JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Selection of Sourdough Lactobacilli with Antifungal Activity for Use as Biopreservatives in Bakery Products

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ABSTRACT: Two hundred and sixteen LAB cultures from sourdoughs and dough for bread and panettone production were screened for in vitro antifungal properties against three indicator cultures ascribed to *Aspergillus japonicus*, *Eurotium repens*, and *Penicillium roseopurpureum*, isolated from bakery environment and moldy panettone. Nineteen preselected isolates were subjected to minimum inhibitory concentration determination against the indicator cultures. Sourdoughs prepared with the two most promising strains, identified as *Lactobacillus rossiae* LD108 and *Lactobacillus paralimentarius* PB127, were characterized. The sourdough extracts were subjected to HPLC analysis coupled with a microtiter plate bioassay against *A. japonicus* to identify the active fractions. MALDI-TOF MS analysis revealed the occurrence of a series of peptides corresponding to wheat α-gliadin proteolysis fragments in the active fraction from *L. rossiae* LD108 sourdough. The ability to prevent mold growth on bread was demonstrated for both strains, whereas *L. rossiae* LD108 also inhibited mold growth on panettone.

KEYWORDS: *lactobacilli, fungal spoilage, sourdough, bakery products, antifungal peptides, biopreservation*

■ INTRODUCTION

Baked goods are easily subject to fungal spoilage, especially when their moisture is quite high, as is the case for bread and sweet leavened goods.[1] Molding leads to relevant economic losses for the bakery industry,[1] causing evident spoilage, off-flavors, and sometimes the production of harmful mycotoxins.[2] Good manufacturing practice can effectively decrease the fungal spore contamination of bakery products, whereas spore germination and fungal growth can be hampered using a wide range of chemical preservatives or different treatments.[3] A preservative-free possible solution may be offered by bio-preservation methods, which are based on the use of protective microorganisms, or their metabolites, naturally occurring or intentionally added to foods.[4] Among the protective micro-organisms, lactic acid bacteria (LAB) are of particular interest because it is well documented that they are capable of producing many molecules with fungistatic or fungicidal effects.[2,4−8] Some studies have demonstrated the LAB antifungal activity under laboratory conditions,[8−10,12] whereas other investigations were aimed at highlighting the capacity of antifungal LAB strains to actually prevent the molding of leavened baked goods.[5,6,11,13−17].

The present study was undertaken to give a useful contribution to the possible exploitation of antifungal LAB cultures as new biopreservatives destined for the bakery industry. With this aim, the two sourdough strains *Lactobacillus rossiae* LD108 and *Lactobacillus paralimentarius* PB127 were selected on the basis of their capacity to inhibit fungal growth, which, to our knowledge, was evidenced herein for the first time for the latter species. Further investigations carried out using the selected strains suggested the involvement of proteinaceous compounds in their antifungal activity, and a series of peptides identified as gluten proteolysis byproducts were then found in the active HPLC fraction from *L. rossiae* LD108 sourdough. The evidence of an indisputable delay of fungal growth on different bakery products following the use of experimental sourdoughs inoculated with the selected strains confirmed the soundness of our results.

■ MATERIALS AND METHODS

Chemicals. All chemicals were purchased as HPLC grade (purity ≥ 99%). Lactic, phenyllactic, butyric, caproic acid and diacetyl were purchased from Sigma (Milan, Italy), acetic acid was from Riadel-de Haën (RdH Laborchemikalien Gmbh, Germany), and propionic acid was from J. T. Baker (Modena, Italy). HPLC grade solvents were purchased from Carlo Erba (Milan, Italy). Milli-Q water was produced by an ultrapure water purification system (Millipore, Milan, Italy).

LAB Isolates. Two hundred and sixteen LAB cultures were used in the present study, which were previously isolated from wheat sourdoughs destined for bread production [18] and from sourdoughs and dough collected during the production of panettone carried out in a local factory.[19]

Fungal Isolates. The isolation campaign was carried out in the above-cited panettone production factory, where the fungal cultures were isolated from either the indoor air or the surfaces of equipment and moldy products. Indoor air was sampled using a Surface Air Sampler (SAS) Super 90 (International PBI, Milan, Italy), whereas the surfaces were analyzed using either contact plates or the swabbing technique.[20] All of the fungal isolates were cultured on Rose-Bengal chloramphenicol (RBC) agar (Oxoid, Basingstoke, UK), at 25 °C for 48−72 h, and maintained on malt extract (ME) agar (Difco Laboratories, Detroit, MI, USA) at 4 °C.

Received: March 19, 2012
Revised: July 12, 2012
Accepted: July 14, 2012
Published: July 14, 2012

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Molecular Identification of Fungal Cultures. The genomic DNA of the fungal cultures was extracted using the procedure proposed by Moller et al.[21] The DNA yield and purity were checked by A_{260} and $A_{260/280}$ measurements, using a Cary 1E spectrophotometer (Varian, Turin, Italy), whereas DNA integrity was assessed by electrophoresis in 0.8% (w/v) agarose gel (Celbio, Milan, Italy).

A DNA fragment of about 1200 nucleotides (including the regions ITS1 and ITS2, the gene coding for the 5.8S rRNA, and about 635 bases coding for the 5′ end of the large subunit rRNA) was amplified using the primer pairs ITS1 [5′ TCC GTA GGT GAA CCT GCG G 3′] and D2R [5′ TTG GTC CGT GTT TCA AGA CG 3′] and following the procedure described by Hinrikson and colleagues[22] with minor modifications. Briefly, 40 ng aliquots of genomic DNA were amplified in a total volume of 50 μL, containing 10 mM Tris-HCl at pH 8.3, 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.2 μM ITS1 primer, 0.2 μM D2R primer, and 1.25 U of Taq polymerase (Applied Biosystem, Milan, Italy). The thermal cycling for the amplification reaction was optimized as follows: initial denaturation at 95 °C for 5 min, followed by 40 cycles at 95 °C for 30 s, 51 °C for 30 s, 72 °C for 2 min, and a final extension at 72 °C for 10 min. The PCR assay was carried out in a Gene Amp PCR System 9700 (Applied Biosystem). The DNA amplification was checked by electrophoresis in 1.5% (w/v) agarose gel. The PCR amplicons were purified with the GFX-PCR-DNA and Gel Band purification kit (Amersham Health, Buckinghamshire, UK) and sent to M-Medical (Milan, Italy) for sequencing. The sequences obtained in FASTA format were compared with those deposited in the GenBank DNA database (http://www.ncbi.nlm.nih.gov/) using the basic BLAST search tools.[23]

Screening of the Antifungal Activity of the LAB Cultures. All of the LAB cultures were preliminarily assayed in a dual culture overlay assay[24] on mSDB agar medium against each of the three fungal strains selected in this study as target cultures (*Aspergillus japonicus* M1, *Eurotium repens* M15, and *Penicillium roseopurpureum* M17). The plates were examined for clear halos of fungal growth inhibition around the bacterial spots, and the results were expressed as follows: (−) for the absence of inhibition halos corresponding to no suppression of fungal growth, and (+), (++), and (+++) for the presence of inhibition halos of 1−2, 2−4, and >4 mm, respectively, corresponding to increasing suppression of fungal growth.

Antifungal Activity of the Preselected LAB Culture Supernatants. The preselected LAB isolates were grown in mSDB broth at 30 °C for 48 h, and 15-fold concentrated cell-free supernatants in 20 mM citrate phosphate buffer (pH 3.6) were prepared as described by Magnusson and Schnürer.[24] Minimum inhibitory concentration (MIC) determinations were performed through serial 2-fold dilutions in microtiter plate wells[24] with *A. japonicus* M1, *Eurotium repens* M15, and *P. roseopurpureum* M17 as target organisms. A 50 μL aliquot of the concentrated cell-free supernatants and 50 μL of ME broth containing 10^4 fungal spores/mL were added to each well. Each microtiter plate included a positive control consisting of 100 μL of ME broth containing 5×10^2 fungal spores and a negative control consisting of 100 μL of sterile ME broth. Fungal growth was monitored at 25 °C with the naked eye. In agreement with Magnusson and Schnürer,[24] the results were recorded after 48 h. Their steadiness was further checked every day, up to 10 days, either with the naked eye or using an inverted microscope at ×40 magnification. The MIC was defined as the lowest concentration causing fungal growth inhibition, expressed as milliliters of native (nonconcentrated) supernatant per milliliter of reaction mixture. All of the assays were carried out in duplicate.

Identification of the Selected LAB Cultures. The LAB isolates, which were selected on the basis of the results of MIC determinations, were subjected to molecular identification by sequencing of the 16S rRNA gene, using primers P27f and P1495r, according to the method of Aquilanti et al.[25]

Effect of Temperature, Proteolytic Enzymes, and pH on the Antifungal Activity of *L. rossiae* LD108 and *L. paralimentarius* PB127. Aliquots (5 mL) of the 15-fold concentrated cell-free supernatants from the selected LAB strains *L. rossiae* LD108 and *L. paralimentarius* PB127 were subjected to the following treatments:[24] heating at 70, 121, and 170 °C for 10 min; enzymatic digestion at 37 °C for 90 min with 100 μg/mL of (i) Pronase E, (ii) α-chymotrypsin, and (iii) trypsin (all enzymes were purchased from Sigma); and pH adjustment to 2.5, 3.0, 4.0, 4.5, 5.0, 6.0, 7.0, and 9.0 with 1.0 M HCl or 2.0 M NaOH. The residual antifungal activity retained after each treatment was assessed against the three selected fungal cultures and expressed as MIC, using the microtiter plate method previously described. All of the treatments were carried out in duplicate.

Characterization of *L. rossiae* LD108 and *L. paralimentarius* PB127 Sourdoughs. The selected strains *L. rossiae* LD108 and *L. paralimentarius* PB127 were inoculated into experimental sourdoughs prepared using wheat (*Triticum aestivum*) flour containing 73% carbohydrate, 9.3% protein, 1.1% lipid, 0.52% ash, and 14.5% moisture. An overnight mSDB culture of each strain was obtained at 30 °C; cells were harvested by centrifugation at 2000*g* for 15 min, washed twice with sterile peptone water (0.1 wt %/vol), and separately resuspended in the same diluent to reach a final load of about 5×10^9 CFU/mL. Eighteen milliliters of each cell suspension was inoculated in a dough prepared with 300 g of wheat flour and 282 mL of tap water, giving a dough yield of 200 g. Each inoculated dough was mixed for 10 min with a homogenizer (Bimby TM31, Vorwerk, Milan, Italy), covered with cotton canvas, and incubated at 30 °C. At 0, 6, 12, 24, 30, and 48 h, sourdough samples were assessed for LAB viable counts on mSDB agar, pH, total titratable acidity (TTA), and content of lactic, acetic, phenyllactic, butyric, caproic, and propionic acids, as previously reported by Zannini et al.[26] Dough prepared by mixing 250 mL of tap water and 250 g of wheat flour was analyzed at the same time points as a noninoculated control.

Antifungal Activity of *L. rossiae* LD108 and *L. paralimentarius* PB127 Sourdough Extracts. Aliquots (500 g) of the two sourdoughs were separately homogenized in 500 mL of distilled water.

The homogenates were centrifuged at 4000*g* for 15 min at 15 °C, freeze-dried, resuspended in 150 mL of 20 mM citrate−phosphate buffer (pH 3.6), and subjected to the following extraction procedure: 5 mL of 1.0 M $HClO_4$ solution was added to 10 mL of each concentrate sourdough homogenate. The mixture was centrifuged at 4000*g* for 15 min at 15 °C, the supernatant was neutralized with 2.0 M KOH, and the volume was adjusted to 25 mL with distilled water. After 30 min on ice, the solution was filtered on 0.45 μm cellulose filter (Millipore). The noninoculated sourdough control prepared as described in the previous paragraph was treated as the inoculated sourdough, in analogy with the noninoculated MRS broth control used by Ström et al.[27] in a similar experiment. The three sourdough extracts were then analyzed by gradient HPLC using an Agilent 1100 HPLC system (Waldronn, Germany), equipped with a reverse phase C18 end-capped column (Purospher Star, 250 × 4 mm, i.d. = 5 μm; Merck Darmstadt, Germany) and a variable-wavelength detector set at 220 nm was used, at a flow rate of 1 mL/min and a column temperature of 50.0 ± 0.1 °C. Aqueous $HClO_4$ (1 mM) solution (A) and acetonitrile containing 5% water (B) were used as mobile phases following the gradient program reported in Table 1. The specific Agilent software (Chemstation Plus rev. A.09) was used for data acquisition and

Table 1. HPLC Gradient Elution Program

	elution gradient[a]	
time (min)	(%) solvent A: 1 mM $HClO_4$	(%) solvent B: CH_3CN/5% water
run time		
0	100	0
8	100	0
10	90	10
25	0	100
equilibration time	100	0
5		

[a]Reverse phase C_{18} end-capped column (250 × 4 mm i.d.; 5 μm; Purospher Star-Merck); flow rate = 1 mL/min; temperature = 50.0 ± 0.1°C.

processing. The HPLC fractions were manually collected at retention times (RTs) corresponding to the appearance of the peaks along the chromatogram. As far as possible, each fraction corresponded to a single peak or clusters of partly overlapping peaks. Fifteen fractions were collected in total. To verify possible interference of the mobile phase with the antifungal activity, a sample-free HPLC run was separately carried out as a control, and 15 sample-free fractions were collected following the previously defined RTs. Each HPLC run was repeated twice to assay the antifungal activity of each fraction (including the sample-free fractions) at two different pH values; this was accomplished by freeze-drying each fraction and redissolving it in a 40 μL aliquot of a 0.5% (w/v) methanol solution[27] preliminarily adjusted to pH 4.0 or 5.5. Each aliquot was transferred to a microtiter plate well, to which 40 μL of ME broth containing 10^4 spores/mL of *A. japonicus* M1 was added. Three negative control wells were included containing (i) 80 μL of sterile ME broth or (ii and iii) 80 μL of a 0.5% methanol solution at pH 4.0 and 5.5, respectively. Three positive control wells were also provided containing (i) 80 μL of ME broth with 4×10^2 fungal spores or (ii and iii) 40 μL of ME broth with 10^4 spores/mL plus 40 μL of a 0.5% methanol solution at pH 4.0 or 5.5, respectively. The microtiter plates were incubated at 25 °C, and fungal growth was monitored as previously described. All of the assays were carried out in duplicate.

Thermoresistance of HPLC Fraction 12 from *L. rossiae* LD108 Sourdough Extract. Aliquots of the HPLC fraction 12 from *L. rossiae* LD108 sourdough extract, prepared and adjusted to pH 5.5 as described above, were treated at 70, 121, and 170 °C for 10 min; the residual antifungal activity was assessed by the microtiter plate assay as described above, using the nonheated fraction as a control.

MALDI-TOF MS Characterization of Fraction 12 from *L. rossiae* LD108 Sourdough Extract. Forty-six milliliters of fraction 12 from *L. rossiae* LD108 sourdough extract was obtained by carrying out 20 HPLC runs. The pooled fraction was freeze-dried and redissolved in 80 μL of 0.1% (v/v) trifluoroacetic acid (TFA). Then a C_{18} reversed phase Zip-Tip microchromatographic (Millipore, Bedford, MA, USA) step was carried out to remove any traces of salt or ionic species that might remain after passage through the HPLC stationary phase, by washing with 0.1% TFA and eluting with 7 μL of 50% acetonitrile (ACN)/0.1% TFA (v/v). Matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) MS analyses were carried out on a Voyager DE-PRO (PerSeptive Biosystems, Framingham, MA, USA) instrument equipped with N_2 laser (337 nm, 3 ns pulse width) operating with an accelerating potential of 20 kV. Mass spectra were acquired in the reflector positive ion mode scanning the m/z 400−7000 range with α-cyano-4-hydroxycinnamic acid (10 mg/mL in 50% ACN/0.1% TFA) as a matrix. To increase the accuracy of the molecular mass measurement, the monoisotopic masses of ions detected in the reflector ion mode were selected for sequence reconstruction. External mass calibration was performed with a mixture of low molecular mass standard peptides (Sigma). Raw data were analyzed using the Data Explorer 4.0 software program supplied with the spectrometer. The peptide sequences reconstructed by computing the differences in MW among contiguous peptide ion signals were used to interrogate by homology analysis the protein and genomic databases (UniProt and GenBank of NCBI, respectively). BLAST and MS-Pattern tools of Protein-Prospector (http://prospector.ucsf.edu) were utilized as search engines. Searches were not restricted taxonomically, and possible ambiguity in the identification of isobaric amino acids (i.e., Ile and Leu; Gln and Lys) was taken into account. Exact matches or single amino acid mismatches were accepted as successful identifications.

Antifungal Activity of Wheat Protein Hydrolysates. A further confirmation of the possible involvement of peptides derived from wheat proteins in the activity of fraction 12 from *L. rossiae* LD108 sourdough extract was researched by assessing the antifungal activity of wheat hydrolysates against the target strain *A. japonicus* M1. Wheat protein fractions were extracted in a stepwise manner, according to the method of Wieser et al.,[28] from 400 mg of the Prolamin Working Group Standard *T. aestivum* flour.[29] Chymotryptic digests from albumins plus globulins and from gliadins were obtained by hydrolyzing 4 mg of the protein fractions with 1/100 (w/w) porcine pancreas α-chymotrypsin (Sigma), in 2 mL of 50 mM NH_4HCO_3, pH 8.5, at 37 °C for 4 h. The peptic−tryptic hydrolysates from albumins plus globulins and from gliadins were obtained using sequencing grade modified trypsin (Promega, Madison, WI, USA), according to the method of Mamone et al.[30] The chymotryptic and peptic−tryptic hydrolysates were resuspended in sterile 0.5% methanol at concentrations of 80 and 66 mg/mL, respectively, depending on their different solubility. The MIC values were determined using the microtiter plate well assay described under Antifungal Activity of the Preselected LAB Culture Supernatants (with 10 μL instead of 50 μL of ME broth containing 10^4 spores/mL of *A. japonicus* M1 being added to each well). Each microtiter plate included a positive control consisting of 60 μL of ME broth with 100 fungal spores and two negative controls, one consisting of 60 μL of sterile ME broth and the other of 60 μL of 0.5% methanol solution. The MIC was defined as the lowest concentration of protein hydrolysates that inhibited the growth of the indicator mold. All of the assays were carried out in duplicate.

Use of *L. rossiae* LD108 and *L. paralimentarius* PB127 Sourdoughs in Bread and Panettone Production. Three sourdoughs, prepared as described under Characterization of L. rossiae LD108 and L. paralimentarius PB127 Sourdoughs were inoculated with *L. rossiae* LD108 and *L. paralimentarius* PB127 either as single cultures or in a 1:1 association. The three types of sourdough were used at a 30% ratio in laboratory-scale breadmaking to manufacture three biologically acidified dough (BAD). These dough were prepared by mixing 770 g of wheat flour, 20 g of NaCl, 15 g of sucrose, 20 g of 7 log CFU/g baker's yeast, 462 g of sourdough, and 340 mL of tap water. As a control, a baker's yeast dough (BYD) was prepared by mixing 2000 g of wheat flour, 40 g of NaCl, 30 g of sucrose, 40 g of 7 log CFU/g baker's yeast, and 1214 mL of tap water. Bread loaves (202 × 102 × 55 mm) were made following the AACC10-09 official method. Four loaves were prepared from BAD, wheras seven loaves were obtained from BYD. All of the loaves were cooled overnight at room temperature, under a laminar flow fan. Subsequently, a loaf of each type was used to determine crumb pH as described by Zannini et al.,[26] and three loaves were sprayed on the top surface with 100 μL of a suspension containing 10^3 spores/mL of *A. japonicus* M1. Three further BYD loaves were sprayed with ethanol (2% vol/wt) before being inoculated with the fungal spore. All of the loaves were packed in polyethylene bags, stored at 25 °C, and monitored with the naked eye on a daily basis for mold growth.[5,6,11,13−17] The shelf life was defined as the number of days before fungal growth became visible on the loaf surface.[14]

An industrial scale production of panettone was carried out at the local factory previously cited, following the ordinary flowchart, described by Garofalo et al.,[19] except that *L. rossiae* LD108 sourdough was introduced at a 10% ratio (w/w on the final dough) into the first dough instead of the currently used sourdough. Standard production panettone cakes were taken as controls. Immediately after baking, all of the experimental and control cakes were transferred to our laboratories, where they were cooled overnight. Afterwards, three experimental and three control cakes were sprayed with the target strain *A. japonicus* M1 and packaged. All of these steps, as well as fungal growth monitoring, were carried out as described above for the bread samples.

RESULTS

Isolation and Identification of Fungal Cultures. The DNA sequence analyses carried out on 17 fungal cultures allowed the isolates to be identified at the species or genus level (Table 2). The strains *A. japonicus* M1, *E. repens* M15, and *P. roseopurpureum* M17 were chosen for subsequent use as target cultures in the antifungal activity assays.

Screening of the Antifungal Activity of LAB Cultures. The dual culture overlay assay carried out on the 216 isolates showed a different level of inhibition against the three fungal target strains, which in turn displayed a different degree of

Table 2. Identity and Source of Fungal Cultures Isolated at the Panettone Production Factory

isolate	closest relative	% identity[a]	accession no.[b]	source
M1	*Aspergillus japonicus*	99	EU021605	packaging conveyor
M2	*Davidiella tassiana*	98	AB492239	packaging conveyor
M3	*Tricoderma atroviride*	99	GQ463683	packaging conveyor
M4	*Tricoderma atroviride*	100	AY864321	cooling conveyor
M5	*Cladosporium* spp.	98	DQ780361	cooling conveyor
M6	*Penicillium roseopurpureum*	99	EU833225	external oven surface
M7	*Eurotium repens*	99	EF652049	flour bag
M8	*Davidiella tassiana*	100	EF679368	packaging conveyor
M9	*Alternaria alternata*	100	GU062279	external oven surface
M10	*Penicillium chrysogenum*	100	AB479305	external oven surface
M11	*Tricoderma citrinoviride*	99	FJ537127	external oven surface
M12	*Mucor racemosus*	99	AY213660	indoor air
M13	*Eurotium rubrum*	100	EF652067	panettone
M14	*Penicillium implicatum*	100	AF033428	panettone
M15	*Eurotium repens*	99	EF652049	panettone
M16	*Penicillium implicatum*	100	AF033428	panettone
M17	*Penicillium roseopurpureum*	99	EU833225	panettone

[a]Percentage of identical nucleotides in the sequence obtained from the isolates and the sequence of the closest relative found in the GenBank database. [b]Accession number of the sequence of the closest relative found by BLAST search.

sensitivity: *P. roseopurpureum* M17 was the most sensitive, being strongly inhibited (+++) by 40% of the LAB isolates, followed by *E. repens* M15, which suffered a moderate (++) inhibition by 34% of the LAB isolates, whereas *A. japonicus* M1 manifested the highest resistance, being subject to low suppression (+) or no suppression at all (−) by 31% of the LAB isolates (data not shown).

The 19 LAB isolates showing the highest level (+++) of antifungal activity against all three target strains were preselected for further investigation.

Antifungal Activity of the Preselected LAB Culture Supernatants. The cell-free supernatants of the culture broths of the 19 preselected LAB isolates showed MIC values ranging from 3.75 to 0.47 mL/mL (data not shown). Small differences were found in the susceptibility of the three target fungal strains, although *A. japonicus* M1 again emerged as the most difficult to inhibit. For all fungal strains the inhibitory effect persisted until the end of the observation time (10 days). The same results were always obtained in the microtiter plate assays carried out in duplicate.

The two isolates LD108 (from panettone sourdough) and PB127 (from bread sourdough) showed the lowest MIC values of 0.47 mL/mL against all three target fungal cultures, and therefore they were finally selected for further investigations.

Identification of the Selected LAB Cultures. The 16S rRNA gene sequences from the selected LAB isolates LD108 and PB127 showed identity percentages ≥99% with GenBank DNA sequences and *e* values lower than 0.05 (data not shown). The two isolates LD108 and PB127 were ascribed to *L. rossiae* (accession no. AM920330; Scherlink et al.[31]) and *L. paralimentarius* (accession no. EU483111; Zhang et al.[32]), respectively.

Effect of Temperature, Proteolytic Enzymes, and pH on the Antifungal Activity of *L. rossiae* LD108 and *L. paralimentarius* PB127. The antifungal activity of *L. rossiae* LD108 was fully maintained at pH ≤5.0, with unchanged MIC of 0.47 mL/mL, whereas it was completely lost at pH ≥6.0, with MIC > 3.75 mL/mL and only partially lost after each heat and enzyme treatment, with MIC of 0.94 mL/mL, irrespective of the type of treatment.

With regard to *L. paralimentarius* PB127, the antifungal activity was stable after all of the heat treatments with an unchanged MIC of 0.47 mL/mL, whereas all of the proteolytic treatments determined a partial suppression of antifungal activity with MIC of 3.75 mL/mL. As far as the variation in pH is concerned, the activity was totally retained at pH ≤4.5, with MIC of 0.47 mL/mL, slightly reduced at pH 5.0, with MIC of 0.94 mL/mL, and completely lost at pH 6.0, with MIC > 3.75 mL/mL. Weak antifungal activity was detected at pH 7.0, with

Table 3. Evolution of Microbiological and Chemical Parameters in *Lactobacillus rossiae* LD108 and *Lactobacillus paralimentarius* PB127 Sourdoughs[a]

					antifungal compound (mM)			
inoculated LAB	time (h)	cell counts (log CFU/g)	pH	TTA[b]	lactic acid	acetic acid	phenyllactic acid	diacetyl
LD108	0	7.5a	6.20 ± 0.04a	2.00 ± 0.02a	nd	nd	nd	nd
PB127	0	7.6a	6.27 ± 0.05a	1.96 ± 0.03a	nd	nd	nd	nd
LD108	6	8.2a	5.94 ± 0.02a	2.00 ± 0.02b	nd	nd	nd	nd
PB127	6	7.8a	5.83 ± 0.03b	2.50 ± 0.05a	nd	nd	nd	nd
LD108	12	9.0a	4.40 ± 0.03a	4.10 ± 0.02b	24.75 ± 1.30b	nd	nd	nd
PB127	12	9.2a	4.15 ± 0.04b	4.90 ± 0.05a	46.61 ± 1.20a	nd	0.09 ± 0.01	0.05 ± 0.01
LD108	24	9.8a	3.76 ± 0.01a	8.90 ± 0.01b	78.80 ± 1.90b	nd	nd	nd
PB127	24	9.3a	3.70 ± 0.03b	9.00 ± 0.04a	86.57 ± 2.03a	nd	0.20 ± 0.03	0.05 ± 0.01
LD108	30	10.4a	3.76 ± 0.05a	9.84 ± 0.02b	99.90 ± 3.05b	nd	nd	nd
PB127	30	10.5a	3.65 ± 0.02b	10.00 ± 0.06a	123.20 ± 2.09a	nd	0.34 ± 0.02	0.06 ± 0.01
LD108	48	9.2b	3.78 ± 0.02a	12.94 ± 0.05b	138.73 ± 3.02b	3.33 ± 0.01b	nd	nd
PB127	48	9.8a	3.63 ± 0.03b	13.10 ± 0.03a	160.00 ± 4.02a	6.66 ± 0.02a	0.54 ± 0.05	0.06 ± 0.02

[a]Mean value ± standard deviation of three replicates. Mean values labeled with different letters within each time point and within the same column are significantly different ($P > 0.05$). nd, not detected. [b]TTA, total titratable acidity (mL of 0.1 M NaOH/10 g of dough).

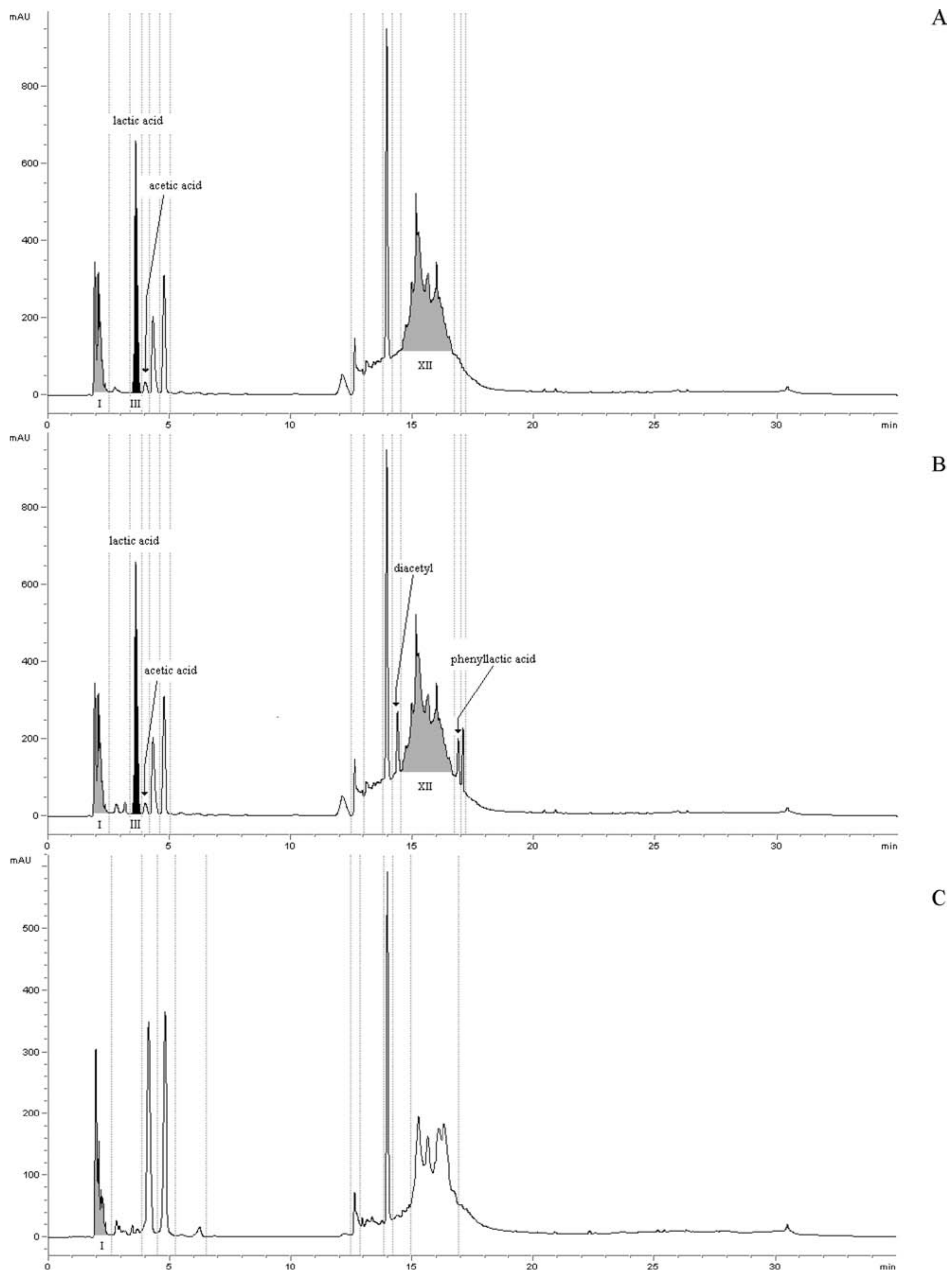

Figure 1. HPLC chromatograms of the extracts from *Lactobacillus rossiae* LD108 (A) and *Lactobacillus paralimentarius* PB127 (B) sourdoughs and from the nonfermented dough (C). The fractions with antifungal activity at both pH 4.0 and 5.5 are marked in light gray, whereas those active only at pH 4.0 are marked in black.

dx.doi.org/10.1021/jf301173u | J. Agric. Food Chem. 2012, 60, 7719−7728

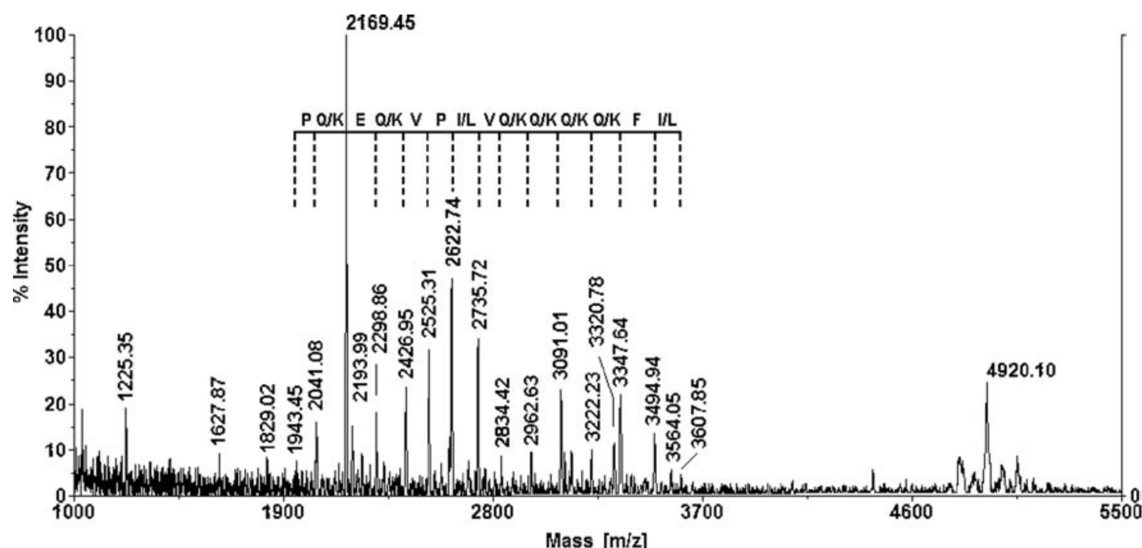

Figure 2. MALDI-TOF MS spectrum of fraction 12 from *Lactobacillus rossiae* LD108 sourdough extract. Each amino acid code corresponds to the mass difference between the two consecutive signals.

MIC of 3.75 mL/mL, whereas greater activity was observed at pH 9.0 with MIC of 0.94 mL/mL.

For both of the selected LAB strains, the effect of the different treatments did not vary depending on the fungal target culture used, and the inhibitory effect always persisted until the end of the observation time (10 days). The same results were always obtained in the microtiter plate assays carried out in duplicate.

Characterization of *L. rossiae* LD108 and *L. paralimentarius* PB127 Sourdoughs. The evolution of the main chemical and microbiological traits of the sourdoughs inoculated with the selected strains *L. rossiae* LD108 and *L. paralimentarius* PB127 is reported in Table 3. The lactic acid content was always higher in the *L. paralimentarius* PB127 sourdough, up to a final value of 160.00 ± 4.02 mM at the end of fermentation. Acetic acid was detected only at 48 h of fermentation, with a value of 6.66 ± 0.02 mM in *L. paralimentarius* PB127 sourdough, which was twice the amount found in *L. rossiae* LD108 sourdough (3.33 ± 0.01 mM). Phenyllactic acid and diacetyl were detected only in the sourdough inoculated with *L. paralimentarius* PB127, where they reached final concentrations of 0.54 ± 0.05 and 0.06 ± 0.02 mM, respectively. No detectable amounts of propionic, butyric, and caproic acids were found in either of the two sourdoughs and, as expected, neither organic acids nor diacetyl were detected in the noninoculated control dough.

Antifungal Activity of *L. rossiae* LD108 and *L. paralimentarius* PB127 Sourdough Extracts. The chromatograms obtained from HPLC analysis of the *L. rossiae* LD108 and *L. paralimentarius* PB127 sourdoughs and the control dough are reported in Figure 1. The fractions exhibiting antifungal activity in the microtiter plate bioassay against *A. japonicus* M1 at either pH 4.0 or 5.5 are highlighted. Both inoculated sourdoughs harbored antifungal activity in fraction 1 (RT 1.8−2.5 min) corresponding to unidentified peaks, fraction 3 (RT 3.5−3.9 min) corresponding to lactic acid, and fraction 12 (RT 14.5−16.8 min) corresponding to unidentified peaks. All of these fractions were active at pH 4.0, whereas only fractions 1 and 12 were active also at pH 5.5. Fraction 1 from the control dough extract was also found to be active, whereas no activity was found for any of the mobile phase HPLC fractions (data not shown). The antifungal activity of fraction 12 at pH 5.5 also proved to be heat stable because it was fully retained after all of the applied heat treatments (data not shown). The inhibitory effect of the active fractions persisted until the end of the observation time (10 days). The same results were always obtained in the microtiter plate bioassays carried out in duplicate.

MALDI-TOF MS Characterization of Fraction 12 from *L. rossiae* LD108 Sourdough Extract. The MALDI-TOF MS spectrum of fraction 12 from the *L. rossiae* LD108 sourdough extract, acquired in the linear ion mode, showed a complex pattern of signals in the range m/z 1200−5000 (Figure 2). Each ion signal corresponded to a peptide, and the mass differences between two consecutive ion signals (in the m/z range from 1943.45 to 3607.85) matched the molecular mass of specific amino acids engaged in peptide bonds. A sequence of 14 amino acids P[Q/K]E[Q/K]VP[I/L]V[Q/K][Q/K][Q/K][Q/K]F[I/L] was thus identified (Figure 2), which was fully included in the peptide with m/z 3607.85. This sequence was utilized for BLAST and MS-Pattern queries, and the sequence was found to be entirely contained in a series of α- and β-gliadin precursor proteins from soft wheat. No more significant matches were retrieved. In particular, the protein region containing the query sequence corresponded to the 17−31 peptide of α-gliadin, which is highly conserved among several homologous α- and β-gliadins. Furthermore, the molecular mass of the most intense signal at m/z 2169.45 matched the theoretical mass of the 1−19 peptide of the reference Q9ZP09 α-gliadin from UniProt, and the minor signal (m/z 1627.87) corresponded to the C-terminal 254−268 peptide of the Q9ZP09 α-gliadin (theoretical m/z 1627.88). Therefore, the 14 peptides observed in fraction 12 correspond to fragments of α-gliadin having sequences from 1−17 (measured m/z 1943.45, theoretical m/z 1943.04) to 1−31 (measured m/z 3607.85, theoretical m/z 3608.09) and differing from each other by a single amino acid. Finally, the low-intensity signals with higher molecular masses ($m/z \sim 4900$) were not definitely identified, although they might most likely correspond to additional gliadin fragments.

Antifungal Activity of Wheat Protein Hydrolysates. MIC values of 0.52, 2.5, 5, and 33 mg/mL were found for the

peptic−tryptic hydrolysates of albumins plus globulins, the chymotryptic hydrolysates of albumins plus globulins, the chymotryptic hydrolysates of gliadins, and the peptic−tryptic hydrolysates of gliadins, respectively. The inhibitory effect of each hydrolysate persisted until the end of the observation time (10 days). The same results were always obtained in the microtiter plate assays carried out in duplicate.

Antifungal Activity of *L. rossiae* LD108 and *L. paralimentarius* PB127 Sourdoughs in Bread and Panettone. Bread samples produced using the three experimental sourdoughs (inoculated with the two selected strains either as single cultures or in a 1:1 association) and experimentally contaminated with *A. japonicus* M1 spores showed different behavior when compared with each other and with the control (Table 4). The shelf life varied from 11 to 32 days, depending on the type of dough used; among the biologically acidified doughs, those inoculated with *L. rossiae* LD108 exhibited the highest inhibiting activity, leading to a shelf life of 32 days, which was notably overcome only by that (39 days) detected for the baker's yeast bread sprayed with ethanol as a preservative. No significant differences were found concerning the pH values of the sourdough breads, whereas, as expected, the baker's yeast bread displayed a significantly higher pH than all of the other samples (Table 4).

Table 4. pH and Shelf Life of the Experimental Bread Loaves in Relation to the Different Types of Doughs Used in Breadmaking Trials[a]

	type of dough[b]	bread pH	bread shelf life[c] (days)
BAD	*L. rossiae* LD108 sourdough	4.35 ± 0.03b	32
	L. paralimentarius PB127 sourdough	4.42 ± 0.02b	19
	L. rossiae LD108 + *L. paralimentarius* PB127 sourdough	4.31 ± 0.02b	30
BYD		6.05 ± 0.04a	11
			39[d]

[a]Mean value ± standard deviation of three replicates. Mean values labeled with different letters within the same column are significantly different ($P > 0.05$). [b]BAD, biologically acidified doughs containing 30% of the different sourdoughs; BYD, baker's yeast dough. [c]Shelf life means the number of days before fungal growth appeared on the surface of bread artificially contaminated with *Aspergillus japonicus* M1 spores. [d]Shelf life of bread sprayed with 2% ethanol before being artificially contaminated with *A. japonicus* M1 spores.

The antifungal efficacy of *L. rossiae* LD108 sourdough was then confirmed by using it in an experimental panettone production trial. In this case, the growth of the *A. japonicus* M1 mycelium from the artificially contaminating spores appeared on the surface of control panettone cakes after 3 months of storage, whereas panettone cakes prepared with *L. rossiae* LD108 sourdough appeared to be mold-free over 4 months of storage.

No differences in terms of shelf life were detected among bread and panettone replicates.

■ DISCUSSION

The objective of this study was to search for sourdough LAB capable of producing antifungal compounds to be used as biopreservatives in bakery products. In the isolation campaign aimed at collecting fungal cultures responsible for bakery product spoilage, species belonging to a wide range of genera were found, namely; *Aspergillus*, *Penicillium*, *Eurotium*, *Trichoderma*, *Davidiella*, *Mucor*, *Alternaria*, and *Cladosporium*, which are all well acknowledged as typical contaminants of cereals, bakery environments, and/or bakery products.[1−3,11] In particular, the species belonging to *Aspergillus* and *Penicillium* are capable of rapid growth on the surface of bread.[22] In this study, *E. repens* and *P. roseopurpureum* occurred both in the factory environment and on the surface of spoiled panettone, thus demonstrating the relevance of a postbaking cross-contamination from the factory environment to the products.[22] For this reason the two strains *E. repens* M15 and *P. roseopurpureum* M17 were chosen in this study as target cultures to assay the LAB antifungal activity. *A. japonicus* M1 was also selected in this study because it was the only isolate belonging to the genus *Aspergillus*. Intriguingly, this strain proved to be the most resistant to the inhibition exerted by our LAB isolates, and it was thus used as the target culture of choice when the antifungal activity was assayed against only one fungal strain.

The preliminary screening carried out against all three fungal target strains allowed 19 LAB cultures with a strong antifungal activity to be preselected. The results concerning the MIC values of the supernatants of these cultures allowed *L. rossiae* LD108 and *L. paralimentarius* PB127 to be recognized as the strains with the strongest antifungal activity. The presence of proteinaceous compounds besides organic acids was hypothesized in the culture supernatants of both strains because their activity was partially lost after proteolytic enzyme treatments. Likewise, the finding that *L. rossiae* LD108 culture supernatant partially lost its activity after all of the heat treatments suggests the presence of proteinaceous compound(s). This hypothesis is consistent with the finding of many antifungal proteinaceous compounds produced by LAB.[4] According to De Muynck et al.,[33] even the antifungal activity of *L. paralimentarius* PB127 at pH >5.0 cannot be ascribed to organic acids, and in particular its peculiar behavior at pH 7.0 and 9.0 can be explained by the presence of proteinaceous compounds for which the activity increases with increasing pH values.[34]

When two dough were separately inoculated with *L. rossiae* LD108 and *L. paralimentarius* PB127, the evolution of the chemical and microbiological parameters were investigated, and our results were consistent with those obtained by Ryan et al.,[6] in terms both of log and steady phase length and of final pH values. Concerning organic acids, in both sourdoughs, inoculated with either the obligate heterofermentative *L. rossiae* LD108 or the facultative heterofermentative *L. paralimentarius* PB127, acetic acid became detectable only at the end of fermentation, and it remained at a quite low concentration (3.33 ± 0.01 and 6.66 ± 0.02 mM, respectively). This was most likely due to the moderate quantity of oxygen available, according to Henry,[35] who found that the acetate produced by obligate heterofermentative lactobacilli in glucose− and maltose−SDB media was much lower under anaerobic and semianaerobic conditions than that detected under aerobiosis. As far as sourdoughs are concerned, the acetate production in our *L. rossiae* LD108 sourdough is very close to that found in the sourdough inoculated with *Lactobacillus brevis* (3.7 mM).[36] By contrast, the much higher values (160−175 mM) found by Zhang et al.[5] in sourdoughs fermented with *Lactobacillus buchneri* and *Lactobacillus diolivorans* could have been, in part, originated together with 1,2-propanediol, as alternative metabolites from lactate. As far as the production of

phenyllactic acid and diacetyl is concerned, the finding of these two antifungal products in only one of the two sourdoughs under study confirmed that their production varies greatly according to different species, strains, and (aerobic/anaerobic) conditions.[35−37] The amounts of phenyllactic acid found in the *L. paralimentarius* PB127 sourdough after 24 h were comparable to the highest values produced by several LAB strains in MRS after 72 h.[37]

Once *L. rossiae* LD108 and *L. paralimentarius* PB127 sourdoughs had been characterized up to 48 h of fermentation, the extracts from the final samples were subjected to HPLC runs and the antifungal activity of the collected fractions was assayed after being freeze-dried and redissolved in methanol at pH 4.0 and 5.5, to reproduce the pH values currently found in mature sourdoughs and bakery products, respectively.[13,16] Antifungal activity at both pH values was found in fraction 1, from both the sourdoughs and the control dough extracts. Therefore, the unknown compounds contained in this fraction, eluting in the early (hydrophilic) zone of the HPLC gradient, are likely to be derived from the flour or from the activity of native microorganisms carried by the flour itself. On the other hand, no activity was found at either pH 5.5 or 4.0 for the fractions corresponding to acetic acid, phenyllactic acid, and diacetyl, which are all acknowledged as fungal growth inhibitors.[4] The highest concentrations found in our study for these three compounds (6.66 ± 0.02, 0.54 ± 0.05, and 0.06 ± 0.02 mM, respectively) were indeed all lower than those reported as inhibitory for different fungal species in in vitro experiments.[4,12,38,39] Analogous remarks can be made about the lack of antifungal activity of the lactic acid contained in fraction 3 from both sourdough extracts at pH 5.5. This may be explained by the levels of lactic acid found in the sourdoughs (138.73 ± 3.02 and 160.00 ± 4.02 mM), which were too low to prevent the growth of *A. japonicus* M1 at this unfavorable pH.[39] On the other hand, irrespective of the low inhibitory capacity of the single antifungal metabolites, it is worth noting that all of these compounds are likely to act in a synergistic way as fungal growth inhibitors, as documented by other authors.[4,9] Interestingly, only fraction 12 from both *L. rossiae* LD108 and *L. paralimentarius* PB127 sourdough extracts exhibited antifungal activity at both pH 4.0 and 5.5, thus highlighting the nonacidic nature of the active compound(s) occurring in this fraction. This finding suggests that other compounds, besides organic acids and diacetyl, could be responsible for the overall synergistic antifungal activity of these strains in a sourdough system. Given that the two HPLC chromatograms from *L. rossiae* LD108 and *L. paralimentarius* PB127 sourdough extracts were practically identical with respect to fraction 12 peaks, only the fraction from *L. rossiae* LD108 sourdough extract was further investigated. First of all, the evidence that the antifungal activity exhibited at pH 5.5 by this fraction was stable after heat treatment up to 170 °C is especially interesting because this implies that the related compound(s) would be still active after baking and therefore able to protect bakery products from mold growth during shelf life. The presence of a cluster of overlapping peaks in the HPLC chromatograms in the correspondence of the broad fraction 12 fully agrees with the MALDI-TOF MS spectrum obtained from this fraction. It indeed proved that the fraction under study contains a series of 14 structurally related peptides with analogous chemical−physical properties, presumably originated as proteolysis byproduct from the N-terminal region of wheat *α*-gliadin. Although MALDI-TOF MS cannot provide quantitative results, the peptide corresponding to the 1−19 sequence of the reference Q9ZP09 *α*-gliadin of *T. aestivum*[40] appeared to be the most abundant in the fraction under study. Our hypothesis is further supported by the fact that gliadin-derived peptides have in general intrinsically much lower ionization yields than peptides from other wheat proteins such as albumins/globulins, due to the lack of basic (protonable) amino acids.[41] Therefore, when the corresponding signals are dominant, as in our case, their prevalence is practically proved, although the occurrence of other peptides (even if in a very low concentration) cannot be ruled out in principle. Moreover, given the soft nature of the MALDI ionization, it was possible to exclude that extensive fragmentation of a precursor peptide might occur during analysis. On the contrary, the simultaneous presence of progressively shorter peptides is most likely attributable to exopeptidase activities of sourdough lactobacilli, which are capable of hydrolyzing gluten proteins[42,43] and specifically *α*-gliadin fragments.[44,45] Accordingly, we hypothesized that the peptides occurring in fraction 12 may play a relevant role in the antifungal effect exerted by this fraction from *L. rossiae* LD108 sourdough extract, although it is clear that because these peptides appear to be originated from *α*-gliadin proteolysis, they should be different from the antifungal proteinaceous compounds produced by this strain in mSDB broth. However, a synergistic antifungal effect between gliadin peptides and these other proteinaceous compounds could be hypothesized. Although a genetic screening and/or biochemical assays involving the use of synthetic peptides, as performed by Coda et al.,[46] are required to provide ultimate confirmation about the mechanisms of the antifungal effect, the antifungal activity of the peptide(s) occurring in fraction 12, most likely originated by *α*-gliadin proteolysis, is further confirmed by the finding that wheat protein hydrolysates were also active against *A. japonicus* M1. Even though the identification of the specific active sequence(s) in the hydrolysates has not been attempted, given the extremely high number of different components which are derived from proteolysis of the entire gliadin fraction, the MIC values were comparable to, or lower than, those detected for cyclic dipeptides against *Penicillium roqueforti* and *Aspergillus fumigatus*[27] and linear peptides against other species of *Penicillium*, *Aspergillus*, and *Eurotium*.[13,17,47] In particular, Rizzello et al.[17] found a series of five antifungal peptides in RP-FPLC fraction 9 of the so-called sourdough fermented wheat germ (SFWG) containing the two selected LAB strains, *L. plantarum* LB1 and *L. rossiae* LB5.

As already done with other LAB culture candidates for use as biopreservatives in bakery products,[5,6,11,13−17] the potential of *L. rossiae* LD108 and *L. paralimentarius* PB127 to act as antifungal agents in real systems was assessed using sourdoughs inoculated with the two strains, either as single cultures or in association, in the production of bread and panettone. According to the procedure currently adopted in the bakery industry, the capacity of contrasting fungal spoilage was evaluated in terms of inhibition of any visible growth of fungal mycelium on the products' surface, which, in our case, had been artificially contaminated with spores of the target culture *A. japonicus* M1. Bread loaves prepared with the three types of sourdough always attained a shelf life much longer than the control ones obtained using baker's yeast, except when these were sprayed with ethanol as a preservative. Both sourdoughs inoculated with *L. rossiae* LD108, either alone or in association with the other selected strain, were able to prolong bread shelf life (32 and 30 days, respectively), compared to baker's yeast

bread (11 days), whereas no synergic effect of the two strains was supposed to occur. It is finally worth noting that the antifungal activity supplied by *L. rossiae* LD108 sourdough allowed fungal growth to be inhibited over 4 months in panettone cakes intentionally contaminated with *A. japonicus* M1 spores, which is, to our knowledge, the first evidence of the ability of sourdough LAB to prevent the molding of leavened products leading to such a prolonged shelf life.

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Notes

The authors declare no competing financial interest.

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