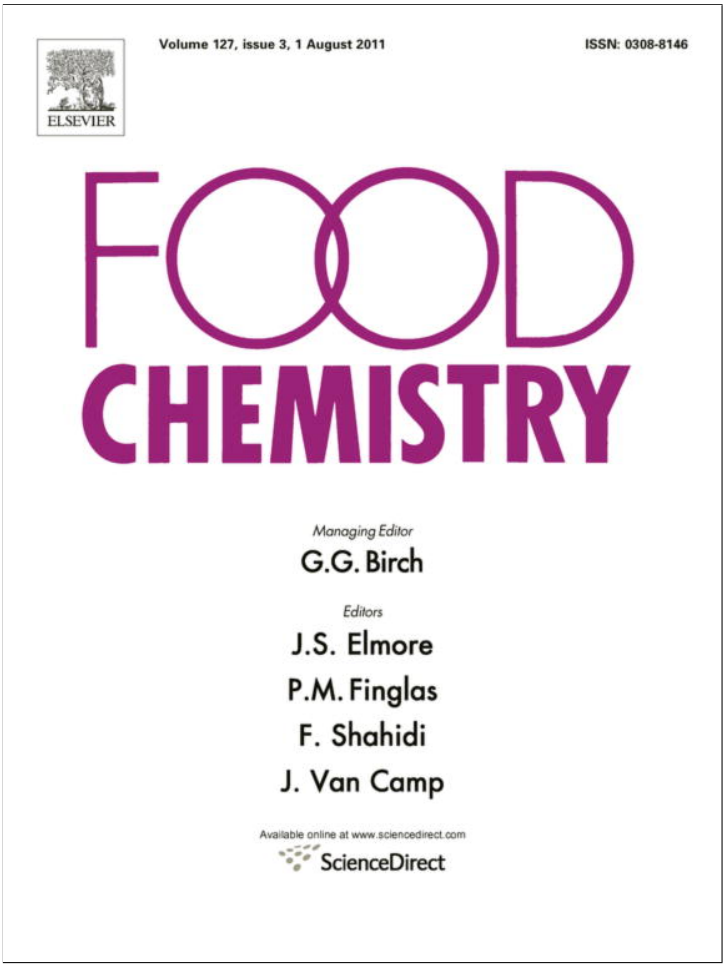

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Antifungal activity of sourdough fermented wheat germ used as an ingredient for bread making

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ABSTRACT

This study aimed at investigating the antifungal activity of sourdough fermented (*Lactobacillus plantarum* LB1 and *Lactobacillus rossiae* LB5) wheat germ (SFWG). Preliminarily, methanol and water/salt-soluble extracts from SFWG were assayed by agar diffusion towards *Penicillium roqueforti* DPPMAF1. As shown by hyphal radial growth rate, the water/salt-soluble extract showed the inhibition of various fungi isolated from bakeries. The antifungal activity was attributed to a mixture of organic acids and peptides which were synthesized during fermentation. Formic (24.7 mM) acid showed the highest antifungal activity. Four peptides, having similarities with well known antifungal sequences, were identified and chemically synthesized. The minimal inhibitory concentration was 2.5–15.2 mg/ml. Slices of bread made by addition of 4% (wt/wt) of freeze dried SFWG were packed in polyethylene bags and stored at room temperature. Slices did not show contamination by fungi until at least 28 days of storage and behaved as the calcium propionate (0.3%, wt/wt).

1. Introduction

Wheat germ, corresponding to 2–3% of the total weight of wheat kernel, is almost systematically removed during milling since it adversely affects the keeping and processing quality of the flour (Rizzello, Nionelli, Coda, De Angelis, & Gobbetti, 2010a). Due to the high concentration of α-tocopherol, vitamins of group B, dietary fiber, polyunsaturated fats, minerals and phytochemicals, wheat germ is one of the most attractive and promising source of vegetable functional compounds (Rizzello et al., 2010a). Nevertheless, the use of wheat germ is still challenging because of the poor stability and the presence of anti-nutritional factors such as: (i) raffinose which is not digested by pancreatic enzymes but metabolized by gas-producing bacteria of the large intestine, thus causing disorders such as flatulence (Rizzello et al., 2010a); (ii) phytic acid which markedly decreases the mineral bioavailability (Febles, Arias, Hardisson, Rodríguez-Alvarez, & Sierra, 2002); and (iii) wheat germ agglutinin (WGA) which is responsible for the hyperplastic and hypertrophic growth of the small bowel and pancreas (Matucci et al., 2003).

Previously, *Lactobacillus plantarum* LB1 and *Lactobacillus rossiae* LB5 were isolated from wheat germ, selected based on the kinetics of acidification and used as starters for the manufacture of sourdough fermented wheat germ (Rizzello, Nionelli, Coda, Di Cagno, & Gobbetti, 2010b). Sourdough fermentation stabilized and enhanced some nutritional properties of the wheat germ. Due to lactic acidification, the lipase activity of the sourdough fermented wheat germ was markedly lower than that found in the raw wheat germ. As shown by SPME/GC/MS analysis, a very low level of volatile compounds deriving from lipid oxidation were found in the freeze dried sourdough fermented wheat germ during 40 days of storage (Rizzello et al., 2010a). The sourdough fermentation was not only effective to partially inhibit the endogenous lipase activity and to increase the shelf-life of wheat germ, but also favoured the increase of the phytase activity and the decrease of the concentration of raffinose (Rizzello et al., 2010a).

Recently, the sourdough fermented wheat germ (SFWG) was used as an ingredient for the manufacture of white bread (Rizzello et al., 2010b). The partial replacement of white wheat flour with 4% (wt/wt) of SFWG following a traditional bread formula led to the improvement in the concentration of free amino acids (Rizzello et al., 2010b) and the in *in vitro* protein digestibility. Phytase and antioxidant activities were also higher in the bread containing SFWG compared to traditional wheat flour bread as well as texture and sensory properties were enhanced (Rizzello et al., 2010b).

The use of sourdough fermentation was already shown to have positive effects on the shelf-life of wheat bread (Clarke, Schober, & Arendt, 2002; Corsetti et al., 2000). Staling and, especially, microbial spoilage by moulds still remain responsible for huge economic losses in the bakery industries (Gray & Bemiller, 2003). The most common spoilage fungi from bakery products belong to the genera *Penicillium*, *Aspergillus*, *Monilia*, *Mucor*, *Endomyces*, *Cladosporium*, *Fusarium* and *Rhizopus* (Coda et al., 2008; Lavermicocca et al.,

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2000). Routinely, salts of propionic, sorbic and benzoic acids are used as the chemical preservatives. The European directive on preservatives (European Union, 1995) decreased the concentration of sorbate (0.2%, wt/wt) and propionate (0.3,% wt/wt) allowed to be used. Ethanol, as an antifungal preservative, is permitted only up to 2% (wt/wt), but the inhibitory effect varied depending on the fungal species and, in some cases, it was not sufficient for preventing contamination (Dantigny, Guilmart, Radoi, Bensoussan, & Zwietering, 2005). Currently biopreservation deserves an increasing interest for food industries, including bakeries. A number of studies considered the use of metabolites from sourdough lactic acid bacteria (e.g., mixture of organic acids, phenyl lactic acid, peptides) (Corsetti, Gobbetti, Rossi, & Damiani, 1998; Lavermicocca et al., 2000; Niku-Paavola, Laitila, Mattila-Sandholm, & Hikara, 1999) and natural preservatives extracted or contained in plant materials (Coda et al., 2008; Rizzello et al., 2009) to inhibit fungal contamination during bread storage. Nevertheless, this problem remains unsolved.

This study aimed at exploiting the potential of sourdough fermented wheat germ (SFWG) to delay the fungal contamination of wheat bread. The mechanism of fungal inhibition by SFWG was described and the inhibitory activity was assayed towards a large spectrum of bakery related fungi, confirming the effect during long-term shelf-life of wheat bread.

2. Materials and methods

2.1. Wheat germ and sourdough fermentation

Six samples of wheat germ were supplied by the industry Tandoi Pellegrino (Corato, Bari, Italy) and pooled before use. The germ was separated from refined flour during milling of *Triticum aestivum* cv. Appulo by a degerminator and a set of rollermills (Bühler AG, Uzwil, Switzerland). Moisture, ash, proteins and fat were determined according to the Approved Methods of the American Association of Cereal Chemists (Corsetti et al., 1998). Total titratable acidity (TTA) was determined by homogenising 10 g of wheat germ with 90 ml of distilled water and expressed as the amount (ml) of 0.1 M NaOH to get a pH of 8.3. The values of pH were determined by a Foodtrode electrode (Hamilton, Bonaduz, Switzerland). *Lactobacillus plantarum* LB1 and *Lactobacillus rossiae* LB5 were previously isolated from raw wheat germ (RWG) and selected based on the kinetics of acidification (Rizzello et al., 2010a).

Lactobacilli were cultivated in modified MRS (mMRS, maltose and fresh yeast extract were added to MRS at 1%and 5%, wt/vol, respectively, and the final pH was 5.6) until the late exponential phase of growth was reached (ca. 10 h), washed twice in 50 mM phosphate buffer, pH 7.0, and re-suspended in tap water. Sourdough fermentation of wheat germ (dough yield of 160) was carried out as previously described (Rizzello et al., 2010a,b). Both lactic acid bacteria were used (final cell density in the dough ca. 10^8 cfu/g), and fermentation was allowed at 30 °C for 24 h. Sourdough fermented wheat germ (SFWG) was freeze dried, and used for bread making or to get methanol and water/salt-soluble extracts. Fermentations were carried out in triplicate.

Serial dilution of freeze dried SFWG were made and plated onto mMRS (Oxoid LTD, Basingstoke, Hampshire, United Kingdom). Enumeration of lactic acid bacteria was carried out at 30 °C for 48 h. DNA was extracted from colonies of the highest plate dilutions of mMRS and used for RAPD–PCR analysis as described by Minervini, Bilancia, Siragusa, Gobbetti, and Caponio (2009). RAPD–PCR analysis was carried out as reported by Coda et al. (2010), using primers P7 (5'-AGCAGCGTGG 3'), and M13 (5'-GAGGGTGGCGGTTCT-3') (Invitrogen, Milan, Italy).

2.2. Fungi, culture media and growth conditions

Penicillium roqueforti DPPMAF1 was used as the indicator microorganism for antifungal assays since it corresponds to one of the more resistant fungi to chemical preservatives (Coda et al., 2008; Suhr & Nielsen, 2004). *Penicillium polonicum* CBS 112490, *Penicillium chrysogenum* CBS 111214, *Penicillium paneum* CBS 101032, *Penicillium albocoremium* CBS 109582, *Penicillium chermesinum* CBS 117279, *Penicillium carneum* CBS 112297, *Eurotium herbarioum* CBS 117336, *Eurotium rubrum* CBS 150.92, *Aspergillus parasiticus* CBS 971.97 *Aspergillus versicolor* CBS 117286 and *Penicillium bialowiezense* CBS 110102 from the Culture Collection of Centraalbureau voor Schimmelcultures (Utrecht, Holland) were also used. All the above species correspond to some of the most relevant spoilage fungi in baked goods (Lavermicocca et al., 2000; Suhr & Nielsen, 2004). Fungi were grown in Potato Dextrose Agar (pH 5.6) (PDA, Oxoid Laboratories, Hampshire, UK) at 25 °C for 24–72 h. When used for agar diffusion assays and for determination of the inhibitory spectrum, the pH of PDA was adjusted to 4.8 with HCl 0.1 N.

Wheat flour hydrolyzate (WFH) was produced as described previously (Coda et al., 2008) and used for the determination of conidia germination. The WFH was sterilized by filtration on 0.22 m membrane filters (Millipore Corporation, Bedford, MA 01730) and stored at 4 °C before use. The pH of the WFH (ca. 5.6) was adjusted to 4.8 with HCl 0.1 N. WFH was chosen as the substrate since it is representative of the chemical composition of wheat flour (Gobbetti, Corsetti, & Rossi, 1994).

2.3. Methanol extracts and total phenols analysis

To determine the antifungal activity of the phenol compounds from RWG and SFWG, methanol extracts were prepared by weighing 5 g of sample and mixing with 50 ml of 80% (vol/vol) methanol following the procedure described by Rizzello et al. (2010a). Fifty milliliters of the extracts were dried on a Speed-Vac centrifuge (Thermo Scientific, Waltham, MA) at 35 °C to concentrate the sample and remove residual organic acids (e.g. formic acid), and re-suspended in 20 ml of 80% methanol.

Analysis of total phenols was according to the method described by Slinkard and Singleton (1997). Gallic acid was the standard. The reaction mixture contained 20 μl of RWG or SFWG extract, 100 μl of Folin–Ciocalteu reagent (Sigma Chemical Co.) and 1.58 ml of distilled water. Three-hundred microliters of saturated sodium carbonate solution were added after a few minutes. The mixture was incubated at 20 °C for 2 h and the absorbance at 765 nm was determined. The concentration of total phenols was calculated as gallic acid equivalents.

2.4. Water/salt-soluble extracts

Water/salt-soluble extracts from RWG and SFWG were prepared according to the method originally described by Osborne (1907) and modified by Weiss, Vogelmeier, and Gorg (1993). Fifteen grams of samples were suspended in 60 ml of 50 mM Tris–HCl (pH 8.8), held at 4 °C for 1 h, vortexing at 15 min intervals, and centrifuged at 20,000*g* for 20 min. The supernatant was used for the antifungal assay. The concentrations of peptides or proteins of the water/salt-soluble extracts and related fractions were determined by the o-phtaldialdehyde (OPA) and Bradford methods (Bradford, 1976; Church, Swaisgood, Porter, & Catignani, 1983), respectively.

2.5. Agar diffusion assay

The agar diffusion assay was carried out as described by Coda et al. (2008). Petri plates (90 mm diameter), containing 10 ml of

PDA (Oxoid), were inoculated with the fungus. The plates were incubated at 25 °C for 72 h. After the mycelial colony had developed, sterile blank paper disks (0.5 cm diameter) were placed at a distance of ca. 0.5 cm away from the rim of the mycelial colony. Ten microliters of the methanol extracts, water/salt-soluble extracts, partially purified fractions and organic acid aqueous solutions (at the concentrations found in the water/salt-soluble extract of SFWG) to be assayed were added to the disks. Plates were incubated at 25 °C for 72 h until the mycelial growth overlaid the negative control paper disk (without sample addition). When the methanol extract of SFWG was assayed, 10 μl of 80% methanol were added to the paper disk as the negative control. At the same time, zones of inhibition were evident in correspondence of paper disks containing samples with inhibitory activity.

2.6. Hyphal radial growth rate

The inhibitory spectrum of the water/salt-soluble extract of SFWG was assayed based on hyphal radial growth rate of fungi (Quiroga, Sampietro, & Vattuone, 2001). The water/salt-soluble extract of SFWG was sterilized by filtration on 0.22 μm membrane filters (Millipore Corporation, Bedford, MA 01730) and added to sterilized PDA. To compare this antifungal activity assays with that under baking test conditions, aliquots (16 ml) of the water/salt-soluble extract from 4 g of SFWG (see above) were added to 84 ml of PDA medium (final concentration 16%, vol/vol).

After mixing, aliquots of 15 ml were poured into Petri plates (90 mm diameter). Control plates contained PDA alone. The assay was carried out by placing a 3 mm diameter plug of growing mycelia onto the centre of Petri dishes containing the culture medium. Plates were incubated aerobically at 25 °C. Three replicates were run simultaneously. The radial growth of mycelia (colony diameter, mm) in all plates was measured 8 days after inoculation. Each datum point is the mean of at least four measurements of a growing colony. The percentage of growth inhibition was calculated from mean values as follows: Percentage of inhibition = [(mycelial growth under control conditions – mycelial growth in the presence of water/salt-soluble extract)/mycelial growth under control conditions] × 100.

2.7. Germination of conidia

The effect of water/salt-soluble extract of SFWG on the germination of conidia was also determined (Gourama, 1997). After growth for 7 days on PDA plates, conidia of *P. roqueforti* DPPMAF1 were harvested in sterile water, containing 0.05% (vol/vol) Tween 80. The count of the conidia in the suspension was carried out using the Petroff-Houser Counting chamber. A fixed number of ca. 10^6–10^7 conidia/ml was added to 5 ml of the mixture of WFH containing 16% vol/vol of SFWG water/salt-soluble extract. The mixture was incubated in 60 mm Petri dishes for 24 h at 25 °C under stirring conditions. WFH alone and WFH with 0.3% (wt/vol) calcium propionate added, were used as the controls. To determine the percentage of germinated conidia (length/width ratio $\geqslant 2$), slides of the suspension were examined under the microscope (40× magnification) at 4 h interval. Three separate replications of at least 100 conidia were used for each assay.

To determine the minimum inhibitory concentration (MIC), concentrated water/salt-soluble extracts and related fractions were freeze dried and re-dissolved in WFH. The mixture was poured into 60 mm Petri dishes, inoculated with a suspension of conidia (10^6–10^7 conidia/ml as the final density) and incubated for 12 h at 25 °C under stirring conditions. After 12 h, slides of the suspension were examined under the microscope and the germinated spores were counted as described above. The MIC was defined as the lowest concentration of water/salt-soluble extract that inhibited fungal growth at 25 °C for 12 h. All assays for antifungal activity were carried out at least in triplicate.

2.8. Proteolysis and heat stability of antifungal compounds

Water/salt-soluble extract of SFWG was treated with trypsin (EC 3.4.21.4, Sigma Aldrich Co.) as described by Atanassova et al. (2003). Trypsin was dissolved in Tris–HCl 0.25 M pH 8 (1%, wt/vol, final concentration). One hundred microliters of the supernatant containing antifungal compounds and 100 μl of the buffered enzyme solution were mixed. After 5 h of incubation at 25 °C, reaction was stopped by boiling the mixture for 3 min. After treatment, the pH of the solution was adjusted to 6.0 and the residual activity determined by agar diffusion assay. Heat stability of the water/salt-soluble extract of SFWG was determined by heating at 100 °C for 5 min. After treatments, the residual activity was determined by agar diffusion assay.

2.9. Purification of antifungal compounds

Water/salt-soluble extract of SFWG was fractionated by ultrafiltration (Ultrafree-MC centrifugal filter units, Millipore) by using three different membrane sizes: 50, 30 and 10 kDa cut-off (fractions A, B and C, respectively). Aliquots of 400 μl of the water/salt-soluble extract were centrifuged at 10,000*g* for 60 min. After ultra-filtration, fractions were used for agar diffusion assay.

Organic acids contained in the 10 kDa partially purified fraction were determined by HPLC (High-Performance Liquid Chromatography), using an ÄKTA Purifier system (GE Healthcare) equipped with an Aminex HPX-87H column (ion exclusion, Biorad) and a UV detector operating at 210 nm. Elution was at 60 °C, with a flow rate of 0.6 ml/min, using H_2SO_4 10 mM as mobile phase (Rizzello et al., 2010a).

Another aliquot of the 10 kDa partially purified fraction was further fractionated (37 fractions) 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). Aliquots containing 0.875 mg/ml of peptides were added to 0.05% (v/v) trifluoroacetic acid (TFA) and centrifuged at 10,000*g* for 10 min. The supernatant was filtered through a 0.22 μm pore size filter and loaded onto the column. Gradient elution was performed at a flow rate of 1 ml/min using a mobile phase composed of water and acetonitrile (CH_3CN) containing 0.05% TFA. The CH_3CN concentration was increased linearly from 5% to 46% between 16 and 62 min, and from 46% to 100% between 62 and 72 min. Solvents were removed from collected fractions by freeze drying. The fractions were re-dissolved in sterile water and subjected to agar diffusion assay. The freeze dried preparation of the fraction with antifungal activity was used for identification.

2.10. Identification and synthesis of antifungal peptides

Identification of peptides was carried out by nano-Liquid Chromatography-Electrospray Ionisation-Mass Spectra/Mass Spectra (nano-LC-ESI-MS/MS), using a Finningan LCQ Deca XP Max ion trap mass spectrometer (ThermoElectron) through the nano-ESI interface. According to manufacturer's instrument settings for nano-LC-ESI-MSMS analyses, MS spectra were automatically taken by Xcalibur software (ThermoElectron), in positive ion mode. MS/MS spectra were processed using the software BioWorks 3.2 (ThermoElectron) generating peaklists suitable for database searches. Peptides were identified using MS/MS ion search of Mascot search engine (Matrix Science, London, England) and NCBInr protein database (National Centre for Biotechnology Information, Bethesda, USA). For identification of peptides the following parameters were

considered: enzyme: "none"; instrument type: "ESI-trap"; peptide mass tolerance: ±0.1% and fragment mass tolerance: ±0.5 Da. Peptide identification results were subjected to a manual evaluation, as described by Chen, Know, Kim, and Zhao (2005), and the validated peptide sequences explained all the major peaks in the MS/MS spectrum. All peptides identified in the active fraction of the water/salt-soluble extract from SFWG were chemically synthesized by NeoSystem Laboratoire (Strasbourg, France). The purity of the synthesized peptides was higher than 95% as determined by high-performance liquid chromatography analysis and certified by the manufacturer.

2.11. Bread making

The characteristics of the wheat flour (*T. aestivum*, cv Appulo) used for bread making were as follows: moisture, 14.2%; protein (N × 5.70), 11.5%, of dry matter (d.m.); fat, 1.6% of d.m.; ash, 0.6% of d.m.; and total soluble carbohydrates, 1.5% of d.m.

According to typical Italian bread making, three breads having a dough yield of 160 were manufactured at the pilot plant of the Department of Biologia e Chimica Agro-Forestale ed Ambientale. The formulas were as follows: (i) wheat flour bread (WFB) made with 250 g flour, 150 g tap water and 6 g of baker's yeast; (ii) wheat flour bread containing calcium proprionate (WFB^{cp}) made with 250 g flour, 150 g tap water, 1.2 g calcium propionate, and 6 g of baker's yeast; and (iii) sourdough fermented wheat germ bread (SFWGB) made with 240 g flour, 10 g of freeze dried SFWG (4%, wt/wt of wheat flour), 150 g tap water and 6 g of baker's yeast. A continuous high-speed mixer (60g, dough mixing time 5 min) was used to prepare the doughs. Fermentation of doughs was allowed at 30 °C for 2.5 h and breads were baked at 220 °C for 40 min (Combo 3, Zucchelli, Verona, Italy). Fermentations were carried out in triplicate and each bread was analysed twice. For each bread, two slices were cut. The size of the slice was 12 cm, height, and 1.5 cm, width. One slice was inoculated by nebulisation with a suspension of 10^2 conidia/ml of *P. roqueforti* DPPMAF1 and the other slice was without inoculum. Slices were packed in polyethylene bags to maintain constant moisture and incubated at room temperature for 28 days. Moisture was determined according to the standard AACC method (2003).

2.12. Bread Structure

Instrumental Texture Profile Analysis (TPA) was performed with a TA.XT2i Texture Analyzer, using a 35 mm flat-end aluminium compression disc (probe P/35), as described by Rizzello et al. (2010b). 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 (area during the withdrawal of the penetration, divided by the area of the first penetration). Triplicate measurements for breads from each storage time were carried out.

2.13. Statistical analysis

Data were subjected to one-way ANOVA; pair-comparison of treatment means was achieved by Tukey's procedure at $P < 0.05$, using the statistical software, Statistica 7.0 for Windows.

3. Results

3.1. Antifungal activity of RWG and SFWG extracts

The averaged values of raw wheat germ (RWG) were the following: moisture 11.08 ± 0.22%, protein (N × 5.70) 28.50 ± 0.72% of dry matter (d.m.); fat 7.95 ± 0.06% of d.m; and ash 3.82 ± 0.009% of d.m. RWG had a pH of 6.36 ± 0.05 and TTA of 18.3 ± 0.11 ml of 0.1 M NaOH/10 g. The concentration of total free amino acids was 15.64 ± 0.13 g/kg.

Lb. plantarum LB1 and *Lb. rossiae* LB5 were monitored during sourdough fermentation of wheat germ by RAPD–PCR analysis. Cell densities were 2.5 ± 0.4 × 10^9 and 7.3 ± 0.5 × 10^9 cfu/g for *Lb. plantarum* LB1 and *Lb. rossiae* LB5, respectively. The pH of the sourdough fermented wheat germ (SFWG) was 4.16 ± 0.03. TTA increased to 24.9 ± 0.07 ml of 0.1 M NaOH/10 g.

The methanol extracts of RWG and SFWG contained 0.49 ± 0.02 and 0.65 ± 0.02 mM of total phenols (expressed as gallic acid equivalent), respectively. No antifungal activity was found in the methanol extracts (Table 1). The water/salt-soluble extracts of RWG and SFWG had a pH of 6.45 ± 0.03 and 5.45 ± 0.05, respectively. The concentration of proteins and peptides was 10.31 ± 0.21 mg/ml, 6.49 ± 0.13 mg/ml, 6.25 ± 0.32 mg/ml and 12.41 ± 0.11 mg/ml for RWG and SFWG, respectively. As shown by agar diffusion assay, the water/salt-soluble extract of SFWG exhibited marked antifungal activity towards the indicator *P. roqueforti* DPPMAF1, whereas no activity was found for the water/salt-soluble extract of RWG (Table 1). After incubation for 12 h at 25 °C on WFH, the mean percentage of spore germination of *P. roqueforti* DPPMAF1 was 10.1%. Incubation in the presence of the water/salt-soluble extract of SFWG induced a moderate but significant ($P < 0.05$) decrease of the spore germination to 8.3%. No significant ($P > 0.05$) decrease of the spore germination was found in the presence of 0.3% (wt/vol) calcium propionate.

The activity of the water/salt-soluble extract of SFWG was assayed against several fungi isolated from bakeries (Table 2) by

Table 1
Inhibitory activity towards *Penicillium roqueforti* DPPMAF1 of methanol and water/salt-soluble extracts, partially purified fractions from raw wheat germ (RWG) and sourdough fermented raw wheat germ (SFWG), and organic acid standard solutions, as determined by agar diffusion assay.

Sample	Activity[c]
Methanol extract of RWG	−
Methanol extract of SFWG	−
Water/salt-soluble extract of RWG	−
Water/salt-soluble extract of SFWG	++ +
Fraction A (cut-off 50 kDa)	+ + +
Fraction B (cut-off 30 kDa)	+ + +
Fraction C (cut-off 10 kDa)	+ + +
Fraction C digested with proteolytic enzymes	+ +
Fraction C after heat treatment	+ + +
FPLC Fractions[a] n.1–8	−
FPLC Fraction n.9	+
FPLC Fractions n.10,11	±
FPLC Fractions n.12–37	−
Organic acid mixture[b]	+ + +
Oxalic acid (1.57 mM)	−
Lactic acid (24.3 mM)	±
Formic acid (24.7 mM)	++
Acetic acid (10.8 mM)	±
Citric acid (3.2 mM)	−
Citric acid (18.2 mM)	−
Phenyl lactic acid (0.4 mM)	+
Valeric acid (0.98 mM)	±

[a] FPLC fractions were obtained from fraction C.
[b] The organic acid concentration of standard solutions correspond to that found in fraction C.
[c] Inhibitory activity was scored visually as follows: −, no inhibition; ±, very weak inhibition (0.5–1.0 mm); +, low inhibition with a little clear zone near the rim of the colony (1.0–2.0 mm); ++ strong inhibition, with large clear zone near the rim of the colony (2.0–3.0 mm); and +++, very strong inhibition, with no growth near the rim of the colony (3.0–4.0 mm).

Table 2
Inhibitory spectrum of the water/salt-soluble extract of sourdough fermented wheat germ (SFWG) (16%, vol/vol, on PDA medium), as determined by hyphal radial growth rate of fungi (Quiroga et al., 2001), after 8 days of incubation at 25 °C.

Fungi	Source of isolation	Inhibitory activity
Penicillium roqueforti DPPMA1	Bread, Italy	40.5%
Penicillium polonicum CBS 112490	Bread, Italy	28.6%
Penicillium chrysogenum CBS 111214	Bread, England	6.8%
Penicillium paneum CBS 101032	Rye bread, Denmark	47.5%
Penicillium albocoremium CBS 109582	Cake factory, Denmark	24.2%
Penicillium chermesinum CBS 117279	Bakery plant, Netherlands	32.2%
Eurotium herbarioum CBS 117336	Chocolate cake, Netherlands	21.4%
Eurotium rubrum CBS 150.92	Cake	No inhibition
Aspergillus parasiticus CBS 971.97	Indian sweets, India	44.6%
Penicillium carneum CBS 112297	Rye bread, Denmark	4.0%
Aspergillus versicolor CBS 117286	Wall in bakery, Netherlands	4.5%
Penicillium bialowiezense CBS 110102	Bread, Italy	No inhibition

Each datum point is the mean of at least four measurements of a growing colony. The percentage of growth inhibition was calculated from mean values as: % Inhibition = [(mycelial growth under control conditions - mycelial growth in the presence of water/salt-soluble extract)/mycelial growth under control conditions] × 100.

measuring the growth inhibition on PDA after 7 days of incubation at 25 °C. Final pH of the medium was 4.8. Among the 12 species of fungi tested, only *E. rubrum* CBS 150.92 and *P. bialowiezense* CBS 110102 were not affected by water/salt-soluble extract (Table 2). The highest values of growth inhibition were found for *P. paneum* CBS 101032 (47.5%) and *A. parasiticus* CBS 971.97 (44.6%) (Fig. 1). The antifungal activity towards *P. roqueforti* DPPMAF1, *P. chermesinum* CBS 150.92, *P. polonicum* CBS 112490 and *P. albocoremium* CBS 109582 was 40.5%, 32.2%, 28.6%, and 24.2%, respectively. A weak antifungal activity was found against *P. chrysogenum* CBS 111214, *A. versicolor* CBS 117286, and *P. carneum* CBS 112297.

3.2. Identification of antifungal compounds

Inhibitory activity towards *P. roqueforti* DPPMAF1 was found in fractions A, B and C obtained by ultra-filtration of the water/salt-soluble extract of SFWG (cut-off 50, 30 and 10 kDa, respectively) (Table 1). This suggested that the molecular mass of the antifungal compounds was lower than 10 kDa. As a consequence, all further determinations were carried out on fraction C. Antifungal activity of the water/salt-soluble extract of SFWG was slightly affected by trypsin digestion, whereas, after heating, the inhibitory activity persisted (Table 1). Oxalic, lactic, formic, acetic, citric, phenyl lactic and valeric acids were found at the following concentrations: 1.57, 24.3, 24.7, 10.8, 3.2, 0.4, and 0.98 mM, respectively. The HPLC analysis of the water/salt-soluble extract of RWG showed the presence of only citric (18.2 mM) and valeric (0.98 mM) acids. Organic acid standard solutions at the concentrations found in the water/salt-soluble extract of SFWG were tested singly or in mixture (Table 1). The mixture of organic acids showed an antifungal activity similar to that of the water/salt-soluble extract of SFWG (Fig. 2). Oxalic and citric acids (1.57 and 3.2 or 18.2 mM, respectively) did not cause inhibition (−), and lactic (24.3 mM), acetic (10.8 mM) and valeric (0.98 mM) acids showed a very weak (±) inhibition. Phenyl lactic acid (0.4 mM) showed a moderate inhibition (+) with a little clear zone near the rim of the colony, whereas formic acid (24.7 mM) caused a marked inhibition (++) with a large clear zone near the rim of the colony of *P. roqueforti* DPPMA1 (Fig. 2). Fraction C was further purified by RP-FPLC, obtaining 37 fractions (peptide concentration in the range 0.30 ± 0.02–5.61 ± 0.04 mg/

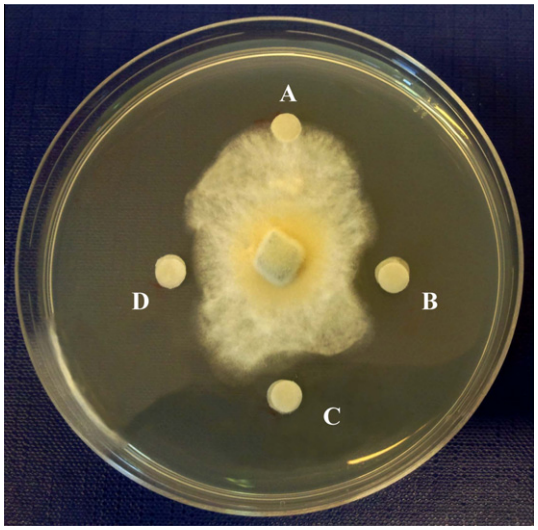

Fig. 2. Antifungal activity as determined by agar diffusion assay using *Penicillium roqueforti* DPPMAF1 as the indicator fungus: A, sterile paper disks without water/salt-soluble extract (control); B, partially purified fraction C from the water/salt-soluble extract of sourdough fermented wheat germ (SFWG); C, organic acid mixture consisting of oxalic, lactic, formic, acetic, citric, phenyl lactic and valeric acids at the concentrations found in the water/salt-soluble extract of SFWG (1.57, 24.3, 24.7, 10.8, 3.2, 0.4, and 0.98 mM, respectively); and D, formic acid (24.7 mM).

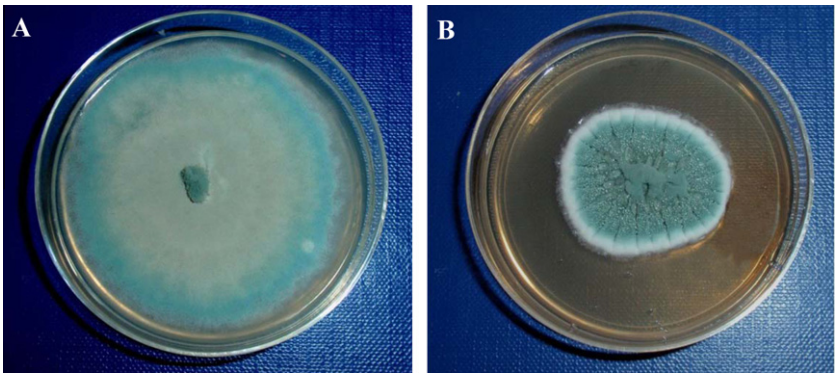

Fig. 1. Antifungal activity of water/salt-soluble extract of sourdough fermented wheat germ (SFWG) towards *Aspergillus parasiticus* CBS 971.97, as determined by hyphal radial growth inhibition (Quiroga et al., 2001) after 8 days of incubation at 25 °C. (A) PDA alone (control); (B) PDA containing water/salt-soluble extract of SFWG (16%, vol/vol). The inhibition corresponded to 44.6%. Details about calculation are explained in Section 2.

Table 3
Sequences of peptides contained in the partially purified fraction (n. 9) of the water/salt-soluble extract of sourdough fermented wheat germ (SFWG).

Fraction	Sequence[a]	Score	Charge	Calculated mass	Expected mass	Delta	Source Protein NCBI accession number
n. 9	VLHEPLF	29	2	853.4698	853.8482	0.3784	FH4_ORYSJ, formin-like protein 4; Q8H8K7
	YNNPIIYVTENGIAEGNNKSLPITEAL	22	3	2946.4974	2946.1642	0.3332	BGL29_ORYSJ, beta-glucosidase 29; A3C053
	ALKAAPSPA	16	3	824.2756	823.9855	0.2901	HOX2_ORYSI, homeobox-leucine zipper protein HOX2; Q84U86
	AILIIVMLFGR	13	2	1244.1679	1243.9586	0.2093	HKT6_ORYSJ, probable cation transporter HKT6; Q6H501
	AAAAVFLSLLAVGHCAAADFNATDADADFAGNGVDFNSSDAAVYWGPWTKAR	13	3	5287.4734	5287.5858	0.1124	EXPB4_ORYSJ, expansin-B4; Q94LR4

[a] Single-letter amino acid code is used.

ml). Although a weak activity was also found in fractions 10 and 11 (1.83 ± 0.02 and 2.04 ± 0.02 mg of peptides/ml), the highest antifungal activity was found in fraction 9 (5.21 ± 0.01 mg of peptides/ml). Five peptides, having 7–52 amino acid residues, were identified (Table 3). VLHEPLF was the only neutral peptide, with an experimental molecular mass of 853.47 Da and total hydrophobic charge of 57%. YNNPIIYVTENGIAEGNNKSLPITEAL (experimental molecular mass of 2946.50 Da) had a total net charge of −2 and total hydrophobic ratio of 33%. ALKAAPSPA (824.28 Da) and AILIIVMLFGR (1244.17 Da), had positive total charge of +1, and hydrophobic ratio of 55% and 81%, respectively. AAAAVFLSLLAVGHCAAADFNATDADADFAGNGVDFNSSDAAVYWGPWTKAR, had a molecular mass of 5287.47 Da, a total net charge of −3, and hydrophobic ratio of 55%. Searching the NCBInr database, these peptides were reported as encrypted into sequences of *Oryza sativa* proteins: formin-like protein 4 (VLHEPLF); beta-glucosidase 29 (YNNPIIYVTENGIAEGNNKSLPITEAL); homeobox-leucine zipper protein HOX2 (ALKAAPSPA); probable cation transporter HKT6 (AILIIVMLFGR); expansin-B4 (AAAAVFLSLLAVGHCAAADFNATDADADFAGNGVDFNSSDAAVYWGPWTKAR). NCBI accession numbers are reported in Table 3.

The partially purified fraction 9 was assayed at concentrations ranging from 0.5 to 20 mg of peptides/ml. No germination of *P. roqueforti* DPPMAF1 conidia was found at a concentration of 3.7 mg peptides/ml.

All peptides were chemically synthesized and assayed at concentrations of 0.875–20 mg of peptides/ml. When used singly VLHEPLF, ALKAAPSPA, AILIIVMLFGR and AAAAVFLSLLAVGHCAAADFNATDADADFAGNGVDFNSSDAAVYWGPWTKAR confirmed the inhibitory activity. The range of MICs varied from 2.5 to 15.2 mg/ml. The other synthesized peptide (YNNPIIYVTENGIAEGNNKSLPITEAL) did not show appreciable inhibition also at the highest concentration assayed. Among all possible combinations, the lowest MIC (ca. 1.05 mg of peptides/ml) was found when ALKAAPSPA and AILIIVMLFGR were used in mixture.

3.3. Characterisation of breads during storage

Three breads (WFB, WFB^{cp} and SFWGB) were manufactured at the pilot plant. The values of pH were 5.45 ± 0.08, 5.44 ± 0.05 and 4.82 ± 0.05 for WFB, WFBcp and SFWGB, respectively (Table 4). SFWGB showed the highest values of TTA (6.5 ± 0.2 ml 0.1 M NaOH/10 g) and specific volume (2.25 ± 0.04 g/cm^3). Hardness is often considered as the index of the total textural attributes. After baking, the value of hardness, corresponding to the peak force of the first compression of the product, was the highest for WFB and WFB^{cp} (2792 ± 12 and 2789 ± 22 g) which significantly (P>0.05) differed from SFWGB (2378 ± 25 g). Resilience indicate how well the bread recovers its original position. The lowest value of resilience was found for SFWGB (0.34 ± 0.01). The fracturability point (the fracture of the bread) corresponds to the first significant peak force during compression of the bread. After baking, the values of fracturability only slightly differed between breads and ranged from 35702 ± 19 (SFWGB) to 39897 ± 28 J (WFB). After 28 days of storage, the hardness of WFB and WFB^{cp} increased to 10312 ± 14 and 10454 ± 21 g, respectively. A lower increase was found for SFWGB (2378 ± 25 to 9521 ± 8 g). At the same time the resilience markedly decreased for WFB and WFB^{cp} (0.42 ± 0.03 and 0.40 ± 0.01, respectively), whereas only a slight decrease was found for SFWGB (0.26 ± 0.01) (Table 4). As a consequence of the moisture decrease, the fracturability increased to 130526 ± 30; 130314 ± 22, and 104407 ± 13 J for WFB, WFB^{cp} and SFWGB, respectively.

3.4. Storage test

The moisture of all the slices was in the range of 29.2–31.4% throughout 28 days of storage. After 7 days of storage, the slice of WFB (control) inoculated with the suspension of spores was colonised by *P. roqueforti* DPPMAF1 (Table 5). No growth of *P. roqueforti* DPPMAF1 was found in WFBcp inoculated with the suspension of

Table 4
Characterization and texture profile analysis of wheat flour (WFB), wheat flour containing calcium propionate (WFB^{cp}), and sourdough fermented wheat germ (SFWGB) breads, packed in polyethylene bags, after baking (0 day) and 28 days of storage.

	WFB		WFB^{cp}		SFWGB	
	0	28	0	28	0	28
pH	5.45 ± 0.08^b	5.45 ± 0.02^b	5.44 ± 0.05^b	5.43± 0.02^b	4.82 ± 0.05^a	4.80 ± 0.02^a
Moisture	31.2 ± 0.3^b	29.4 ± 0.3^a	31.4 ± 0.1^b	29.2 ± 0.3^a	31.0 ± 0.2^b	29.3 ± 0.2^a
Total titratable acidity (TTA)	3.5 ± 0.1^a	3.5 ± 0.2^a	3.3 ± 0.2^a	3.4 ± 0.1^a	6.5 ± 0.2^b	6.6 ± 0.1^b
Specific volume (g/cm^3)	1.92 ± 0.02^a	1.89 ± 0.02^a	1.90 ± 0.03^a	1.89 ± 0.03^a	2.25 ± 0.04^b	2.20 ± 0.04^b
Density (cm^3/g)	0.52 ± 0.02^b	0.52 ± 0.04^b	0.53 ± 0.02^b	0.53 ± 0.01^b	0.45 ± 0.02^a	0.44 ± 0.02^a
Hardness (g)	2792 ± 12^b	10,454 ± 21^d	2789 ± 22^b	10,312 ± 14^d	2378 ± 25^a	9521 ± 8^c
Resilience	0.65 ± 0.02^d	0.42 ± 0.03^c	0.64 ± 0.03^d	0.40 ± 0.01^c	0.34 ± 0.01^b	0.26 ± 0.01a
Fracturability (J)	39,897 ± 28^b	130,526 ± 30^d	39,901 ± 24^b	130,314 ± 22^d	35,702 ± 19^a	104,407 ± 13^c

Details of the formulas for bread making are indicated in Section 2.
Data are the mean of three independent samples twice analysed.
[a–d]Values in the same row with different superscript letters differ significantly (P<0.05).

Table 5
Fungal contamination of slices of wheat flour bread (WFB), wheat flour bread containing calcium propionate (WFB^{cp}), and sourdough fermented wheat germ bread (SFWGB), packed in polyethylene bags and incubated at room temperature for 28 days.

Days	Breads	Inoculated slices			Non inoculated slices		
		WFB	WFB^{cp}	SFWGB	WFB	WFB^{cp}	SFWGB
7		++	–	–	–	–	–
14		+++	+	–	+	–	–
21		++++	+	+	++	+	–
28		++++	+++	+	++++	++	–

Contamination was scored as follows: –; 0% of contamination of the slice surface; +/–: 10% of contamination; +: 20% of contamination; ++: 40% of contamination; +++: 80% of contamination; ++++: 100% of contamination.
For each bread, a slice was inoculated by nebulisation with a suspension of 10^2 conidia/ml of *Penicillium roqueforti* DPPMAF1 and the other slice was without inoculum. Fermentations were carried out in triplicate and each bread was twice analysed.

spores before 14 days of storage. After 21 days, mycelial growth appeared in the inoculated slice of SFWGB.

After 14 days, mycelial growth appeared in the slice of WFB without inoculum of the spore suspension, whereas no fungal growth was evident for WFB^{cp} before 21 days. No contamination by fungi was visible for SFWGB at 28 days of storage (Table 5). After 28 days, the moisture of the slices gradually decreased (values below 29.0%), indicating that no growth of *P. roqueforti* DPPMAF1 was probably due to the decrease of the water activity.

4. Discussion

Recently (Rizzello et al., 2010a; Rizzello et al., 2010b), it was shown that nutritional, texture and sensory characteristics of breads enriched with sourdough fermented wheat germ (SFWG) were better than those of white breads made with or without raw (non fermented) wheat germ (Rizzello et al., 2010b). This study showed that SFWG possesses a marked antifungal activity which may extend the microbial shelf-life of leavened baked goods. No antifungal activity was found for raw wheat germ (RWG), thus hypothesising that the inhibitory activity was strictly related to sourdough fermentation.

Since the effectiveness of many antifungal compounds (especially organic acids) was shown to be pH dependent (Suhr & Nielsen, 2004), all antifungal assays were carried out at pH 4.8, which corresponded to the value previously reported for breads containing SFWG (Rizzello et al., 2010b). Aiming at purifying the antifungal compounds contained in SFWG, the methanol extract, mainly consisting of phenol compounds, was preliminarily assayed. Several studies reported that growth rate and spore germination of spoilage fungi are inhibited by phenol compounds (López-Malo, Alzamora, & Palou, 2005). Under the experimental conditions of this study, the methanol extract of SFWG did not show activity. On the contrary, the water/salt-soluble extract of SFWG had antifungal activity towards several fungal species (e.g., *P. paneum* CBS 101032 and *A. parasiticus* CBS 971.97) which are commonly isolated from contaminated baked goods. A weak effect against spore germination was found, which suggested the fungistatic activity of the extract (Coda et al., 2008). As shown by other authors (Coda et al., 2008; Lavermicocca et al., 2000), also calcium propionate at a concentration of 0.3% (wt/vol) had very poor activity in inhibiting the spore germination.

The preliminarily purification of the water/salt-soluble extract of SFWG showed that antifungal compounds had molecular masses lower than 10 kDa and thermal stability. After enzyme digestion, the antifungal activity slightly decreased. Threfore, it was hypothesised an inhibitory effect not only due to proteinaceous compounds. Several organic acids were found in the water/salt-soluble extract. Among these, especially acetic, formic, phenyl lactic and valeric acids might exert antifungal activity (Corsetti et al., 1998; Lavermicocca et al., 2000). The major inhibition by formic and phenyl lactic acid (24.7 and 0.4 mM) was confirmed with further assays. In particular, formic acid might be synthesized during the stationary phase of growth as an alternative catabolism of the piruvate via the piruvate–formate–lyase system (Corsetti et al., 1998). The concentration of formic acid found in this study was similar to the concentration previously reported as inhibitory for *F. graminearum* (Corsetti et al., 1998). On the contrary, the concentration found for phenyl lactic acid seemed to be too low to cause a marked antifungal activity (Lavermicocca et al., 2000). As previously shown (Corsetti et al., 1998), the highest inhibitory activity was found in the presence of the mixture of organic acids suggesting a synergistic effect. The concentration of organic acid synthesized by lactic acid bacteria during fermentation of wheat germ in part differed from that usually found in sourdough (Corsetti et al., 1998). This could also be due to the different chemical composition of wheat germ compared to the wheat flour (Rizzello et al., 2010b).

Complex and synergistic activities between organic acids and peptides were already found to be responsible for the antifungal activity of sourdough lactic acid bacteria (Corsetti et al., 1998; Niku-Paavola et al., 1999). Indeed, the partially purified fraction from the water/salt-soluble extract of SFWG contained a mixture of five peptides. The peptide ALKAAPSPA showed 35% homology with the antimicrobial peptide Temporin-LT2 (Wang et al., 2009) which contains the same motif ALKA. The sequence of the peptide AILIIVMLFGR showed 40% homology with the following antifungal peptides: Temporin-SHa (Abbassi et al., 2008), Temporin 1Bya (Conlon et al., 2003) and Temporin-1Gc (Kim et al., 2000). The peptide AAAAVFLSLLAVGHCAAADFNATDADADFAGNGVDFNSSDAAVYWGPWTKAR is encrypted in the cereal protein expansin-B4 (Q94LR4). It was hypothesised that expansins could be involved in the degradation of the cell walls of fungi during the defense response of the host plants (Zhou, Norioka, Li, & Norioka, 2003). The MIC of the partially purified fraction from the water/salt-soluble extract of SFWG was 3.7 mg/ml which compared well with the antifungal activities of similar proteinaceous substances (Coda, et al., 2008; Rizzello et al., 2009; Suhr & Nielsen, 2004). Furthermore, the MIC of the mixture of the two chemically synthesized peptides ALKAAPSPA and AILIIVMLFGR was 1.05 mg/ml. The synergistic enhancement of the anti-fungal activity of antimicrobial peptides was previously documented (Egorov, Odintsova, Pukhalsky, & Grishin, 2005). The diluted extracts used for the *in vitro* antifungal assays were chosen to mimic the concentration of antifungal compounds in breads where the wheat flour was in part replaced by SFWG. Indeed, the addition of SFWG in the bread formula delayed the fungal growth at least until 28 days of storage at room temperature and showed the same activity as the 0.3% (wt/wt) calcium propionate.

Bread making did not consider the addition of the water/salt-soluble extract of SFWG but the use of the freeze dried SFWG

(4%, wt/wt) as the ingredient, thus applying a simpler technology compared to those reported for other plant extracts (Coda et al., 2008; Rizzello et al., 2009). As previously demonstrated (Rizzello et al., 2010b), this addition improved texture properties and delayed staling of the bread. Because of the antistaling effect and the synthesis of antifungal compounds, wheat germ fermented by sourdough lactic acid bacteria may be considered as an ingredient to naturally extend the bread shelf-life.

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