d. Shaded bars, control bread; black bars, 25% unfermented teff-enriched bread; empty bars, 25% fermented teff-enriched bread. Standard deviation is given for each sample (n = 6).

mixtures enriched with fermented teff had lower hardness than bread made from mixtures enriched with unfermented teff. As discussed above, unfermented teff bread had lower volume than fermented teff-enriched flour bread, and this could lead to increased crumb firmness.

Firmness was monitored during storage to assess the rate of bread hardening and, therefore, of textural shelf-life. During the three-day test period teff-enriched breads retained higher crumb firmness than control, but the staling rate of teff-enriched bread was lower than control, in agreement with Hager et al. (2012b). Teff starch has a lower tendency to retrograde than maize starch (Bultosa et al., 2002) that is the main ingredient of many gluten-free commercial mixes, including the one used in this study. Bread enrichment with fermented teff did not compromise crumb softness during storage.

## 4. Conclusions

This study indicates that it is possible to produce a gluten-free bread enriched with a significant amount of teff (25%), improving the nutritional properties of control gluten-free bread. In this frame, fermented teff flour appears to exert a beneficial effect on the texture properties of the enriched bread - also during storage - with respect to the untreated teff flour.

Fermentation of teff flour is accompanied by a significant increase in nutritionally relevant soluble fiber, and by a decrease in free sugars. Whereas the lipid fractions remain essentially unaffected, proteins in teff flour are a target for the LABs mainly responsible of fermentation, as reported for sorghum flour. However, fermentation-related proteolytic events are altogether limited, and do not affect extensively those teff proteins that are most relevant to forming a stable network with other proteins in the system. These effects may contribute positively to the overall structure of maize-based gluten-free bread.

Thus, fermented teff flour may represent a suitable supplement for gluten free bread, also in consideration of the improved nutritional quality of the dietary fiber component. Even within the intrinsic limitations of this study, the findings reported here underscore the possibility of testing novel uses of teff also outside the limited geographical areas where teff-based foods nowadays represent a major staple food.

## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.lwt.2016.12.042.

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