



Investigation of the nutritional, functional and technological effects of the sourdough fermentation of sprouted flours

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ABSTRACT

In recent years, recommendations on whole grains consumption have been added to the overall dietary guidelines of many countries around the world. Despite the many benefits on human health, whole grains contain several anti-nutritional factors which decrease their nutritional quality leading to a poor use in human diet. Here, an integrate biotechnological approach, combining germination and sourdough fermentation with selected lactic acid bacteria, was set-up in order to improve the functional and nutritional quality of wheat, barley, chickpea, lentil and quinoa grains. Sourdough fermentation with *Lactobacillus rossiae* LB5, *Lactobacillus plantarum* 1A7 and *Lactobacillus sanfranciscensis* DE9 further enhanced the nutritional and functional features of sprouted flours by means of increased peptides, free amino acids and γ -aminobutyric acid concentrations, and decreased phytic acid, condensed tannins, raffinose and trypsin inhibitory activity. Sensory appreciable wheat breads fortified with the fermented sprouted flours were manufactured and characterized, showing high protein digestibility and low starch availability.

1. Introduction

Grains, mainly represented by cereals and legumes, are global dietary staples that, when consumed in whole form, offer considerable health benefits. Whole grains provide energy, nutrients, fibres, and bioactive compounds that may synergistically contribute to their protective effects. Despite their benefits, the intake of whole grains appears to be lower than recommended by international agencies and organization (Slavin et al., 2013) in many Countries. Besides the use as such or after milling, cereals and legumes have usually been treated by germination, fermentation or selectively heat treated to increase the amount or availability of nutrients. Although sprouting of legumes and some cereals is an ancient and well known practice, its application has recently been of emerging interest due to the significant enhancement of the nutritional and bioactive content of grains, as well as improved palatability. Germination may therefore offer a practical, natural, dietary intervention to increase the health benefits and acceptability of whole grains, with potentially widespread effects across populations in attenuating adverse lifestyle disease outcomes (Nelson et al., 2013).

Germination starts when the dry seed begins to take up water (imbibition) and is completed when the embryonic axis elongates. During germination, reserves within the storage tissues of the seed are hydrolysed in low molecular weight compounds and mobilised to support seedling growth (Bewley, 2001). From the moment the seed breaks dormancy, protective responses emerge through the synthesis of bioactive compounds, such as riboflavin, thiamine, biotin, pantothenic acid, niacin, vitamin C, tocopherols and phenolic compounds, and also increase their availability (Donkor et al., 2012). Germination triggers the enzymatic activity of sprouting seeds, leading to the breakdown of starch and non-starch polysaccharides, as well as proteins, the increase in reducing sugars, soluble dietary fibres, peptides and amino acids, as well as the release of the insoluble phenolic compounds covalently bound to cell wall polysaccharides (Hung et al., 2012). The degree of the changes depends on various germination conditions, such as temperature, humidity, steeping (soaking) and length (Koehler et al., 2007).

The incorporation of germinated cereals and legumes in bread for nutritional enhancement have been recently proposed (Mäkinen and

Abbreviations: LAB, lactic acid bacteria; rF, raw flour; sF, sprouted flour; rS, raw sourdough made with rF; sS, sprouted sourdough made with sF; WSE, water/salt-soluble extract; TTA, total titratable acidity; DY, dough yield; FQ, fermentation quotient; TFAA, total free amino acids; FAA, free amino acids; GABA, γ -aminobutyric acid; IVPD, in vitro protein digestibility; ANFs, antinutritional factors; TIA, trypsin inhibitor activity; rS-B, sourdough bread containing 20% (w/w) of rS; sS-B, sourdough bread containing 20% (w/w) of sS; GI, glycemic index; IDF, insoluble dietary fibres; SDF, soluble dietary fibres

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Arendt, 2015); this appears to be easily applicable thanks to the positive effect of germination on the sensory properties of grains, since it confers a typical flavour and odour generally perceived as pleasant (Mäkinen and Arendt, 2015). Moreover, the reducing sugars and amino acids released during germination markedly and positively affect the profile of Maillard reaction products released during baking (Kaukovirta-Norja et al., 2004).

A combination of germination and fermentation for nutritional modification of grains has been explored by several authors, showing a relevant hydrolysis of protein and starch, and an efficient enhancement of mineral solubility than fermentation alone (Katina et al., 2007; Sripriya et al., 1997; Valencia et al., 1999).

Sourdough fermentation, involving lactic acid bacteria (LAB) besides yeasts, is recognized as a reliable tool to improve nutritional, functional, technological and sensory properties of wheat and non-wheat flours, nevertheless, the effects on flours obtained from germinated seeds still remains unexplored.

In this work, wheat, barley, lentil, chickpea and quinoa seeds were germinated, and fermented with a selected pool of LAB. The effects of germination and fermentation on the main nutritional and functional features were investigated. Experimental breads fortified with germinated flours sourdoughs were also characterized.

2. Materials and methods

2.1. Grains, germination process and milling

Five different grains were considered in this study. Legumes (*Cicer arietinum* var. Pascià and *Lens culinaris*) and cereals (*Triticum durum* var. Simeto and *Hordeum vulgare* var. Nure) were provided by Caporal Grani S.a.s. (Gravina di Puglia, Bari, Italy); *Chenopodium quinoa*. Was supplied by Quinoa Marche Srls (Jesi, Ancona, Italy).

Grains were germinated according to the protocol described by Donkor et al. (2012) with some modifications (Fig. S1). Briefly, grain samples were submerged in 1.25% sodium hypochlorite (NaClO) (seed:water ratio of 1:5, w/v) at room temperature for 30 min to disinfect the grain surface prior the germination process. In order to remove residual NaClO, grains were washed under tap water, at approximately 10 °C, for 20–30 min. Surfaces of seeds were assessed for sterility by blotting them tightly on 1/10-strength tryptic soy agar and incubating plates at 30 °C for 48 h (Minervini et al., 2015). The disinfected grains were then steeped in tap water for 24 h at 16.5 °C. For sprouting, batches of grains (ca. 100 g) were placed in a household germination system (BioSnacky, Biokosma GmbH, Konstanz, Germany) at 16.5 °C in the dark. The grains were sprinkled with tap water for 15 min at 12 h-intervals and aerated by hand once every 24 h. The sprouting time of germination was different depending on seed type. In particular, the optimal time was chosen based on the speed of grains to achieve good rootlets length (ca. 3/4 of the medium seed length). After sprouting, the grains were carefully washed with distilled water (3 × 800 ml) and dried in drying chambers (Binder GmbH, Tuttlingen, Germany) at 50 °C for 25 h. The rootlets were not separated from the grains. The drying conditions used in this work were comparable to those used for industrial malting of barley and are known to result in enhanced enzyme activity (Laitila et al., 2006).

Flours were obtained from raw and germinated grains by a laboratory mill Ika-Werke M20 (GMBH, and Co. KG, Staufen, Germany). After milling all the flours were sieved (mesh size 500 µm) to remove the coarse fraction.

2.2. Chemical characterization of flours

Protein (total nitrogen × 5.7), lipids, ash, starch and moisture were determined according to the AACC approved methods 46-11A, 30–10.01, 08–01, 76–13.01 and 44-15A, respectively (AACC, 2010). Carbohydrates were calculated as the difference

[100 – (proteins + lipids + ash + total dietary fibres + starch)]. Proteins, lipids, carbohydrates and ash were expressed as % of dry matter (d.m.).

Insoluble (IDF) and soluble (SDF) dietary fibres were determined according to the procedure previously described by Goñi et al. (2009) for solid samples. Briefly, 300 mg of homogenized dry samples were weighed into 50 ml centrifuge tubes and 10 ml phosphate buffer and 0.2 ml pepsin solution (2000 U/g) were added. Samples were incubated at 40 °C for 1 h. Pancreatin (5 mg/ml) was added to the sample mixtures and pH adjusted to 7.5. Samples were incubated at 37 °C for 6 h. Then, α-amylase (110 U/ml) was added and samples (pH adjusted at 6.9) incubated at 37 °C for 16 h under stirring conditions. Then, samples were centrifuged (15 min, 3000 × g) and supernatants discarded. Residues were washed twice with 5 ml of distilled water and all supernatants mixed. Residues were dried overnight at 105 °C, cooled in desiccator and weighed to determine residue weight, corresponding to IDF. Sodium acetate buffer (pH 4.75) was added to the supernatants followed by 0.1 ml amyloglucosidase (140 U/ml), and then incubated at 60 °C, for 45 min, in water bath under stirring conditions. The reaction mixtures were transferred into dialysis tubes (cut-off 14,000 Da) and dialyzed against water at 37 °C for 48 h. Dialysis retentates were freeze-dried. The residues, corresponding to SDF, were quantified gravimetrically.

Water/salt-soluble extracts (WSE) of flours were prepared according to Weiss et al. (1993) and used to analyse peptides, free amino acids (FAA) and GABA (γ-aminobutyric acid) concentrations. The peptide concentration was determined by reversed-phase fast performance liquid chromatography (RP-FPLC), using a Resource RPC column and an ÄKTA FPLC equipment with the UV detector operating at 214 nm (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). FAA and GABA were analysed by a Biochrom 30 series Amino Acid Analyzer (Biochrom Ltd., Cambridge Science Park, England) with a Na-cation-exchange column (20 by 0.46 cm internal diameter), as described by Rizzello et al. (2010a).

2.3. Microbiological characterization of flours

For microbiological analysis, 10 g of flour was homogenized with 90 ml of sterile peptone water (1% [w/v] of peptone and 0.9% [w/v] of NaCl) solution. LAB were enumerated using De Man, Rogosa and Sharpe (MRS) (Oxoid, Basingstoke, Hampshire, UK) agar medium, supplemented with cycloheximide (0.1 g/l). Plates were incubated, under anaerobiosis (AnaeroGen and AnaeroJar, Oxoid), at 30 °C for 48 h. Cell densities of yeasts and moulds were estimated on Yeast Peptone Dextrose Agar medium (YPDA) (Sigma-Merck, Darmstadt, Germany) supplemented with chloramphenicol (0.1 g/l), through pour and spread plate enumeration, respectively, and incubated at 30 °C for 72 h. The identification of yeast or mould was done by visual analysis of colony morphology. Total mesophilic aerobic bacteria were determined on Plate Count Agar (PCA, Oxoid) at 30 °C for 48 h, and total *Enterobacteriaceae* were determined on Violet Red Bile Glucose Agar (VRBGA, Oxoid) at 37 °C for 24 h.

2.4. Sourdough fermentation and acidification kinetics

Lactobacillus rossiae LB5, *Lactobacillus plantarum* 1A7 and *Lactobacillus sanfranciscensis* DE9, belonging to the Culture Collection of the Department of Soil, Plant and Food Sciences (University of Bari, Italy) previously isolated from wheat germ and flour were used in this study as starters for sourdough fermentation. The strains were previously characterized and selected based on the metabolic adaptability, pro-technological properties (kinetics of growth and acidification), proteolytic activity and sensory characteristics (Coda et al., 2013; Rizzello et al., 2010a, 2012). Moreover, *L. plantarum* 1A7 and *L. rossiae* LB5 were found to have antifungal activity and to be able to grow on non-wheat substrates rich in fibre and polyphenols, respectively. Strains

were used as mixed starter (ratio 1:1:1) for fermentation of either raw (rF) and sprouted (sF) flours, obtaining raw (rS) and sprouted (sS) sourdoughs. Cell suspensions were prepared as described by Rizzello et al. (2010a), the DY (dough yield, dough weight \times 100 / flour weight) was 160 and the initial cell density of LAB was ca. 7.0 Log cfu/g. Fermentation was carried out in triplicate at 30 °C for 24 h.

The kinetic of acidification was determined on-line by a pHmeter (Model 507, Crison, Milan, Italy) with a food penetration probe. Acidification data were modelled according to the Gompertz equation (Zwietering et al., 1990): $y = k + A \exp\{-\exp[(V_{max}/A)(\lambda - t) + 1]\}$; where y is the acidification extent expressed as dpH/dt (units of pH/h) at the time t ; k is the initial level of the dependent variable to be modelled; A (ΔpH) is the difference in pH (units) between the initial value (pH₀) and the value reached in the stationary phase (pH_t) of the dough fermentation; V_{max} is the maximum acidification rate dpH/h , λ is the length of the latency phase of acidification expressed in hours, and t is the time. The experimental data were modelled through the nonlinear regression procedure of the statistic package Statistics 12.0 (StatSoft Inc., Tulsa, USA). Total titratable acidity (TTA) was measured on 10 g of sample (T_0 and T_f), which was homogenized with 90 ml of distilled water for 3 min in a Bag Mixer 400P (Interscience, St Nom, France), and expressed as the amount (ml) of 0.1 M NaOH to achieve the pH of 8.3.

2.5. Biochemical characterization of sourdough

Organic acids were determined by High Performance Liquid Chromatography (HPLC), using an ÅKTA Purifier system (GE Healthcare, Buckinghamshire, UK) equipped with an Aminex HPX-87H column (ion exclusion, Biorad, Richmond, CA), and an UV detector operating at 210 nm. Elution was at 60 °C, with a flow rate of 0.6 ml/min, using H₂SO₄ 10 mM as mobile phase (Rizzello et al., 2010a). The fermentation quotient (FQ) was determined as the molar ratio between lactic and acetic acids. Raffinose was determined by using the Megazyme kit Raffinose/D-Galactose Assay Kit K-RAFGA (Megazyme International Ireland Limited, Bray, Ireland), following the manufacturer's instructions. Amino acids and peptides concentrations were determined as reported above. Phytic acid concentration was measured using Megazyme test kit K-PHYT 05/07 (Megazyme) following the manufacturer's instructions. Phytase activity was determined on the WSE of flours and sourdoughs, by monitoring the rate of hydrolysis of *p*-nitrophenyl phosphate (*p*-NPP) (Sigma, 104-0). The assay mixture contained 200 μ l of 1.5 mM *p*-NPP (final concentration) in 0.2 M Na-acetate, pH 5.2, and 400 μ l of WSE. The mixture was incubated at 45 °C and the reaction was stopped by adding 600 μ l of 0.1 M NaOH. The *p*-nitrophenol released was determined by measuring the absorbance at 405 nm (Rizzello et al., 2010a). One unit (U) of activity was defined as the amount of enzyme required to liberate 1 μ mol/min of *p*-nitrophenol under the assay conditions. Condensed tannins were determined using the acid butanol assay, as described by Hagerman (2002). All data are expressed on dry weight basis.

2.6. Total phenols and antioxidant activity

Total phenols were determined on the methanolic extract (ME) of flours and sourdoughs. Five grams of each sample were mixed with 50 ml of 80% methanol to get ME. The mixture was purged with nitrogen stream for 30 min, under stirring condition, and centrifuged at 4600 \times g for 20 min. MEs were transferred into test tubes, purged with nitrogen stream and stored at ca. 4 °C before analysis. The concentration was determined as described by Slinkard and Singleton (1977) and expressed as gallic acid equivalent. The radical DPPH \cdot was used for determining the free radical scavenging activity (Rizzello et al., 2010a). The synthetic antioxidant butylated hydroxytoluene (BHT) was included in the analysis as the reference (75 ppm).

2.7. Trypsin inhibitor activity

Trypsin inhibitors were determined as described by Alonso et al. (2000), using α -N-benzoyl-DL-arginine-*p*-nitroanilidehydrochloride (BAPNA) as the substrate for trypsin. Trypsin inhibitor activity (TIA), expressed as trypsin inhibitor units/mg sample, was calculated from the absorbance read at 410 nm against a reagent blank. One trypsin unit was determined as the increase by 0.01 absorbance unit at 410 nm of the reaction mixture.

2.8. Bread making

Experimental breads (DY, 160) were manufactured at the pilot plant of the Department of Soil, Plant and Food Science of the University of Bari (Italy), according to the two-stage protocol commonly used for sourdough bread making (Rizzello et al., 2016). In particular, rF and sF obtained from the grains were fermented for 24 h at 30 °C with the mixed starters as described before (step I); then, rS and sS were mixed with wheat flour, water, and baker's yeast at 60 \times g for 5 min with an IM 58 high-speed mixer (Mecnosud, Flumeri, Italy) and incubated for 1.5 h at 30 °C (step II). The characteristics of the flour (*T. aestivum*, cv Appulo) used were the following: moisture, 14.2%; protein (N \times 5.70), 11.5% of d.m.; fat, 1.6% of d.m.; ash, 0.6% of d.m. and total soluble carbohydrates, 1.5% of d.m. In details, rS and sS were used at the percentage of 20% (w/w) of the total dough weight (Nionelli et al., 2014) and baker's yeast was added at the percentage of 1.5% w/w, corresponding to a final cell density of ca. 9 Log cfu/g in all the doughs only for the step II. At the end of step II, doughs were baked at 210 °C for 30 min (Combo 3, Zucchelli, Verona, Italy), obtaining breads fortified with raw and sprouted sourdoughs (rS-B and sS-B, respectively).

2.9. Textural and colour characteristics of breads

Instrumental Texture Profile Analysis (TPA) was carried out with a TVT-300XP Texture Analyzer (TexVol Instruments, Viken, Sweden), equipped with a cylinder probe P-Cy25S. For the analysis, boule shaped loaves (300 g) were baked, packed in polypropylene micro perforated bags and stored for 24 h at room temperature. Crust was not removed. The selected settings were the following: test speed 1 mm/s, 30% deformation of the sample and one compression cycle. TPA was carried out (Rizzello et al., 2012), using Texture Analyzer TVT-XP 3.8.0.5 software (TexVol Instruments). The following textural parameters were obtained by the texturometer software: hardness (maximum peak force), fracturability (the first significant peak force during the probe compression of the bread), and resilience (ratio of the first decompression area to the first compression area). Specific volume of breads was measured by the BVM-test system (TexVol Instruments). The chromaticity co-ordinates of the bread crust (obtained by a Minolta CR-10 camera) were also reported in the form of a colour difference, dE^*_{ab} , as follows:

$$dE^*_{ab} = \sqrt{(dL)^2 + (da)^2 + (db)^2}$$

where dL , da , and db are the differences for L , a , and b values between sample and reference (a white ceramic plate having $L = 93.4$, $a = -0.39$, and $b = 3.99$).

2.10. Nutritional characterization

The in vitro protein digestibility (IVPD) of flours, sourdoughs and breads was determined by the method proposed by Akeson and Stahmann (1964) with some modifications (Rizzello et al., 2016). Samples were subjected to a sequential enzyme treatment mimicking the in vivo digestion in the gastro intestinal tract and IVPD was expressed as the percentage of the total protein which was solubilized after enzyme hydrolysis. The concentration of protein of digested and

non-digested fractions was determined by the Bradford method (Bradford, 1976).

The analysis of starch hydrolysis was carried out on breads. The procedure mimicked the in vivo digestion of starch (De Angelis et al., 2009). Aliquots of breads, containing 1 g of starch, were subjected to enzymatic process and the released glucose content was measured with D-Fructose/D-Glucose Assay Kit (Megazyme). The degree of starch digestion was expressed as the percentage of potentially available starch hydrolysed after 180 min. Wheat flour bread (WB) leavened with baker's yeast was used as the control to estimate the hydrolysis index (HI = 100). The predicted GI was calculated using the equation: $GI = 0.549 \times HI + 39.71$ (Capriles and Arêas, 2013).

2.11. Sensory analysis

Sensory analysis of breads was carried out by 10 trained panellists (5 male and 5 female, mean age: 30 years, range: 18–54 years), according to the method described by Haglund et al. (1998) (Rizzello et al., 2010b). Elasticity, colour of crust and crumb, acidic taste, acidic smell, sweetness, dryness, and taste were considered as sensory attributes using a scale from 0 to 10, with 10 the highest score. Salty taste, previously described as another wheat sourdough bread attribute, was also included (Rizzello et al., 2010b). According to a preliminary sensory evaluation of breads (data not shown), the attribute “mouldy”, defined as the aroma associated with damp closed air spaces (Elia, 2011), was also evaluated. The sensory attributes were discussed with the assessors during the introductory sensory training sessions. Samples were served in random order and evaluated in two replicates by all panellists. Before the sensory evaluation, the loaves were thawed at room temperature for 5–6 h, then cut into slices 1.5 cm thick. Slices were cut into 4 pieces and each panellist received 2 pieces per sample.

2.12. Statistical analysis

All data of biochemical analyses were obtained at least in duplicates and each replicate was analysed twice. Data were subject to one-way ANOVA, using the IBM SPSS Statistics 26 (IBM Corporation, New York City, NY, United States) software.

3. Results

3.1. Raw and sprouted flours

3.1.1. Germination

Germinability was higher than 96% in almost all grains, with the exception of chickpea for which it was 90%. The optimal germination time was established based on the length of rootlets (ca. 3/4 of the medium seed length), before the seedling development (Fig. 1). According to different rate of germination, it was different among grains. In particular, quinoa reached the good rootlets length in one day and wheat, barley and lentil in three days. Chickpea completed this stage in five days.

3.1.2. Proximate composition and microbiological characterization

Proximate composition of raw and sprouted flours is reported in Table 1. As expected, large differences were found in protein concentration, that was the highest in chickpea and lentil flours, followed by quinoa and the two cereals. No significant differences were found in total protein concentration after germination, while an increase of sugars (up to ca. 10 folds) was always found (Table 1). In particular, quinoa had the highest concentration of sugars after germination. As expected, total starch decreased during germination reaching the lowest value in quinoa sF (Table 1). The concentration of TDF of legumes was higher than those found in other grains (Table 1). IDF were the main fraction of TDF, representing up to 90% of TDF in all grains. The IDF/SDF ratio ranged from 1.6 to 8.5. Germination led to a

decrease of IDF and an increase of SDF, improving the ratio IDF/SDF (0.8–6.7). A slight but not significant decrease of ash concentration was observed, probably due to the leaking of salts from grains during the soaking.

Mesophilic bacteria in rF were higher than 4 Log cfu/g, while moulds and yeasts were lower than 3 Log cfu/g. *Enterobacteriaceae* were markedly higher than 2.65 Log cfu/g only in lentil and quinoa rF (Table 1). Microbial profiles changed as consequence of the germination. These changes had different magnitudes depending on the grains and microbial group investigated. Mesophilic bacteria and yeasts number increased up to 3 Log cycles, while *Enterobacteriaceae* and presumptive LAB grown up to 4 Log cycles. The highest increase was observed in lentil (presumptive LAB) and chickpea (*Enterobacteriaceae*). Moulds remained almost constant during the germination in all grains (Table 1).

3.1.3. Protein derivatives and IVPD

Aiming at investigating the proteolysis occurring during germination, the profile of the organic nitrogen compounds in rF and sF was evaluated based on the analysis of peptides and TFAA concentrations. According to the peptide total peak area, derived from the RP-FPLC chromatograms (data not shown), lentil rF had the highest concentration of peptides, while in chickpea and quinoa it was 25 and 35%, respectively lower. Wheat and barley rF were characterized by the lowest peptide concentrations (data not shown). Overall, peptides increased during germination; in particular, sprouted lentil flour had the highest final concentration, while in barley sF the highest increase (ca. 1.6 folds) was observed in comparison with the corresponding rF. However, barley showed the lowest concentration of peptides in both raw and sprouted flours. Overall, the germination also caused significant increase of TFAA in all the grains considered (Table 2). Wheat had the highest increase; indeed, TFAA concentration in sF was ca. 10 folds higher than the raw one. The same was observed for quinoa, legumes and barley sF, that showed concentrations of ca. 4-, 2- and 1-fold higher than corresponding rF. Similar trend was found for the concentration of GABA (Table 2). All sF were characterized by a higher concentration of GABA than the corresponding rF. Although wheat flour was subjected to the most relevant increase (ca. 30 times), chickpea sF had the highest concentration of GABA (510 mg/kg) (Table 2). Quinoa showed the lowest increase of concentration of GABA during germination (ca. 8 folds).

According to the increase of peptides and TFAA, the IVPD was higher in sF compared to rF (Table 2), with the exception of chickpea where the IVPD was not affected by germination. Quinoa and barley showed the highest increases (ca. 80%) of IVPD during germination.

3.1.4. Total phenols and antioxidant activity

The effect of germination on total phenols concentration was examined after methanolic extraction. Chickpea and wheat rF had the lowest concentrations, while significantly higher values were found in lentil, and, especially, in barley and quinoa rF. When the ME of sF was analysed (Table 2) total phenols concentration was higher than the corresponding raw ones. The highest concentrations (> 5 mmol/kg) were observed in barley and quinoa sF. The antioxidant activity, determined on ME as radical DPPH scavenging activity, was relevant (> 80%) in barley, lentil and quinoa rF, and lower than 40% in wheat and chickpea. Together with the increase of the total phenols concentration in ME, also the antioxidant activity increased with the germination (Table 2). The highest radical scavenging activity was observed for quinoa, barley and lentil sF (> 90%). Although wheat and chickpea sF had the lowest activities, increases of ca. 2 fold and 60% were respectively found compared to the corresponding rF (Table 2). BHT, used as positive reference, gave a radical scavenging activity of 78%.



Fig. 1. Sprouted wheat (A), barley (B), chickpea (C), lentil (D) and quinoa (D) seeds.

3.1.5. Condensed tannins, trypsin inhibitory activity, and raffinose

Condensed tannins were found at concentration lower than 0.36 ± 0.02 mg/g in all the rF, with the only exception of lentil flour, characterized by concentration ca. 3-fold higher (Table 3). Overall, germination caused the decrease of condensed tannins concentration, with different magnitudes depending on the grains. In particular, lentil flour was subject to the highest decrease (ca. 66%), however it still contains the greater amount of condensed tannins. On the contrary, condensed tannins in quinoa, chickpea and barley were only weakly affected by germination, although in these flours they were at the lowest concentration (Table 3).

Cereal rF were characterized by the lowest values of trypsin inhibitory activity, while in lentil and chickpea rF ten times higher values were observed (Table 3). Germination caused a marked and significant decrease of the inhibitory activity in legumes and quinoa flours.

Together with the high concentration of condensed tannins, chickpea and lentil rF also contained the highest amount of raffinose (concentrations higher than 5.83 ± 0.05 g/100 g of flours). During germination raffinose significantly decreased in all the flours, with the exception of wheat, however characterized by the lowest initial concentration. Despite the degradation occurring during germination (corresponding to ca. 68% in chickpea and ca. 90% in lentil), legumes sF still contained the highest concentration of raffinose.

3.1.6. Phytic acid and phytase activity

Phytic acid in rF ranged from 0.19 ± 0.03 to 1.30 ± 0.05 g/100 g (Table 3). Overall, lower concentrations were found after germination, with the only exception of quinoa, in which no significant differences in phytic acid concentration were found between rF and sF. Lentil contained the highest amount in both rF and sF, while the highest decrease (ca. 50%) was observed in both the cereals. Accordingly, an increased phytase activity was found in all the sF compared to the corresponding

rF, with the exception of quinoa, in which the phytase activity remained stable. The highest increase of phytase activity was found in barley (ca. 4 U, Table 3).

3.2. Sourdough fermentation with selected LAB

3.2.1. Acidification

The parameters of the kinetics of acidification obtained by using a mixed starter including *L. rossiae* LB5, *L. plantarum* 1A7 and *L. sanfranciscensis* DE9 are reported in Table 4. Mixed starter showed Δ pH in the range of 1.96–2.39 and 1.49–1.90 in rS and sS, respectively. Similarly, λ was in the range of 5.43–6.96 and 7.52–8.06 in rS and sS, respectively. Overall, the use of sF as substrate for LAB fermentation corresponded to a decrease of the Δ pH and the increase of λ values. Final pH values of sourdoughs were in the range of 3.85–4.44 with no significant differences between rS and sS (Table 4), nevertheless significant differences in TTA values were found. TTA was higher in almost all of sS compared to the corresponding rS, with the exception of barley. As expected, the fermentation led to an increase of the TTA in both the sourdough types. Regardless the type of flour, the highest values were found in quinoa, while cereals showed the lowest values. Fermentation with mixed selected starter caused an increase of the concentration of both lactic and acetic acids. The magnitude of these changes depended on the grain species. With the exception of legumes, when sF were used as substrate for LAB fermentation, the concentration of lactic acid was lower than the corresponding rS. No significant differences were found for the acetic acid concentration between rS and sS (Table 4). FQ ranged from 4.8 ± 0.1 to 7.4 ± 0.2 , which is in accordance with values usually found in wheat type-I sourdough (Rizzello et al., 2010b).

Table 1
Proximate composition and microbiological characterization of raw (rF) and sprouted (sF) flours. Data are expressed on dry weight basis.

	rF					sF				
	Wheat	Barley	Chickpea	Lentil	Quinoa	Wheat	Barley	Chickpea	Lentil	Quinoa
Chemical composition										
Protein (%)	17.46 ± 0.45 ^d	14.02 ± 0.36 ^c	25.20 ± 0.48 ^b	30.07 ± 0.38 ^a	20.64 ± 0.39 ^e	17.34 ± 0.29 ^d	13.61 ± 0.32 ^e	24.82 ± 0.34 ^b	31.57 ± 0.43 ^a	18.25 ± 0.33 ^c
Fat (%)	1.11 ± 0.06 ^b	0.88 ± 0.04 ^b	3.28 ± 0.06 ^a	0.66 ± 0.03 ^c	3.31 ± 0.07 ^a	0.87 ± 0.03 ^b	1.50 ± 0.05 ^b	3.85 ± 0.08 ^a	0.76 ± 0.04 ^a	3.33 ± 0.10 ^a
Sugars (%)	5.54 ± 1.12 ^f	7.95 ± 1.02 ^f	14.4 ± 0.94 ^e	18.33 ± 0.95 ^d	6.4 ± 0.98 ^f	40.58 ± 1.05 ^b	48.61 ± 1.18 ^a	24.52 ± 0.78 ^e	22.48 ± 0.85 ^c	49.61 ± 0.96 ^a
Starch (%)	60.4 ± 1.1 ^a	55.4 ± 1.1 ^b	32.5 ± 1.08 ^c	27.6 ± 1.07 ^d	52.2 ± 1.21 ^b	31.5 ± 0.94 ^c	23.5 ± 1.05 ^e	24.2 ± 1.09 ^e	24.9 ± 1.17 ^e	15.7 ± 1.22 ^f
Total fibres (%)	15.35 ± 0.36 ^e	21.52 ± 0.32 ^b	24.30 ± 0.29 ^a	23.07 ± 0.37 ^a	17.22 ± 0.26 ^d	9.60 ± 0.21 ^g	12.64 ± 0.18 ^f	22.43 ± 0.27 ^a	20.08 ± 0.31 ^c	12.86 ± 0.19 ^f
SDF (%)	2.42 ± 0.11 ^e	2.93 ± 0.11 ^d	2.60 ± 0.10 ^e	2.44 ± 0.09 ^e	6.69 ± 0.21 ^a	3.13 ± 0.12 ^d	3.79 ± 0.11 ^e	5.53 ± 0.15 ^b	2.58 ± 0.07 ^e	7.02 ± 0.16 ^a
IDF (%)	12.93 ± 0.34 ^e	18.59 ± 0.25 ^b	21.70 ± 0.27 ^a	20.63 ± 0.32 ^a	10.53 ± 0.19 ^f	6.47 ± 0.13 ^f	8.85 ± 0.12 ^e	16.90 ± 0.23 ^b	17.50 ± 0.24 ^b	5.84 ± 0.15 ^f
Ash (%)	1.45 ± 0.10 ^d	2.46 ± 0.16 ^c	2.85 ± 0.15 ^b	3.24 ± 0.16 ^a	2.49 ± 0.10 ^c	1.31 ± 0.09 ^d	2.12 ± 0.10 ^c	2.64 ± 0.11 ^b	3.02 ± 0.12 ^a	1.93 ± 0.13 ^c
Microbiological characterization										
Mesophilic aerobic bacteria (Log cfu/g)	4.80 ± 0.12 ^e	4.82 ± 0.11 ^e	4.01 ± 0.11 ^f	5.86 ± 0.13 ^d	6.62 ± 0.13 ^c	6.86 ± 0.12 ^c	6.78 ± 0.14 ^c	7.32 ± 0.14 ^b	8.64 ± 0.13 ^a	7.21 ± 0.11 ^b
Yeast (Log cfu/g)	0.90 ± 0.11 ^d	2.03 ± 0.12 ^c	0.48 ± 0.09 ^e	2.30 ± 0.11 ^c	0.70 ± 0.06 ^d	3.22 ± 0.10 ^b	3.44 ± 0.10 ^b	2.31 ± 0.12 ^c	5.36 ± 0.15 ^a	2.55 ± 0.09 ^e
Moulds (Log cfu/g)	2.65 ± 0.13 ^b	3.27 ± 0.18 ^a	1.60 ± 0.07 ^d	2.88 ± 0.09 ^d	2.99 ± 0.10 ^a	2.00 ± 0.08 ^c	3.03 ± 0.12 ^a	1.93 ± 0.10 ^c	1.85 ± 0.09 ^e	1.85 ± 0.12 ^c
LAB (Log cfu/g)	0.85 ± 0.05 ^g	1.30 ± 0.11 ^f	1.71 ± 0.09 ^e	3.05 ± 0.12 ^d	0.78 ± 0.06 ^g	3.44 ± 0.11 ^c	3.09 ± 0.09 ^d	3.65 ± 0.11 ^b	7.04 ± 0.20 ^a	3.29 ± 0.14 ^c
Enterobacteriaceae (Log cfu/g)	2.08 ± 0.05 ^f	2.66 ± 0.08 ^e	2.65 ± 0.09 ^e	4.97 ± 0.11 ^d	5.70 ± 0.10 ^c	5.47 ± 0.12 ^c	5.69 ± 0.18 ^c	6.51 ± 0.14 ^b	7.65 ± 0.13 ^a	6.36 ± 0.21 ^b

SDF, soluble dietary fibres; IDF, insoluble dietary fibres; LAB, lactic acid bacteria. The data are the means of three independent experiments ± standard deviations (n = 3).
a–g Values in the same row with different superscript letters differ significantly (p < 0.05).

Table 2
Total free amino acids (TFAA), GABA and total phenols concentrations, radical scavenging activity and in vitro protein digestibility (IVPD) of raw and sprouted flours (rF and sF, respectively) and corresponding sourdoughs (rS and sS, respectively) (DY 160) started with *L. rossiae* LB5, *L. plantarum* 1A7 and *L. sanfranciscensis* DE9 and fermented at 30 °C for 24 h.

	rF					sF				
	Wheat	Barley	Chickpea	Lentil	Quinoa	Wheat	Barley	Chickpea	Lentil	Quinoa
TFAA (mg/kg)	760 ± 54 ^b	1475 ± 100 ^g	4747 ± 84 ^d	4205 ± 99 ^e	2262 ± 87 ^f	7033 ± 74 ^c	8119 ± 95 ^b	8250 ± 97 ^b	9515 ± 86 ^a	8034 ± 112 ^b
GABA (mg/kg)	5 ± 1 ^g	32 ± 2 ^e	63 ± 6 ^d	15 ± 1 ^f	59 ± 6 ^d	168 ± 12 ^c	352 ± 25 ^b	510 ± 41 ^a	151 ± 15 ^c	350 ± 29 ^b
Total phenols (mmol/kg)	0.80 ± 0.07 ^e	2.70 ± 0.27 ^c	0.49 ± 0.05 ^f	1.42 ± 0.10 ^d	3.28 ± 0.30 ^b	2.54 ± 0.20 ^c	5.21 ± 0.50 ^a	1.01 ± 0.10 ^d	2.90 ± 0.30 ^c	5.44 ± 0.50 ^a
Radical scavenging activity (%)	38 ± 2 ^c	84 ± 7 ^b	23 ± 2 ^d	80 ± 5 ^b	81 ± 7 ^b	82 ± 8 ^b	94 ± 9 ^a	37 ± 2 ^e	90 ± 8 ^a	94 ± 7 ^a
IVPD (%)	52.6 ± 2.0 ^f	54.4 ± 1.9 ^f	88.4 ± 1.1 ^a	61.7 ± 1.5 ^e	42.0 ± 1.7 ^g	78.9 ± 2.1 ^c	83.7 ± 1.6 ^b	87.7 ± 1.8 ^a	72.2 ± 1.9 ^d	75.8 ± 1.5 ^{c,d}
Sourdoughs										
	rS					sS				
	Wheat	Barley	Chickpea	Lentil	Quinoa	Wheat	Barley	Chickpea	Lentil	Quinoa
TFAA (mg/kg)	3670 ± 74 ^b	3193 ± 100 ^f	8455 ± 84 ^f	8027 ± 99 ^g	9984 ± 87 ^e	14,148 ± 74 ^b	13,603 ± 95 ^c	12,212 ± 97 ^d	13,873 ± 86 ^c	14,693 ± 112 ^a
GABA (mg/kg)	355 ± 35 ^g	538 ± 52 ^f	831 ± 83 ^c	272 ± 27 ^h	642 ± 65 ^e	1010 ± 54 ^b	969 ± 94 ^b	1195 ± 61 ^a	748 ± 65 ^d	878 ± 84 ^e
Total phenols (mmol/kg)	2.06 ± 0.20 ^e	1.92 ± 0.10 ^e	1.26 ± 0.10 ^f	2.82 ± 0.25 ^d	5.79 ± 0.50 ^b	3.46 ± 0.40 ^c	5.36 ± 0.50 ^b	2.18 ± 0.30 ^d	3.48 ± 0.30 ^c	6.63 ± 0.60 ^a
Radical scavenging activity (%)	75 ± 5 ^c	82 ± 7 ^b	37 ± 3 ^e	95 ± 8 ^a	90 ± 7 ^{a,b}	84 ± 3 ^b	97 ± 5 ^a	67 ± 2 ^d	97 ± 8 ^a	97 ± 9 ^a
IVPD (%)	79.5 ± 0.6 ^e	66.6 ± 0.5 ^f	89.6 ± 0.8 ^b	87.5 ± 0.8 ^c	66.8 ± 0.8 ^f	78.6 ± 0.9 ^e	85.5 ± 0.8 ^d	91.8 ± 1.1 ^a	87.0 ± 0.8 ^c	89.1 ± 0.7 ^b

The data are the means of three independent experiments ± standard deviations (n = 3). a–h Values in the same row with different superscript letters differ significantly (p < 0.05).

Table 3

Condensed tannins, trypsin inhibitor activity, raffinose, phytic acid and phytase activity of raw (rF) and sprouted (sF) flours. Data are expressed on dry weight basis.

	rF					sF				
	Wheat	Barley	Chickpea	Lentil	Quinoa	Wheat	Barley	Chickpea	Lentil	Quinoa
Condensed tannins (mg/g)	0.36 ± 0.02 ^c	0.26 ± 0.02 ^d	0.22 ± 0.02 ^e	1.07 ± 0.04 ^a	0.28 ± 0.02 ^d	0.20 ± 0.01 ^f	0.24 ± 0.02 ^d	0.18 ± 0.01 ^f	0.47 ± 0.02 ^b	0.18 ± 0.01 ^f
TIA*	0.51 ± 0.08 ^a	0.82 ± 0.2 ^b	11.21 ± 0.52 ^g	7.08 ± 0.64 ^f	3.45 ± 0.4 ^d	0.50 ± 0.08 ^a	0.78 ± 0.08 ^b	5.82 ± 0.5 ^e	5.80 ± 0.6 ^e	2.30 ± 0.3 ^c
Raffinose (g/100 g)	0.13 ± 0.05 ^e	0.71 ± 0.06 ^d	6.53 ± 0.07 ^a	5.83 ± 0.05 ^b	0.61 ± 0.03 ^d	0.11 ± 0.04 ^e	0.04 ± 0.03 ^f	2.14 ± 0.04 ^c	0.58 ± 0.03 ^d	0.06 ± 0.02 ^f
Phytic acid (g/100 g)	0.77 ± 0.03 ^d	0.73 ± 0.04 ^d	0.91 ± 0.05 ^c	1.30 ± 0.05 ^a	0.19 ± 0.03 ^g	0.39 ± 0.04 ^f	0.36 ± 0.03 ^f	0.58 ± 0.04 ^e	1.13 ± 0.05 ^b	0.16 ± 0.02 ^g
Phytase activity (U)**	4.21 ± 0.2 ^f	1.82 ± 0.1 ^{d,e}	0.22 ± 0.1 ^a	0.83 ± 0.1 ^b	1.73 ± 0.1 ^{d,e}	6.22 ± 0.2 ^b	5.90 ± 0.1 ^g	1.10 ± 0.1 ^c	1.93 ± 0.1 ^e	1.61 ± 0.1 ^d

The data are the means of three independent experiments ± standard deviations (n = 3).

^{a-h}Values in the same row with different superscript letters differ significantly (p < 0.05).

* Trypsin inhibitor activity, expressed as trypsin inhibitor units/mg sample.

** One unit (U) of activity was defined as the amount of enzyme required to liberate 1 μmol/min of p-nitrophenol under the assay conditions.

3.2.2. Protein derivatives and IVPD

The effect of the sourdough fermentation on the protein fraction and IVPD of rF and sF was investigated. The peptide concentration in rS slightly increased during fermentation (up to 30% compared to T₀), with the only exception of wheat sourdough, in which the final concentration was 1.3 folds higher than that found before the fermentation. When sF was used, a similar trend was found: indeed, the fermentation caused increases ranging from 25 to 37%, with the only exception of wheat, in which the final concentration was ca. 2 fold higher than the corresponding T₀. TFAA and GABA concentrations increased during fermentation in both type of sourdoughs (Table 2). In particular, the relative increases were higher when rF were fermented, although the highest final concentrations were found in the sS (Table 2). Among the rS, the highest increase of TFAA during fermentation was found for wheat (ca. 5 folds higher than T₀), while the highest concentration was observed in quinoa. TFAA in sS was, in all the cases, in the narrow range 12–14 g/kg (Table 2).

Overall, GABA concentration markedly increased during fermentation of both types of flour. In rS, GABA was in the range 272 to 831 mg/kg, and the highest value was found for chickpea, followed by quinoa. GABA in sS was higher than 748 mg/kg, and also in this case chickpea

had the highest concentration (Table 2).

The fermentation affected the IVPD, which increased for almost all the flours, with the exception of wheat sF in which it did not vary significantly. The highest increases were observed for lentil (26% and 15%, respectively for rF and sF) and quinoa (24% and 14%, respectively for rF and sF) (Table 2).

3.2.3. Total phenols and antioxidant activity of fermented doughs

The concentration of total phenols in the ME of rS and sS are reported in Table 2. Overall, fermentation led to a further increase of the concentration and sS had significantly higher concentration than rS (from 1.2 to 2.8 folds). The highest increase of total phenols (ca. 3 folds) was observed when wheat rF was fermented, while a slightly lower concentration was found in barley rF (Table 2). When sF were fermented, significant increases were found compared to T₀. Quinoa sS had the highest concentration of total phenols, while chickpea had the lowest (Table 2). Antioxidant activity of the sourdoughs is reported in Table 2. Overall, sS showed antioxidant activity higher than rS. Among the rS, the highest increases during fermentation were observed for wheat (ca. 2 folds) and chickpea (ca. 70%). Lower increases were found for lentil and quinoa, while the radical scavenging activity of wheat rF

Table 4

Parameters of the kinetics of acidification, pH, TTA, concentration of lactic and acetic acids and fermentation quotient (FQ), of raw (rF) and sprouted (sF) flours sourdoughs (DY 160) started with *L. rossiae* LB5, *L. plantarum* 1A7 and *L. sanfranciscensis* DE9 and fermented at 30 °C for 24 h. pH and TTA of doughs before fermentation (T₀) were also reported.

	rS					sS				
	Wheat	Barley	Chickpea	Lentil	Quinoa	Wheat	Barley	Chickpea	Lentil	Quinoa
Kinetics of acidification										
ΔpH (pH units)	2.39 ± 0.09 ^a	1.97 ± 0.11 ^b	1.84 ± 0.08 ^c	1.96 ± 0.07 ^b	1.97 ± 0.08 ^b	1.90 ± 0.09 ^b	1.70 ± 0.06 ^d	1.49 ± 0.05 ^e	1.62 ± 0.05 ^d	1.55 ± 0.04 ^e
Vmax (ΔpH/h)	0.45 ± 0.04 ^b	0.30 ± 0.02 ^c	0.27 ± 0.03 ^c	0.31 ± 0.02 ^c	0.29 ± 0.02 ^c	0.29 ± 0.02 ^c	0.27 ± 0.02 ^c	0.31 ± 0.02 ^c	1.21 ± 0.07 ^a	0.28 ± 0.01 ^c
λ (h)	5.43 ± 0.12 ^f	5.90 ± 0.11 ^e	6.22 ± 0.13 ^d	6.96 ± 0.12 ^c	6.37 ± 0.13 ^d	7.81 ± 0.15 ^a	8.06 ± 0.14 ^a	7.57 ± 0.12 ^b	7.52 ± 0.13 ^b	7.55 ± 0.09 ^b
Acidification capacity										
pH (T ₀)	6.24 ± 0.09 ^a	5.90 ± 0.07 ^b	6.20 ± 0.08 ^a	6.18 ± 0.07 ^a	6.12 ± 0.06 ^a	5.98 ± 0.09 ^b	5.69 ± 0.10 ^c	5.95 ± 0.07 ^b	5.99 ± 0.06 ^b	5.67 ± 0.08 ^c
pH (TF)	3.85 ± 0.06 ^d	3.95 ± 0.05 ^c	4.39 ± 0.04 ^a	4.23 ± 0.07 ^b	4.18 ± 0.04 ^b	3.98 ± 0.05 ^d	4.00 ± 0.06 ^c	4.44 ± 0.05 ^a	4.39 ± 0.11 ^b	4.13 ± 0.05 ^b
TTA (T ₀)	4.0 ± 0.3 ^f	12.0 ± 0.5 ^a	4.2 ± 0.3 ^f	6.6 ± 0.4 ^e	2.8 ± 0.2 ^g	9.6 ± 0.5 ^c	8.4 ± 0.4 ^d	10.8 ± 0.4 ^b	8.2 ± 0.2 ^d	7.8 ± 0.3 ^d
TTA (TF)	12.8 ± 0.7 ^f	14.5 ± 0.6 ^f	19.8 ± 0.5 ^c	17.6 ± 0.6 ^e	26.6 ± 0.8 ^a	18.0 ± 0.4 ^d	16.0 ± 0.4 ^e	21.4 ± 0.5 ^c	21.0 ± 0.6 ^c	24.2 ± 0.5 ^b
Lactic acid (mmol/kg)	72 ± 2 ^d	83 ± 3 ^b	84 ± 3 ^{a,b}	79 ± 2 ^{b,c}	83 ± 3 ^b	70 ± 2 ^d	74 ± 3 ^{c,d}	95 ± 4 ^a	91 ± 3 ^a	74 ± 3 ^{c,d}
Acetic acid (mmol/kg)	15 ± 1 ^{a,b}	13 ± 2 ^{b,c}	17 ± 1 ^a	15 ± 2 ^{a,b}	15 ± 2 ^{a,b}	10 ± 1 ^c	10 ± 1 ^c	15 ± 3 ^{a,b}	17 ± 2 ^a	12 ± 1 ^c
FQ	4.8 ± 0.1 ^e	6.4 ± 0.2 ^c	4.9 ± 0.2 ^c	5.3 ± 0.1 ^e	5.5 ± 0.2 ^d	7.0 ± 0.2 ^b	7.4 ± 0.2 ^a	6.3 ± 0.3 ^d	5.3 ± 0.2 ^d	6.1 ± 0.3 ^c

The data are the means of three independent experiments ± standard deviations (n = 3).

^{a-f}Values in the same row with different superscript letters differ significantly (p < 0.05).

FQ, fermentation quotient, molar ratio between lactic and acetic acids.

Table 5

Condensed tannins, trypsin inhibitor activity, and raffinose, phytic acid and phytase activity of raw (rS) and sprouted (sS) flours sourdoughs (DY 160) started with *L. rossiae* LB5, *L. plantarum* 1A7 and *L. sanfranciscensis* DE9 and fermented at 30 °C for 24 h. Data are expressed on dry weight basis.

	rS					sS				
	Wheat	Barley	Chickpea	Lentil	Quinoa	Wheat	Barley	Chickpea	Lentil	Quinoa
Condensed tannins (mg/g)	0.10 ± 0.01 ^d	0.20 ± 0.01 ^b	0.15 ± 0.01 ^c	0.72 ± 0.01 ^a	0.18 ± 0.01 ^b	0.14 ± 0.01 ^c	0.21 ± 0.01 ^b	0.14 ± 0.01 ^c	0.20 ± 0.01 ^b	0.14 ± 0.01 ^c
TIA*	0.25 ± 0.02 ^f	0.20 ± 0.03 ^f	2.57 ± 0.21 ^a	1.92 ± 0.09 ^b	0.93 ± 0.07 ^c	nd	nd	0.91 ± 0.08 ^c	0.75 ± 0.06 ^d	0.42 ± 0.05 ^e
Raffinose (g/100 g)	0.03 ± 0.01 ^e	0.11 ± 0.01 ^d	2.32 ± 0.05 ^b	2.75 ± 0.04 ^a	nd	nd	nd	0.23 ± 0.03 ^c	0.03 ± 0.01 ^e	nd
Phytic acid (g/100 g)	0.33 ± 0.01 ^d	0.37 ± 0.02 ^d	0.78 ± 0.05 ^b	1.07 ± 0.04 ^a	0.14 ± 0.02 ^c	0.31 ± 0.02 ^d	0.34 ± 0.02 ^d	0.48 ± 0.03 ^c	1.00 ± 0.03 ^a	0.11 ± 0.01 ^e
Phytase activity (U)**	4.59 ± 0.21 ^b	2.85 ± 0.08 ^c	1.93 ± 0.11 ^d	4.26 ± 0.41 ^b	1.77 ± 0.08 ^d	6.32 ± 0.03 ^a	6.00 ± 0.02 ^a	1.04 ± 0.12 ^e	1.92 ± 0.16 ^d	1.40 ± 0.05 ^e

The data are the means of three independent experiments ± standard deviations (n = 3). nd: not detected.

^{a-f}Values in the same row with different superscript letters differ significantly (p < 0.05).

* Trypsin inhibitor activity, expressed as trypsin inhibitor units/mg sample.

** One unit (U) of activity was defined as the amount of enzyme required to liberate 1 μmol/min of *p*-nitrophenol under the assay conditions.

did not change during fermentation (Table 2). When sF were fermented, antioxidant activity slightly increased (< 10%), and only for chickpea an increase of ca. 80% occurred.

3.2.4. Condensed tannins, trypsin inhibitor activity, and raffinose

Although with different magnitudes, changes of antinutritional factors (ANFs) were found during the fermentation (Table 5). Condensed tannins slightly decreased in all sourdoughs regardless the type of flour used. The highest concentration was found in lentil rS, but the sS had a significantly lower final concentration.

Fermentation affected the trypsin inhibitor activity, which decreased in all sourdoughs, especially in those obtained with legume rF, characterized by the highest concentration before fermentation. When sF were fermented, TIA was not detectable in cereals, and it was lower than 1 U/mg in the other sS (Table 5).

A significant decrease of the raffinose concentration was observed during the fermentation of all the flours. Legume rS contained the highest concentration of raffinose, in both cases higher than 2 g/100 g of doughs. In sS, raffinose was not detectable in wheat, barley and quinoa, while it was lower than 0.51 g/100 g in chickpea and lentil (Table 5).

3.2.5. Phytic acid and phytase activity

Phytic acid slightly decreased during fermentation, and only when wheat and barley were used, decreases were higher than 50% compared to not fermented rF (Table 5). Lentil rS and sS showed the highest amount, while the lowest concentration was observed in quinoa rS and sS. No significant differences were found in final concentration of phytic acid in rS and sS of wheat, barley and quinoa. After fermentation of rF, the phytase activity significantly increased in chickpea (ca. 1.7 U), lentil (ca. 3.4 U) and, to a lesser extent in cereals (ca. 0.3–1.0 U), whereas it remained constant in quinoa. On the contrary, phytase activity in sS was similar to that found in corresponding sF (Tables 3 and 5).

3.3. Breads characterization

rS and sS were used as ingredient to fortify a wheat flour bread. No significant differences in the specific volume of breads were found (Table 6). However, the value of hardness, corresponding to the peak force of the first compression of the product, was up to 23% higher in sS-B compared to rS-B (Table 6). Among the breads containing rS, the lowest hardness corresponded to wheat rS-B, while quinoa led to the highest. The same was found when breads containing sS were considered. The use of sS led to a decrease of the resilience values

compared to the corresponding rS-B, with the exception of quinoa rS-B and sS-B, in which similar values were found (Table 6). Overall, the use of chickpea rS and sS led to the lowest resilience values.

Similarly to hardness, fracturability of breads made with sS was higher compared to breads fortified with the corresponding rS (Table 6). In particular, fermented quinoa corresponded to the highest values for fracturability, while the use of cereals led to the lowest values. Nevertheless, slight differences of fracturability among the breads were found when sS were added.

Overall, final IVPD of the breads was markedly affected by the digestibility of the sourdough used as ingredient. Among the breads fortified with rS, those containing quinoa and barley had the lowest values, while chickpea and lentil addition led to IVPD higher than 73%. As expected, digestibility was higher when sS were used (Table 6) compared to the correspond rS-B; in particular, the highest increases (ca. 16%) were found for breads enriched with barley and quinoa sS.

The predicted GI of all the breads was lower than the reference bread. Overall, the values of those made with rS were lower than the corresponding including sS. In particular, the use of rF allowed the obtaining of breads having GI lower than 60%, and the use of rS gave GI slightly but significantly higher (Table 6). Fermented quinoa led to the highest GI in both the formulations, but also when sF was used, the value was lower than ca.77% (Table 6).

The results for bread sensory analysis are shown in Fig. 2A–B. Overall, elasticity and dryness (perceived as how much the bread crumbles in the mouth) of the sS-B were slightly lower than rS-B. The scores for the colour of the crust and the crumb were significantly higher in sS-B compared to rS-B. The same was found for the acidic attributes. In particular, a markedly higher perception of both acidic smell and taste was observed in chickpea sS-B compared to the corresponding rS-B, and the same was observed for the salty taste. No significant differences were found in sweetness and salty taste of the other rS-B in comparison with the corresponding sS-B.

The overall taste, considered as a global index of the palatability, was higher in wheat, barley, and lentil sS-B, compared to the corresponding rS-B and lower in chickpea and quinoa sS-B. In particular, the most appreciated bread was the wheat sS-B, while the lowest score corresponded to chickpea sS-B. This latter was probably affected by the mouldy aroma perception.

4. Discussion

Cereals and legumes are the most important contributors to the balanced human diet worldwide, providing significant amount of macro- and micro-nutrients. Besides such grains, pseudocereals (e.g.,

Table 6

Specific volume, textural analysis, IVPD and predicted glycemic index of raw (rS-B) and sprouted (sS-B) sourdough breads stated with 20% (w/w) of raw (rS) and sprouted (sS) sourdoughs (DY 160). Baker's yeast was added at the percentage of 1.5% w/w.

	rS-B					sS-B				
	Wheat	Barley	Chickpea	Lentil	Quinoa	Wheat	Barley	Chickpea	Lentil	Quinoa
Technological properties										
Specific volume (cm ³ /g)	1.94 ± 0.03 ^a	1.97 ± 0.05 ^a	1.93 ± 0.02 ^a	1.98 ± 0.06 ^a	1.89 ± 0.06 ^a	1.99 ± 0.05 ^a	1.92 ± 0.04 ^a	1.94 ± 0.04 ^a	1.96 ± 0.6 ^a	1.90 ± 0.04 ^a
Hardness (g)	4283 ± 32 ^d	4934 ± 21 ^c	5199 ± 13 ^c	5471 ± 23 ^b	5985 ± 17 ^c	5256 ± 21 ^c	5678 ± 33 ^b	5834 ± 15 ^b	5607 ± 46 ^c	6420 ± 43 ^a
Resilience	0.44 ± 0.05 ^a	0.39 ± 0.06 ^{a,b}	0.19 ± 0.03 ^c	0.29 ± 0.04 ^b	0.22 ± 0.02 ^c	0.31 ± 0.02 ^b	0.21 ± 0.02 ^c	0.18 ± 0.03 ^c	0.19 ± 0.02 ^c	0.22 ± 0.02 ^c
Fracturability (J)	4189 ± 67 ^f	4015 ± 54 ^f	5032 ± 78 ^e	5300 ± 88 ^c	2485 ± 45 ^e	5239 ± 49 ^d	5203 ± 38 ^d	5581 ± 47 ^b	5318 ± 35 ^c	5833 ± 40 ^a
Nutritional properties										
IVPD (%)	68.6 ± 1.1 ^{b,c}	65.5 ± 0.9 ^c	74.8 ± 1.3 ^a	73.3 ± 1.2 ^a	64.6 ± 1.0 ^c	70.5 ± 0.9 ^b	72.8 ± 1.2 ^{a,b}	76.8 ± 1.2 ^a	74.0 ± 1.0 ^a	75.1 ± 0.9 ^a
GI	63.2 ± 0.6 ^d	61.3 ± 0.5 ^c	56.5 ± 0.6 ^f	57.7 ± 0.7 ^f	72.2 ± 0.8 ^b	66.2 ± 0.7 ^c	63.3 ± 0.6 ^d	63.6 ± 0.5 ^d	65.2 ± 0.7 ^c	76.4 ± 0.5 ^a

The data are the means of three independent experiments ± standard deviations (n = 3).

^{a-d}Values in the same row with different superscript letters differ significantly (p < 0.05).

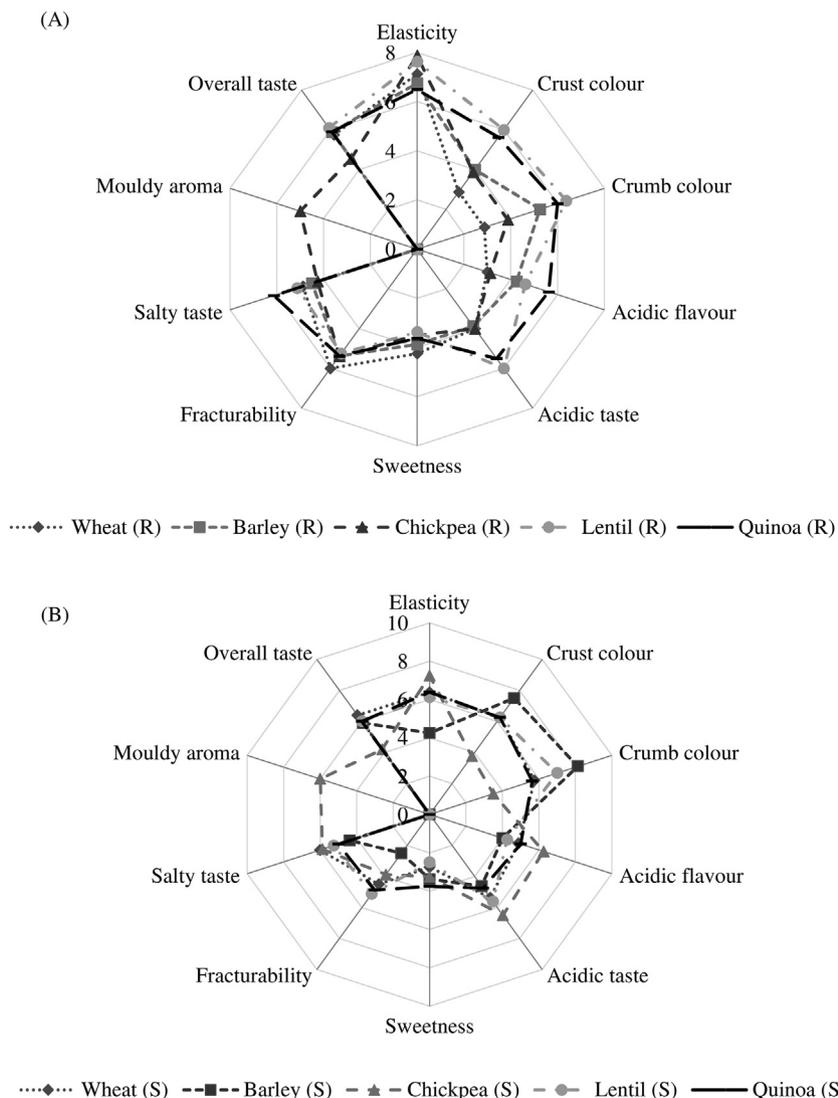


Fig. 2. Spider web chart of the sensory analysis data for experimental wheat, barley, chickpea, lentil and quinoa raw (rS-B) (panel A) and sprouted (sS-B) (panel B) sourdough breads produced with 20% (w/w) of raw (rS) and sprouted (sS) sourdoughs (DY 160). Baker's yeast was added at the percentage of 1.5% w/w.

quinoa, amaranth, buckwheat), which already had an important role in human nutrition in the past, are gaining popularity thanks to their remarkable technological and nutritional properties (Alvarez-Jubete et al., 2009). Despite their large use as food ingredient, the nutritional quality of grains and derived products is often affected by lacking of some components (e.g. dietary fibres, high biological value proteins) and sometimes it is lowered by the presence of ANFs (such as phytic acid, condensed tannins, raffinose and trypsin inhibitors) (Chavan and Kadam, 1989). Due to the raised attention in healthy nutrition and the large growth of the market of foods designed for vegetarian, vegan, and gluten-free diets (Gobbetti et al., 2017; McGirr et al., 2017), there has been an increased interest in improving the nutritional quality of grains-derived ingredients to be used in food preparation.

Germination has been recognized as an effective method to enhance the nutritional quality of whole grains, since significant changes in nutritional and physicochemical properties occur in seeds due to the activation of dormant enzymes (Donkor et al., 2012). Germinated seeds are considered nutritionally superior (Donkor et al., 2012), however, the effects of such process on the nutritional, technological and sensory properties of seeds and derived flours, dependent on the species considered and the process conditions, are only partially investigated.

Similarly to germination process, sourdough fermentation, thanks to the role played by LAB within its microbial consortium, offers a large number of advantages, including enhancement of nutritional value and functional properties and decrease of ANFs of wheat and non-wheat flours (Curriel et al., 2015; Gobbetti et al., 2014; Rizzello et al., 2016; Verni et al., 2017).

In this study, the combined effect of germination and sourdough fermentation was investigated on five species, representative of the three main categories of grain crops: cereals (wheat and barley), legumes (chickpea and lentil), pseudocereals (quinoa). The germination conditions used in this study were comparable to those used for industrial malting of barley, indeed it was extended until the rootlets length was ca.75% of the medium seed length, thus when a proper hydrolytic enzymatic activity is achieved (Katina et al., 2007; Gupta et al., 2010). Depending on grains used, one to five days were necessary. Aiming at keeping low the variable number, the same germination conditions were applied to all the grains, although a further optimization of the process parameters, i.e., temperature and humidity, may affect the enzymatic activities accordingly.

Drying conditions, mainly temperature and treatment time, affect colour, enzymatic activity and sensory profile of the processed grains. In this study, a low temperature drying was employed for all sprouted grains, to limit the temperature-related outcomes, preserve enzymatic activity and to avoid intense caramelization and Maillard reaction (Katina et al., 2007). Flours obtained from milled whole germinated grains were characterized for the main microbiological and chemical features. Microbial cell density of rF was lower than ca. 6.0 Log cfu/g and due to the disinfection of the grain surface prior the germination, their microbiota was mainly represented by endophytes. Optimal conditions for microbial growth occurred during the germination (Yang et al., 2013) leading to an increase of microbial cell density. *Enterobacteriaceae* reached values higher than 6.0 Log cfu/g in chickpea, lentil and quinoa sF. Indeed, it can be hypothesized that drying conditions were not critical for microbial viability. Several authors already observed microbial loads in seeds between 3.0 and 6.0 Log cfu/g; and 2 or 3 Logs greater counts in germinated grains (Yang et al., 2013). National Advisory Committee on Microbiological Criteria for Foods (NACMCF) recommendations to improve the microbial safety of sprouted seeds and flours should be applied if sprouted grains are intended for the market (Ding et al., 2013).

Although the activation of proteases characterizes the germination (Bewley, 2001) no significant differences in total crude protein content was found in comparison with the corresponding raw whole flours. Nevertheless, the Kjeldahl estimation, based on a total organic nitrogen calculation, is not suitable to investigate the degree of proteolysis

occurred. For this reason, the peptide and TFAA were further analysed and their concentrations were found higher after the germination. The increase of TFAA during germination was already reported for barley, wheat, oat, and rice (Singh et al., 2015). Mainly due to the protein proteolysis and polypeptides solubilisation, which usually occur during sprouting (Bewley, 2001), IVPD of sF was significantly higher than rF (up to ca. 80%). Among FAA, relevant increases of GABA were found. GABA is a non-protein amino acid that is primarily produced from the decarboxylation of L-glutamic acid which possesses well-known physiological functions such as neurotransmission, induction of hypotension, diuretic and tranquilizer effects (Wong et al., 2003). Increases of GABA during germination were already observed in cereals, pseudocereals, and legumes (Donkor et al., 2012; Kuo et al., 2004). Under the study conditions, GABA was higher than 300 mg/kg in barley, chickpea and quinoa.

Many health benefits related to the regular consumption of whole cereals and legumes have been attributed to their high content of DF, and their inclusion in diet is recommended aiming at increasing the daily DF intake (Slavin et al., 2013). During germination, the ratio between IDF and SDF changed (Benítez et al., 2013; Donkor et al., 2012; Mäkinen and Arendt, 2015). In particular SDF, able to slow intestinal transit, delay gastric emptying, and reduce glucose and sterol absorption by the intestine (Charalampopoulos et al., 2002) increased, while IDF and TDF decreased due to the activity of endogenous enzymes. Among others, germination causes an increase of the α -amylase activity (Ghavidel and Prakash, 2007) which lead to a total starch loss. This may explain the significant increases of sugars found in sF samples.

The concentration of total phenols significantly increase during germination in all flours, probably due to a better extractability of phenolic compounds from the seed altered tissues (Singh et al., 2015) and as the consequence of increased activity of cell wall degrading enzymes such as xylanases and cellulases (Katina et al., 2007). As previously reported (Cevallos-Casals and Cisneros-Zevallos, 2010), the antioxidant activity increased during the germination, according to the higher solubilisation of total phenols. Germination has been identified as an inexpensive and effective technology to enhance legumes and cereals digestibility (Mubarak, 2005; Tian et al., 2010), through the decrease of ANFs impact (Benítez et al., 2013). Indeed, the bioavailability of different compounds of the whole grains with nutritional and functional importance is still negatively affected by the presence of condensed tannins, phytic acid, trypsin inhibitors and flatulence-causing oligosaccharides (Curriel et al., 2015; Khattab and Arntfield, 2009; Singh et al., 2015). According to the most recent literature (Singh et al., 2015) and regardless the type of flour, germination caused a decrease of all ANFs. Condensed tannins inhibit digestive enzymes and thereby lower digestibility of most nutrients, especially protein (Khattab and Arntfield, 2009). Similarly, trypsin inhibitors strongly inhibit trypsin activity reducing the digestion and absorption of dietary protein (Norton, 1991). Trypsin inhibitors were found in all the rF with the highest concentration in chickpea and lentil. Condensed tannins concentration was significantly lower in almost all the sF compared to the corresponding rF, and a relevant decrease of the TIA was observed for all flours during germination. The partial degradation of tannins during germination as results of endogenous enzyme degradation was already reported (Helmalatha et al., 2007), while the trypsin inhibitors degradation, although still debated (Sangronis and Machado, 2007) can be observed during the early stages of germination (Burbano et al., 1999).

Although with different magnitudes, germination caused increase of phytase activity and led to a significant degradation of phytic acid concentration. Phytic acid acts as ANF binding essential dietary minerals as well as proteins and starch, and consequently reducing their bioavailability (Gobbetti et al., 2014). Enhanced endogenous phytase activity and reduced concentration of phytic acid were already reported in germinated cereals, pseudocereals and legumes (Egli et al., 2002). It can be hypothesized that the degradation of condensed tannins, trypsin

inhibitors and phytic acid contributed to the increase of IVPD occurred during germination. Similarly, raffinose was significantly lower in sS compared to rF. As expected (Curriel et al., 2015), legumes contained the highest amount prior and after the germination and its presence is one of the major limitation to their use in animal and human nutrition (Wang et al., 2003). Raffinose is not digested by pancreatic enzymes but metabolized by gas-producing bacteria in the large intestine, thus causing disorders such as flatulence (Rizzello et al., 2010a). Nevertheless, its concentration decreased up to 90% in legumes sF and undetectable levels were observed in barley and quinoa. The intensity of the degradation among flours can be attributed to the different endogenous α -galactosidase activity commonly observed in seeds of different species and cultivar (Shimelis and Rakshit, 2007).

Sourdough-like fermentation, carried out by the use of selected LAB, has largely been reported as a suitable tool to enhance nutritional and functional properties of different flours (Coda et al., 2014; Curriel et al., 2015; Gobetti et al., 2014), and to improve the technological, sensory, and nutritional features of fortified wheat-based foods like bread and pasta (Coda et al., 2017; Rizzello et al., 2016). In this study, the combination of germination and fermentation has been evaluated. In particular, rF and sF were fermented with selected LAB and changes of nutritional and functional properties of flours were determined. Strains of *L. plantarum*, *L. rossiae* and *L. sanfranciscensis* were chosen since these species are frequently reported as dominant in sourdough products (Gobetti et al., 2016). Along with the commonly chemical changes typical of the sourdough fermentation (e.g., decrease of pH and increase organic acids concentration), functional and nutritional improvements were observed. The combined effect of the flour endogenous proteases and LAB peptidases (Gänzle, 2014) led to a TFAA concentration higher in fermented than not fermented flours. As previously found, also GABA significantly increased (Curriel et al., 2015; Rizzello et al., 2016). Overall, sS were characterized by higher concentration of the TFAA and GABA compared to the corresponding rS. The markedly higher concentration of peptides and TFAA reflected on IVPD which was higher in sS than rS and, in all cases, higher in sourdoughs compared to the corresponding not fermented rF and sF.

Similar trend was found for total phenols, as quantified in the ME extract. LAB fermentation was already suggested as tool to improve the total phenols bioavailability of cereals (Gobetti et al., 2014), pseudocereals (Coda et al., 2014) and legumes (Curriel et al., 2015). The acidification improves their extraction, and feruloyl-esterase and β -glucosidase activities, already described for LAB (Nionelli et al., 2014), contributes to the release of phenols from bound and more complex forms. Higher concentration of total phenols was found in sS compared to rS; this difference may be due to the increased activity of cell wall degrading enzymes and enzyme active microbes occurring during germination (Katina et al., 2007; Laitila et al., 2006). Decreases of ANFs in flours of different origin have largely been achieved with sourdough fermentation (Coda et al., 2015; Nionelli et al., 2018; Rizzello et al., 2010a, 2012, 2016). Trypsin inhibitors and tannins are, among other ANFs, responsible for the low bioavailability of grains proteins. According to the results reported for fermented legumes (Coda et al., 2015; Curriel et al., 2015), cereals (Roger et al., 2015) and other non-wheat flours (Nionelli et al., 2018) microbial activities and, especially, LAB fermentation consistently decreased the levels of condensed tannins. Together with the proteolytic activity of the LAB, this may explain the increase of the protein digestibility. TIA decreased during fermentation more in sS than rS, thus confirming a contribution of the germination on the degradation of the trypsin inhibitors. The TIA decrease was already observed in fermented cereals, legumes (Starzyńska-Janiszewska and Stodolak, 2011), milling by-products (Rizzello et al., 2010a), pseudocereals (Rizzello et al., 2016) and other non-conventional flours (Nionelli et al., 2018), depending on the specific capability of the LAB strains involved in fermentation (Waters et al., 2015).

Different α -galactosides, such as raffinose, which are abundant in legumes, can be enzymatically hydrolysed by LAB during fermentation

(Coda et al., 2017), thus increasing product digestibility and reducing digestive discomfort (Waters et al., 2015). Fermentation with selected LAB significantly decreased the raffinose in both rF and sF, however an higher decrease was observed when sF were used as substrate.

Despite the improvement in nutritional and functional features, good sensory and texture properties still remain an essential requisite for baked goods. For this reason, sourdoughs obtained with rF and sF were used to fortify wheat breads that were subjected to a sensory analysis. According to the most recent literature (Coda et al., 2017; Nionelli et al., 2018; Rizzello et al., 2016) breads fortified with non-wheat rS were more appreciated than wheat rS-B, and all received scores for acidic taste and smell comparable to those found in literature for common sourdough breads (Nionelli et al., 2018). When sS were used, almost all the breads (with the exception of chickpea and wheat sS-B) obtained a similar evaluation to that obtained for the corresponding rS-B, for all the attributes considered. The sensory profile of chickpea sS-B was negatively affected by the perception of unpleasant off-flavours (mouldy), while that of wheat sS-B was judged as the more appreciated and balanced by the panellists.

IVPD of the fortified wheat breads were analysed, founding higher values in sS-B compared to rS-B since it is affected by the more intense proteolysis and degradation of the protein-ANF complexes allowed by the synergistic effects of germination and LAB fermentation.

Also the predicted GI was investigated. It is well known that sourdough fermentation can decrease the starch hydrolysis during digestion thanks to biological acidification (De Angelis et al., 2009). Due to the appreciable increase of sugars during the germination, sS-B showed higher GI as compared to the corresponding rS-B.

5. Conclusion

Germination and fermentation are safe, cheap, and traditional methods used to improve the functional and nutritional properties of whole grains and flours allowing a pleasant sensory improvement. This study demonstrates that, thanks to the LAB metabolism, fermentation further enhanced the nutritional and functional features of sprouted flours, through the release of peptides and free amino acids, phenolic compounds and soluble fibres, and leading to the intense decrease of several whole grains ANFs. Breads fortified with five fermented sprouted flours showed peculiar sensory profiles, and, compared to conventional wheat flour bread, they were overall characterized by high protein digestibility and low starch availability.

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