Antifungal Activity of *Wickerhamomyces anomalus* and *Lactobacillus plantarum* during Sourdough Fermentation: Identification of Novel Compounds and Long-Term Effect during Storage of Wheat Bread[▽]

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This study aimed at investigating the antifungal activity of *Wickerhamomyces anomalus* and sourdough lactic acid bacteria to extend the shelf life of wheat flour bread. The antifungal activity was assayed by agar diffusion, growth rate inhibition, and conidial germination assays, using *Penicillium roqueforti* DPPMAF1 as the indicator fungus. Sourdough fermented by *Lactobacillus plantarum* 1A7 (S1A7) and dough fermented by *W. anomalus* LCF1695 (D1695) were selected and characterized. The water/salt-soluble extract of S1A7 was partially purified, and several novel antifungal peptides, encrypted into sequences of *Oryza sativa* proteins, were identified. The water/salt-soluble extract of D1695 contained ethanol and, especially, ethyl acetate as inhibitory compounds. As shown by growth inhibition assays, both water/salt-soluble extracts had a large inhibitory spectrum, with some differences, toward the most common fungi isolated from bakeries. Bread making at a pilot plant was carried out with S1A7, D1695, or a sourdough started with a combination of both strains (S1A7-1695). Slices of the bread manufactured with S1A7-1695 did not show contamination by fungi until 28 days of storage in polyethylene bags at room temperature, a level of protection comparable to that afforded by 0.3% (wt/wt) calcium propionate. The effect of sourdough fermentation with *W. anomalus* LCF1695 was also assessed based on rheology and sensory properties.

The major problem for long-term shelf life of baked goods is contamination with fungi. Although they are used as the main chemical preservatives, salts of propionic, sorbic, and benzoic acids have shown several drawbacks. Reports on the appearance of cancer-like tumors in rats fed with propionic acid at concentrations of up to 4% led to prohibition of the use of calcium propionate in some European countries (31). Prolonged preservation of breads with high concentrations of calcium propionate stimulated the growth of resistant strains of *Penicillium roqueforti* (42). Since a few years ago, the European directive on preservatives (18) decreased the permitted concentrations of sorbate and propionate to 0.2 and 0.3% (wt/wt), respectively. Ethanol is also permitted as an antifungal preservative (2% [wt/wt]). Its inhibitory effect varies depending on the fungal species, and in some cases, it is not sufficient to prevent contamination (14).

Several studies have focused on the antifungal activity of compounds from natural sources and on biopreservation as effective alternatives to chemical preservatives. Several antimicrobial compounds have been identified, especially proteins or peptides from plants, as well as metabolites derived from fermentation by lactic acid bacteria (12, 23, 25, 42, 43). Besides acidification during sourdough fermentation, lactic acid bacteria also synthesize a wide range of low-molecular-weight compounds (27), peptides (28), and proteins (10, 37) with antifungal activity. Unfortunately, only a few studies have assessed their inhibitory activity under bread-making conditions. Recent studies proposed a combined effect of sourdough fermentation and extracts from plants which extended the shelf life of bread. The use of the water-soluble extract of *Phaseolus vulgaris* cv. Pinto together with sourdough fermentation by *Lactobacillus brevis* AM7 delayed mold growth during 21 days of storage at room temperature (10). The water-soluble extract of amaranth seeds was used as an ingredient for the manufacture of gluten-free and wheat flour breads, and inhibitory activity was achieved during long-term storage under pilot plant conditions (40). Recently, breads enriched with sourdough-fermented wheat germ delayed fungal growth for at least 28 days (37).

Besides these promising results, further efforts seem to be needed to achieve a long shelf life for leavened baked goods by use of convenient and simple biotechnology. Biopreservation using combinations of yeasts and bacteria has already been suggested as an alternative for storage of fruits and cereal grains (34). The capacity of antagonistic yeasts to control fungal contamination has been studied extensively, and several species, such as *Candida oleophila*, *Cryptococcus albidus*, and *Metschnikowia fruticola*, are included in commercially available biocontrol products (18). The mechanism for fungal inhibition varies depending on the species of yeast, features of the target fungi, and physical parameters of the biocontrol system (30, 34). Species belonging to the genus *Pichia* (e.g., *Pichia guilli-*

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ermondii, *Pichia angusta*, and *Pichia anomala*) are well known to control a range of postharvest fungi and to decrease sporulation and mycotoxin production (32). In particular, *P. anomala* (*Wickerhamomyces anomalus*, according to the latest taxonomic system) was shown to inhibit the growth of fungi in airtight-stored cereal grains (32). The biocontrol effect in a model grain silo with moist wheat (water activity of 0.96) was attributed to the antifungal action of metabolites derived from glycolysis rather than to competition for nutrients or activity of cell wall lytic enzymes (16).

This work aimed at investigating the mechanism of inhibition and the antifungal effect of a mixed starter consisting of selected yeasts and lactic acid bacteria during sourdough fermentation. A protocol for making bread with an extended shelf life was set up.

MATERIALS AND METHODS

Microorganisms, culture media, and growth conditions. *Lactobacillus brevis* AM7 (previously selected for antifungal activity) (10) and 40 strains of sourdough lactic bacteria belonging to *Lactobacillus rossiae* (6 strains), *Lactobacillus sanfranciscensis* (9 strains), *Lactobacillus plantarum* (6 strains), *Lactobacillus paraplantarum* (1 strain), *Lactobacillus pentosus* (2 strains), *Lactobacillus paralimentarius* (2 strains), *L. brevis* (5 strains), *Lactobacillus curvatus* (2 strains), *Lactobacillus sakei* (1 strain), *Lactobacillus fermentum* (1 strain), *Lactobacillus hilgardii* (1 strain), *Lactobacillus alimentarius* (1 strain), *Lactobacillus fructivorans* (1 strain), *Lactobacillus farciminis* (1 strain), and *Lactobacillus frumenti* (1 strain) were from the Culture Collection of the Department of Biologia e Chimica Agro-Forestale ed Ambientale (University of Bari, Italy). *W. anomalus* LCF1694 and *W. anomalus* LCF1695 were from the personal culture collection of G. Cardinali (Dipartimento di Biologia Applicata, University of Perugia, Italy). Lactic acid bacteria were propagated for 24 h at 30°C in MRS (Oxoid Laboratories, Hampshire, United Kingdom) with the addition of fresh yeast extract (5% [vol/vol]) and 28 mM maltose to a final pH of 5.6 (mMRS). Yeasts were propagated for 48 h at 30°C in YEPG (10 g/liter yeast extract, 10 g/liter peptone, 20 g/liter glucose). Enumeration of lactic acid bacteria and yeasts in dough was carried out by plating on mMRS and YEPG, respectively, at 30°C for 48 h.

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 (10). *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 herbariorum* CBS 117336, *Eurotium rubrum* CBS 150.92, *Aspergillus parasiticus* CBS 971.97, *Aspergillus versicolor* CBS 117286, and *Penicillium bialowiezense* CBS 110102 were from the culture collection of the Centraalbureau voor Schimmelcultures (Utrecht, Netherlands). All of the above species correspond to some of the most relevant spoilage fungi in baked goods (23). Fungi were grown on potato dextrose agar (pH 5.6) (PDA; Oxoid Laboratories) at 25°C for 24 to 72 h.

Wheat flour hydrolysate (WFH), used for the determination of conidial germination, was produced as described previously (19). WFH was chosen as the substrate because it is representative of the chemical composition of wheat flour.

Dough fermentation. The characteristics of the flour (*Triticum aestivum* cv. Appulo) used were as follows: moisture, 14.2%; protein ($N \times 5.70$), 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.

Preliminarily, strains of lactic acid bacteria and yeasts were used singly for dough fermentation. Cells were cultivated until the late exponential phase of growth was reached. After growth, cells were harvested by centrifugation at $10,000 \times g$ for 10 min at 4°C and were washed with 20 mM phosphate buffer, pH 7.0. Two hundred grams of flour, 115 ml of tap water, and 5 ml of the cell suspension (final cell density in the dough of ca. 10^8 and 10^6 CFU/g for lactic acid bacteria and yeasts, respectively) were used to produce 320 g dough (dough yield of 160) by use of a continuous high-speed mixer ($60 \times g$; dough mixing time, 5 min). A sourdough containing both *Lactobacillus plantarum* 1A7 and *W. anomalus* LCF1695 (final cell density in the dough of ca. 10^8 and 10^6 CFU/g, respectively) was also manufactured (S1A7-1695). Dough fermentation was carried out at 30°C for 16 h. After fermentation, doughs were subjected to water/salt-soluble extraction or used directly for bread making. Fermentations were carried out in triplicate and analyzed twice.

Water/salt-soluble extracts. Water/salt-soluble extracts from doughs were prepared according to the method originally described by Osborne (29) and modified by Weiss et al. (48). The supernatant was used for antifungal assay. The concentrations of peptides and proteins of the water/salt-soluble extracts and related fractions were determined by the o-phthaldialdehyde (OPA) and Bradford methods (6, 9), respectively.

Assays of antifungal activity. Three different methods were used to determine the antifungal activity. The agar diffusion assay was carried out as described by Coda et al. (10). Petri plates (90-mm diameter) containing 10 ml of PDA (Oxoid) were inoculated with 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 each sample to be assayed was 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). At the same time, zones of inhibition were evident in corresponding paper disks containing samples with inhibitory activity.

The inhibitory spectrum of the water/salt-soluble extracts was assayed based on the hyphal radial growth rates of fungi (36). The water/salt-soluble extracts were sterilized by filtration through 0.22-μm membrane filters (Millipore Corporation, Bedford, MA) and added (30% [vol/vol] final concentration) to sterilized PDA. After mixing of the samples, aliquots of 15 ml were poured into petri plates (90-mm diameter). Control plates contained PDA alone. The assay was carried out by placing 3-mm-diameter plugs of growing mycelia onto the center 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, in mm) on all plates was measured 8 days after inoculation. Each datum point is the mean for at least four measurements of a growing colony. The percent growth inhibition was calculated from the mean values as follows: percent inhibition = [(mycelial growth under control conditions − mycelial growth in the presence of water/salt-soluble extract)/mycelial growth under control conditions] × 100.

The effect of water/salt-soluble extracts on the germination of conidia was also determined (24). 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. Counts of the conidia in the suspension were carried out using a Petroff-Hausser counting chamber. A fixed number of ca. 10^6 to 10^7 conidia/ml was added to 5 ml of the mixture of WFH containing 30% (vol/vol) water/salt-soluble extract. The mixtures were incubated in 60-mm petri dishes for 24 h at 25°C under stirring conditions. WFH alone and WFH supplemented with 0.3% (wt/vol) calcium propionate were used as the controls. To determine the percentage of germinated conidia (length/width ratio of ≥2), slides of the suspension were examined under a microscope (magnification, ×40) at 4-h intervals. Three separate replicates of at least 100 conidia were used for each assay.

To determine the MIC, samples were serially diluted in WFH. The mixture was poured into 60-mm petri dishes, inoculated with a suspension of conidia (10^6 to 10^7 conidia/ml as the final density), and incubated for 12 h at 25°C under stirring conditions. After 12 and 24 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 extracts, related fractions, or ethanol/ethyl acetate aqueous solutions that inhibited fungal growth at 25°C for 12 h. All assays for antifungal activity were carried out at least in triplicate.

Proteolysis and heat stability of antifungal compounds. Water/salt-soluble extracts were treated with trypsin (EC 3.4.21.4; Sigma Aldrich Co.) as described by Atanassova et al. (2). Heat stability was determined by heating extracts at 100°C for 5 min. After treatments, the residual activity was determined by agar diffusion assay.

Purification of antifungal compounds. Water/salt-soluble extracts were fractionated by ultrafiltration (Ultrafree-MC centrifugal filter units; Millipore) using three different membrane sizes (50-, 30-, and 10-kDa cutoffs). Aliquots of 400 μl were centrifuged at $10,000 \times g$ for 60 min. After ultrafiltration, fractions were used for agar diffusion assay.

Ethanol and ethyl acetate were detected by high-performance liquid chromatography (HPLC), using an Äkta Purifier system (GE Healthcare) equipped with an Aminex HPX-87H column (ion-exclusion column; Bio-Rad), a UV detector operating at 210 nm, and a Perkin Elmer 200a refractive index detector operating at 32°C. Elution was done at 60°C, with a flow rate of 0.6 ml/min, using 10 mM H_2SO_4 as the mobile phase (40).

Samples containing peptides were partially purified by reversed-phase fast-performance liquid chromatography (RP-FPLC), using a Resource RPC column and an Äkta FPLC instrument with a UV detector operating at 214 nm (GE Healthcare BioSciences AB, Uppsala, Sweden). Aliquots containing 0.875 mg/ml of peptides were added to 0.05% (vol/vol) trifluoroacetic acid (TFA) and cen-

TABLE 1. Formulas for bread making

Ingredient	Amt in bread[d]				
	WB	WB[cp]	SB1A7	D1695	SB1A7-1695
Wheat flour (g)	250	250	175	175	175
Tap water (ml)	150	150	105	105	105
S1A7[a] (g)			120		
D1695[b] (g)				120	
S1A7-1695[c] (g)					120
Calcium propionate (g)		1.2			
Baker's yeast (g)	8	8	8	8	8

[a] Sourdough containing 62.5% flour and 37.5% water (dough yield of 160) and inoculated with *Lactobacillus plantarum* 1A7 (10^8 CFU/g).
[b] Dough containing 62.5% flour and 37.5% water (dough yield of 160) and inoculated with *Wickerhamomyces anomalus* LCF1695 (10^6 CFU/g).
[c] Sourdough containing 62.5% flour, 37.5% water (dough yield of 160) and inoculated with *Lactobacillus plantarum* 1A7 (10^8 CFU/g) and *Wickerhamomyces anomalus* LCF1695 (10^6 CFU/g).
[d] The dough yield of all breads was 160.

trifuged at $10,000 \times g$ 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 redissolved in sterile water and subjected to agar diffusion assay. The freeze-dried preparation of the fraction with antifungal activity was used for identification.

A mixture of standard organic acids (lactic, acetic, caproic, propionic, butyric, *n*-valeric, phenyl lactic, and formic acids) with presumptive antifungal activity that might be synthesized by lactic acid bacteria during sourdough fermentation (10) was analyzed by RP-FPLC under the same conditions.

Identification and synthesis of antifungal peptides. Identification of peptides was carried out by nano-liquid chromatography–electrospray ionization–tandem mass spectrometry (nano-LC-ESI-MS/MS), using a Finnigan LCQ Deca XP Max ion trap mass spectrometer (ThermoElectron) through the nano-ESI interface. MS spectra were automatically taken by Xcalibur software (ThermoElectron) in positive ion mode according to the manufacturer's instructions. MS/MS spectra were processed using the software BioWorks 3.2 (ThermoElectron), generating peak lists suitable for database searches. Peptides were identified using MS/MS ion searches of the Mascot search engine (Matrix Science, London, England) and the NCBI nr protein database (National Center for Biotechnology Information, Bethesda, MD). 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 manual evaluation as described by Chen et al. (8), and the validated peptide sequences explained all the major peaks in the MS/MS spectrum. Identified peptides 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.

Bread making. Breads with a dough yield of 160 were manufactured at the pilot plant of the Department of Biologia e Chimica Agro-Forestale ed Ambientale, University of Bari, Italy. The formulas for bread making are reported in Table 1. Preliminarily, the formula for SB1A7 was used to make breads with sourdoughs fermented by *L. rossiae* 3D, *L. plantarum* 1A7, *L. brevis* AM7 or 5S, or *L. fructivorans* DSM20203. A continuous high-speed mixer ($60 \times g$; dough mixing time, 5 min) was used to prepare the doughs. Fermentation was allowed at 30°C for 2.5 h, and breads were baked at 220°C for 40 min (Combo 3 oven; Zucchelli, Verona, Italy). For each bread, 2 slices were cut. The size of the slice was 12 cm (height) by 1.5 cm (width). One slice was inoculated by nebulization with a suspension of 10^2 conidia/ml of *P. roqueforti* DPPMAF1, and the other slice was not inoculated. Slices were packed in polyethylene bags to maintain constant moisture and then incubated at room temperature for 28 days. Moisture was determined according to the standard American Association of Cereal Chemistry (AACC) method (1).

Bread characterization. The pH values were determined online by use of a pH meter (model 507; Crison, Milan, Italy) with a food penetration probe. Instrumental texture profile analysis (TPA) was carried out with a TA.XT2i

texture analyzer, using a 35-mm flat-end aluminum compression disk (probe P/35). The selected settings were as follows: test speed of 1 mm/s, 30% deformation of the sample, and one compression cycle. TPA was carried out using Texture Export Exceed software (39). Specific volume was measured by the TA.XT2i system. 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). The crumb grain of breads was evaluated after 24 h of storage, using image analysis technology. Images of the sliced breads were captured using an image scanner (Amersham Pharmacia Biotech, Uppsala, Sweden). Images were scanned full-scale at 300 dots per inch and analyzed in gray scale (0 to 255). Image analysis was performed using the UTHSCSA ImageTool program (version 2.0; University of Texas Health Science Centre, San Antonio, TX [available by anonymous FTP from ftp://maxrad6.uthscsa.edu]). A threshold method was used for differentiating gas cells and noncells. The threshold was determined according to the method of Crowley et al. (13). Analysis was carried out on two subimages of 500 by 500 pixels (field of view) selected from within the bread slice. Two slices were analyzed per treatment. The crumb cell features recovered were number, area, perimeter, and ratio of gas cell area to total area.

Color was measured at three different positions of the bread surface by use of a Minolta CR-10 camera (2). The $L*a*b*$ color space analysis method was used, where $L*$ represents lightness (white-black) and $a*$ and $b*$ represent the chromaticity coordinates (red-green and yellow-blue, respectively). Results were reported in the form of a color difference, $dE*_{ab}$, as follows:

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

where dL, da, and db are the differences for L, a, and b values between the sample and the reference (a white ceramic plate having an L value of 93.4, an a value of -1.8, and a b value of 4.4).

Sensory analysis of breads was carried out by 10 panelists (5 male and 5 female; mean age, 35 years; age range, 18 to 54 years) according to the method described by Haglund et al. (21). Elasticity, color, acid taste, acid flavor, sweetness, dryness, and taste were the considered sensory attributes for breads (21), using a scale of 0 to 10, with 10 being the highest score. The sensory attributes were discussed with the assessors during the introductory sensory training sessions. Samples were served in random order and evaluated in two replicates by all panelists. In preparation for the sensory evaluation, the loaves were thawed at room temperature for 5 to 6 h and then cut into 1.5-cm-thick slices. Slices were cut into 4 pieces, and each assessor received 2 pieces per sample.

Statistical analysis. Data were subjected to one-way analysis of variance (ANOVA). Pairwise comparison of treatment means was achieved by Tukey's procedure with a significance level of P values of <0.05, using the statistical software Statistica 7.0 for Windows.

RESULTS

Selection of lactic acid bacteria and yeasts. As shown by agar diffusion assay, only 5 of the 40 water/salt-soluble extracts from sourdoughs had marked antifungal activity toward the indicator organism *P. roqueforti* DPPMAF1. These sourdoughs were fermented by *L. rossiae* 5A, *L. plantarum* 1A7, *L. brevis* AM7 or 5S, or *L. fructivorans* DSM20203. These sourdoughs were used for bread making. After 7 days of storage, only the slices of bread manufactured with the sourdough fermented by *L. plantarum* 1A7 (S1A7) did not show fungal contamination by *P. roqueforti* DPPMAF1, as determined by a visual inspection.

Regarding yeasts, the water/salt-soluble extract from the dough fermented by *W. anomalus* LCF1695 (D1695) showed the most inhibition of the indicator organism *P. roqueforti* DPPMAF1 (ca. 3 mm of clear zone near the rim of the colony).

Doughs fermented by *L. plantarum* 1A7 and *W. anomalus* LCF1695 were selected and used for further characterization.

Identification of antifungal compounds. The buffered water/salt-soluble extract of S1A7 had a pH of 7.34 ± 0.03. The concentrations of proteins and peptides were 2.38 ± 0.21 and

TABLE 2. Sequences of peptides identified in partially purified fractions of water/salt-soluble extract of sourdough fermented with *Lactobacillus plantarum* 1A7

Fraction	Sequence[a]	Score	Charge	Calculated mass (Da)	Expected mass (Da)	Delta	Source protein (NCBI accession no.)
12	PPDVLTKLTAVPAAQQLDEADGHPR	24	2	2,638.3715	2,638.2228	−0.1487	KNOS8_ORYSJ, homeobox protein knotted-1-like 8 (Q10ED2)
	SAFEFADEHKGAYS	17	2	1,557.6736	1,558.0624	0.3888	GUN23_ORYSJ, endoglucanase 23 (Q69NF5)
	AAIIFGSIFWNVGMKR	17	2	1,808.9760	1,808.4154	−0.5606	PDR13_ORYSJ, pleiotropic drug resistance protein 13 (Q8S628)
	ALGFEMTPEQIHQMI	16	2	1,743.8324	1,744.5257	0.6953	CML8_ORYSJ, probable calcium-binding protein CML8 (Q338P8)
13	IGRDVQNQSLFSPQVDSSSLLYNMVP NLTSNVSDGNLSTIPSGSTYLQN AMYG	18	3	5,678.7145	5,679.5927	0.8782	ARFL_ORYSI, auxin response factor 12 (Q258Y5)
	SAFEFADEHKGAYS	17	2	1,557.6736	1,558.0624	0.3888	GUN23_ORYSJ, endoglucanase 23 (Q69NF5)
	AEGEVILEDVQPSSVQS	14	2	1,785.8632	1,785.2057	−0.6576	COPD1_ORYSJ, coatomer subunit delta-1 OS (*Oryza sativa* subsp. *japonica*) (Q0DJA0)
21	EGTYLDYIQNNGKTLGAEDSTNEFG WDNK	31	3	3,278.4640	3,278.8756	0.4117	GUN23_ORYSJ, endoglucanase 23 (Q69NF5)
	DEEIMLDITTCAMAFRLLR	13	2	2,240.1003	2,239.8321	−0.2682	KS1_ORYSJ, ent-kaur-16-ene synthase, chloroplastic (Q0JA82)
	WFVELTGIPVTTTLMGLGNFPSDDPL SLRMLGMHGTVYANYAVDK	13	3	4,926.4435	4,925.9840	−0.4595	ILVB1_ORYSJ, acetolactate synthase 1, chloroplastic (Q6K2E8)

[a] The single-letter amino acid code is used.

1.92 ± 0.13 mg/ml, respectively. As shown by agar diffusion assays, the antifungal activity was almost completely lost by trypsin digestion, while it was unaffected by heating at 100°C for 5 min. Preliminarily, the water/salt-soluble extract of S1A7 was fractionated by ultrafiltration. Inhibitory activity toward *P. roqueforti* DPPMAF1 was found in all three fractions (cutoffs of 50, 30, and 10 kDa). As a consequence, further characterization was carried out only with the fraction showing a molecular mass of <10 kDa. This fraction was subjected to partial purification through RP-FPLC, and 37 fractions were separated (the concentrations of peptides varied from 0.30 ± 0.02 to 5.61 ± 0.04 mg/ml). Although weak antifungal activity was found in fractions 2, 3, and 21 (0.25 ± 0.01, 0.47 ± 0.02, and 2.04 ± 0.02 mg of peptide/ml, respectively), the most inhibition was found for fractions 12 and 13 (3.25 ± 0.03 and 4.21 ± 0.01 mg of peptide/ml, respectively). As expected, the mixture of organic acids used as the standard eluted in the hydrophilic zone of the acetonitrile gradient, which corresponded to fractions 1, 2, and 3. Therefore, the inhibitory activity of fractions 12, 13, and 21 was presumptively related to compounds different from organic acids.

The peptides contained in fractions 12, 13, and 21 were identified by nano-LC-ESI-MS/MS followed by MS/MS ion searches with the Mascot search engine. Nine peptides of 14 to 55 amino acid residues were identified (Table 2). Peptides PPDVLTKLTAVPAAQQLDEADGHPR, SAFEFADEHKG AYS, AAIIFGSIFWNVGMKR, and ALGFEMTPEQIHQMI

were identified in fraction 12. These peptides had experimental molecular masses of 2,638.37, 1,557.67, 1,808.98, and 1,743.83 Da, respectively. Except for AAIIFGSIFWNVGMKR, which had a positive charge (+2) and a total hydrophobic ratio of 62%, the other peptides had a negative charge. SAFEFADE HKGAYS was also identified in fraction 13, which contained two other peptides, with molecular masses of 5,678.71 and 1,785.86 Da, a net negative charge, and total hydrophobic ratios of 30 and 35%. The peptides EGTYLDYIQNNGKTL GAEDSTNEFGWDNK, WFVELTGIPVTTTLMGLGNFPS DDPLSLRMLGMHGTVYANYAVDK, and DEEIMLDITT CAMAFRLLR were identified in fraction 21. They had total net charges of −4, −1, and −2 and hydrophobic ratios of 20, 42, and 57%, respectively. By searches of the NCBI nr database, all peptides were reported as encrypted in sequences of *Oryza sativa* proteins. NCBI accession numbers of source proteins are listed in Table 2.

Partially purified fractions 12, 13, and 21 were freeze-dried, redissolved in WFH to have a range of concentrations of 0.5 to 20.0 mg of peptide/ml, and used to determine the MIC based on conidial germination. No germination of *P. roqueforti* DPPMAF1 conidia was found at concentrations of 3.9, 4.5, and 9.6 mg peptide/ml for fractions 12, 13, and 21, respectively. Peptides identified in the most active fractions (12 and 13) were chemically synthesized and used to determine the MIC. When used singly, SAFEFADEHKGAYS, AAIIFGSIFWNV GMKR, AEGEVILEDVQPSSVQS, and PPDVLTKLTAVP

AAQQLDEADGHPR confirmed the inhibitory activity. The range of MICs varied from 2.5 to 11.2 mg/ml. The other synthesized peptide also did not show appreciable inhibition at the highest concentration assayed. Among all possible combinations, the lowest MIC (ca. 2.05 mg of peptides/ml) was found when SAFEFADEHKGAYS and AAIIFGSIFWNVGMKR were used in a mixture.

The antifungal activity of the water/salt-soluble extract of D1695 (pH 7.82 ± 0.02) persisted after trypsin digestion or after heating at 100°C for 5 min. Protein derivatives were therefore excluded as the source of inhibitory activity. HPLC analysis of the water/salt-soluble extract of D1695 showed concentrations of ethanol and ethyl acetate of 1.48 (35.1 mM) and 6.50 (73.8 mM) mg/ml, respectively. Various aqueous solutions, containing ethanol and ethyl acetate at a molar ratio of 35:74, were used to determine the MIC. No germination of *P. roqueforti* DPPMAF1 conidia was found at concentrations of ethanol and ethyl acetate of 1.69 (36.6 mM) and 6.81 (77.0 mM) mg/ml, respectively.

Characterization of antifungal activity. The dough fermented with *W. anomalus* LCF1695 (D1695) and the sourdoughs started with *L. plantarum* 1A7 (S1A7) and with strains 1A7 and LCF1695 (S1A7-1695) were characterized. After fermentation, *L. plantarum* 1A7 was found at cell densities of 6.5 × 10⁹ and 3.4 × 10⁹ CFU/g in S1A7 and S1A7-1695, respectively. *W. anomalus* LCF1695 was found at cell densities of 6.2 × 10⁷ and 2.2 × 10⁷ CFU/g in D1695 and S1A7-1695, respectively. After 16 h of fermentation at 30°C, the pH values were 3.90 ± 0.02, 5.05 ± 0.01, and 3.79 ± 0.02 for S1A7, D1695, and S1A7-1695, respectively. As shown by HPLC analysis, the concentrations of ethanol and ethyl acetate in the water/salt-soluble extract of S1A7-1695 were 1.65 (35.8 mM) and 5.07 (57.5 mM) mg/ml, respectively.

After incubation for 12 h at 25°C on WFH, the mean percentage of spore germination of *P. roqueforti* DPPMAF1 was 16.9% ± 0.9% (Fig. 1). Incubation in the presence of the water/salt-soluble extracts of S1A7, D1695, and S1A7-1695 caused significant (P < 0.05) decreases of conidial germination, to values below 9.0%. In particular, the highest inhibitory activity was obtained with the water/salt-soluble extract of D1695 (7.0% ± 0.1%). The inhibitory activity of 0.3% (wt/vol) calcium propionate was slightly higher (7.8% ± 0.3%). After 24 h, the percentage of conidial germination in WFH reached 90% ± 0.7%. The inhibitory activity of 0.3% (wt/vol) calcium propionate (26% ± 0.3%) was lower than those found in the water/salt-soluble extracts of the selected dough and sourdoughs. The lowest value for conidial germination was still found for D1695 (9.7% ± 0.5%).

The activities of the water/salt-soluble extracts of S1A7, D1695, and S1A7-1695 were assayed against several fungi isolated from bakeries (Table 3). The growth inhibition was measured on PDA after 8 days of incubation at 25°C (Fig. 2). All fungi were variously affected. Regarding S1A7, the highest percentages of inhibition were found for *A. parasiticus* CBS 971.97 (58.2% ± 0.7%) and *P. polonicum* CBS 112490 (56.6% ± 1.5%). Weak antifungal activity was found against *P. albocoremium* CBS 109582, *A. versicolor* CBS 117286, *E. herbariorum* CBS 117336, and *P. bialowiezense* CBS 110102. Except for assays with *P. bialowiezense* CBS 110102 and *Eurotium rubrum* CBS 150.92, the activities of the water/salt-soluble ex-
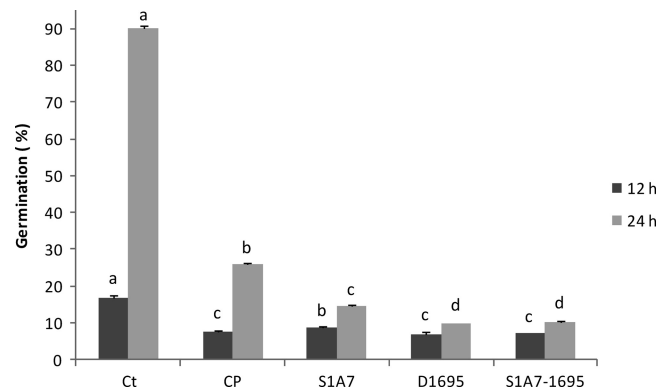

FIG. 1. Conidial germination of *Penicillium roqueforti* DPPMAF1 incubated in WFH for 12 and 24 h at 25°C under different conditions. Treatments were as follows: Ct, WFH alone (control); CP, WFH supplemented with 0.3% (wt/vol) calcium propionate; S1A7, WFH supplemented with 30% water/salt-soluble extract of sourdough fermented with *Lactobacillus plantarum* 1A7; D1695, WFH supplemented with 30% water/salt-soluble extract of dough fermented with *Wickerhamomyces anomalus* LCF1695; and S1A7-1695, WFH supplemented with 30% water/salt-soluble extract of sourdough fermented with *L. plantarum* 1A7 and *W. anomalus* LCF1695. Data are the means for three independent experiments, and the values within series marked with different letters are statistically significantly different (P < 0.05).

tract of D1695 were significantly (P < 0.05) higher than those of S1A7. The highest values of inhibition were found for *P. polonicum* CBS 112490 (61.5% ± 1.1%), *A. parasiticus* CBS 971.97 (58.2% ± 0.9%), and *P. polonicum* CBS 112490 (56.6% ± 0.3%). *P. chrysogenum* CBS 111214, *P. albocoremium* CBS 109582, and *A. versicolor* CBS 117286 were significantly (P < 0.05) more inhibited by the water/salt-soluble extract of S1A7-1695 than by those of S1A7 and D1695, whereas the growth inhibition was intermediate for the other fungi tested (Table 3).

Bread making. Five breads were manufactured at pilot plant scale. The pH values were 5.45 ± 0.08, 5.44 ± 0.05, 4.82 ± 0.05, 5.24 ± 0.05, and 4.83 ± 0.02 for wheat flour bread (WB), wheat flour bread containing calcium propionate (WBᶜᵖ), SB1A7, B1695, and SB1A7-1695, respectively (Table 4). The specific volumes of breads for which sourdough fermentation was used were always the highest (2.25 ± 0.04 and 2.40 ± 0.04 g/cm³ for SB1A7 and SB1A7-1695, respectively). Overall, for all parameters considered, no significant (P > 0.05) differences were found between WB and WBᶜᵖ. The concentrations of ethanol and ethyl acetate were determined in the water/salt-soluble extracts of B1695 and SB1A7-1695. A slightly larger amount of ethyl acetate was found in the water/salt-soluble extract of SB1A7-1695 (20.4 mM versus 19.5 mM), while both extracts contained ca. 21.7 mM ethanol.

The value of hardness, corresponding to the peak force of the first compression of the product, was the highest for B1695 (2,889 ± 22 g), and it differed significantly (P < 0.05) between SB1A7, SB1A7-1695, and WB (Table 4). Resilience indicates how well a product fights to regain its original position. The lowest value for resilience was found for SB1A7-1695 (0.32 ± 0.01), followed by SB1A7 (0.36 ± 0.01). The highest value (0.67 ± 0.03) was found for B1695. The fracturability point corresponds to the first significant peak force during compres-

TABLE 3. Inhibitory spectra of water/salt-soluble extracts of sourdoughs or dough fermented with *Lactobacillus plantarum* 1A7 (S1A7), *Wickerhamomyces anomalus* LCF1695 (D1695), or a combination of strains 1A7 and 1695 (S1A7-1695) (30% [vol/vol] on PDA)

Fungal strain	Source, country of isolation	Inhibitory activity (%)[a]		
		S1A7	D1695	S1A7-1695
Penicillium roqueforti DPPMAF1	Bread, Italy	42.6 ± 1.2^b	47.2 ± 0.8^a	45.6 ± 1.0^a
Penicillium polonicum CBS 112490	Bread, Italy	56.6 ± 1.5^c	61.5 ± 1.1^a	58.5 ± 0.5^b
Penicillium chrysogenum CBS 111214	Bread, England	23.6 ± 0.4^c	26.3 ± 0.5^b	34.2 ± 1.1^a
Penicillium paneum CBS 101032	Rye bread, Denmark	43.9 ± 0.6^c	59.7 ± 0.5^a	47.6 ± 0.9^b
Penicillium albocoremium CBS 109582	Cake factory, Denmark	4.3 ± 0.3^c	10.8 ± 0.4^b	13.9 ± 0.5^a
Penicillium chermesinum CBS 117279	Bakery plant, Netherlands	43.6 ± 0.7^b	56.3 ± 1.1^a	43.6 ± 0.9^b
Eurotium herbariorum CBS 117336	Chocolate cake, Netherlands	7.7 ± 0.5^c	46.1 ± 0.5^a	23.2 ± 0.6^b
Eurotium rubrum CBS 150.92	Cake	18.4 ± 0.3^a	10.5 ± 0.6^c	15.3 ± 0.6^b
Aspergillus parasiticus CBS 971.97	Indian sweets, India	58.2 ± 0.7	58.5 ± 0.3	58.3 ± 0.9
Penicillium carneum CBS 112297	Rye bread, Denmark	45.6 ± 0.6^c	59.6 ± 0.8^a	48.3 ± 0.9^b
Aspergillus versicolor CBS 117286	Wall in bakery, Netherlands	5.3 ± 0.4^c	12.3 ± 0.5^b	14.8 ± 0.5^a
Penicillum bialowiezense CBS	Bread, Italy	9.5 ± 0.6^a	4.7 ± 0.6^b	8.7 ± 0.4^a

[a] Inhibitory activity was determined by hyphal radial growth rates of fungi (36) after 8 days of incubation at 25°C. The percentage of growth inhibition was calculated from mean values as follows: % inhibition = [(mycelial growth under control conditions − mycelial growth in the presence of water/salt-soluble extract)/mycelial growth under control conditions] × 100. Data are means and standard deviations for at least four measurements of a growing colony. Means within a row with different superscript letters are significantly different ($P < 0.05$).

sion of the bread. In this case, the highest value was also found for B1695 (41,101 ± 24 J). Significant ($P < 0.05$) differences were also found between SB1A7 and SB1A7-1695 (35,702 ± 19 and 35,002 ± 19 J, respectively).

The crumb grain of the breads was determined by image analysis technology. Digital images were preprocessed to detect crumb cell total area by a binary conversion (black/white pixels) (Table 4). The cell total area (corresponding to the black pixel total area) was highest for SB1A7-1695 (44.18% ± 0.20%), followed by SB1A7 (42.28% ± 0.20%). The cell total area for B1695 was lower than that found for WB (34.76% ± 0.15% and 39.23% ± 0.1%, respectively). Crumb cell detection with bread slice portions showed some differences in the numbers of gas cells. The number of cells for SB1A7-1695 (231 ± 5) was the highest, followed by SB1A7 (188 ± 5). The mean areas and mean perimeters of cells for SB1A7-1695 (2.22 ± 0.02 mm² and 1.25 ± 0.01 mm, respectively) and SB1A7 (2.16 ± 0.02 mm² and 1.25 ± 0.01 mm, respectively) differed markedly ($P < 0.05$) from those found for B1695 and WB.

The crust showed a decrease in lightness (L) from 61.31 ± 0.10 to 56.73 ± 0.08, which corresponded to B1695 and SB1A7-1695, respectively (Table 4). The color difference (dE), calculated based on the chromaticity coordinates L, a, and b, was not significantly different for WB, WB[cp], or B1695. Significant ($P < 0.05$) differences were found for SB1A7 and SB1A7-1695 (dE values of 46.81 and 48.47, respectively).

A few hours after bread baking, sensory analysis was carried out (Table 5). SB1A7 and SB1A7-1695 received high scores for elasticity. In addition, the same two breads received the highest scores for acid taste and flavor. The scores for color were similar for WB, WB[cp], and B1695. They differed significantly ($P < 0.05$) between SB1A7 (6.6 ± 0.6) and SB1A7-1695 (7.6 ± 0.6). Sweetness scores were slightly higher for WB and WB[cp]. As previously reported (11, 38), the scores for dryness (how much the bread crumbles in the mouth) for sourdough-fermented SB1A7 and SB1A7-1695 were lower than that for baker's yeast bread (Table 5). The overall perception of taste was the highest for SB1A7-1695 (7.7 ± 0.5), followed by SB1A7, B1695, and WB and WB[cp].

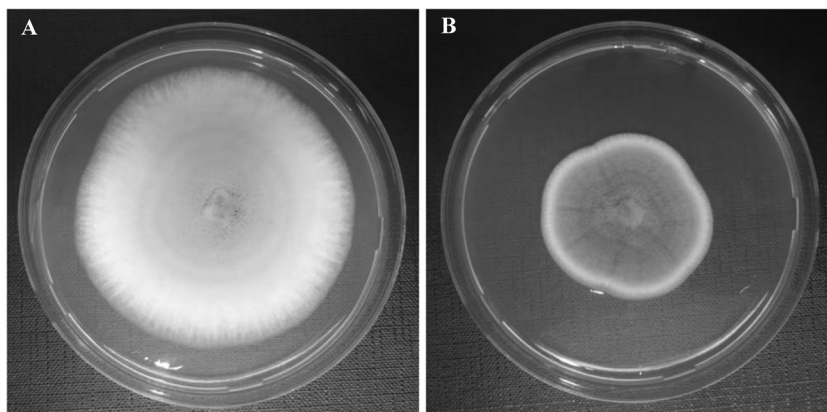After the bread was baked, 2 slices of each bread were cut

FIG. 2. Antifungal activity of water/salt-soluble extract of dough fermented with *Wickerhamomyces anomalus* LCF1695 (D1695), as determined by hyphal radial growth inhibition (44) after 8 days of incubation at 25°C. (A) PDA alone (control); (B) PDA containing water/salt-soluble extract of D1695 (30% [vol/vol]).

TABLE 4. Characterization and texture profile analysis of wheat flour bread (WB), wheat flour bread containing calcium propionate (WB[cp]), sourdough bread fermented with *Lactobacillus plantarum* 1A7 (SB1A7), bread fermented with *Wickerhamomyces anomalus* LCF1695 (B1695), and sourdough bread fermented with strains 1A7 and LCF1695 (SB1A7-1695)

Parameter	Value[a]				
	WB	WB[cp]	SB1A7	B1695	SB1A7-1695
pH	5.45 ± 0.08^c	5.44 ± 0.05^c	4.82 ± 0.05^a	5.24 ± 0.05^b	4.83 ± 0.02^a
Moisture	31.2 ± 0.3	31.4 ± 0.1	31.0 ± 0.2	31.4 ± 0.1	31.0 ± 0.2
TPA					
Sp vol (g/cm^3)	1.92 ± 0.02^b	1.90 ± 0.03^b	2.25 ± 0.04^c	1.84 ± 0.03^a	2.40 ± 0.04^d
Density (cm^3/g)	0.52 ± 0.02^c	0.53 ± 0.02^c	0.45 ± 0.02^b	0.56 ± 0.02^d	0.39 ± 0.02^a
Hardness (g)	$2,792 \pm 12^c$	$2,799 \pm 22^c$	$2,578 \pm 25^b$	$2,889 \pm 22^d$	$2,378 \pm 25^a$
Resilience	0.65 ± 0.02^c	0.64 ± 0.03^c	0.36 ± 0.01^b	0.67 ± 0.03^d	0.32 ± 0.01^a
Fracturability (J)	$39,897 \pm 28^c$	$39,901 \pm 24^c$	$35,702 \pm 19^b$	$41,101 \pm 24^d$	$35,002 \pm 19^a$
Image analysis					
Black pixel area (%)	39.23 ± 0.1^b	38.98 ± 0.1^b	42.28 ± 0.20^c	34.76 ± 0.15^a	44.18 ± 0.20^d
No. of cells detected	118 ± 3^a	120 ± 3^a	188 ± 5^c	135 ± 4^b	231 ± 5^d
Mean area (mm^2)	3.59 ± 0.02^c	3.58 ± 0.02^c	2.16 ± 0.02^a	3.20 ± 0.03^b	2.22 ± 0.02^a
Mean perimeter (mm)	1.52 ± 0.01^c	1.49 ± 0.01^c	1.25 ± 0.01^a	1.35 ± 0.02^b	1.25 ± 0.03^a
Color analysis					
L	61.27 ± 0.10^c	61.09 ± 0.10^c	58.93 ± 0.08^b	61.31 ± 0.10^c	56.73 ± 0.08^a
a	11.66 ± 0.02^b	11.62 ± 0.02^b	15.33 ± 0.02^c	11.55 ± 0.02^a	15.35 ± 0.02^c
b	33.8 ± 0.02^b	33.6 ± 0.01^b	31.03 ± 0.02^a	33.2 ± 0.03^b	31.05 ± 0.02^a
dE	45.58^a	45.57^a	46.81^b	45.14^a	48.47^c

[a] Triplicate measurements were made for each bread, and means $\pm$ standard deviations are reported. Means within a row with different superscript letters are significantly different ($P < 0.05$). The ingredients used for bread making are listed in Materials and Methods.

and inoculated with a suspension of *P. roqueforti* DPPMAF1 (10^2 conidia/ml). Slices were packed with polyethylene film and stored at room temperature. The fungal contamination of the bread slices was observed throughout 28 days (Table 6). The moisture of all slices was in the range of 30.5 to 31.4% during the 28 days.

Among the inoculated slices, only the WB slices were colonized by *P. roqueforti* DPPMAF1 after 7 days of storage. The growth of *P. roqueforti* DPPMAF1 was observed in WB[cp] and SB1A7 after 14 days of storage. At 28 days, only SB1A7-1695 showed weak fungal growth, while in all other slices the fungal contamination was higher than 20% (Table 6). Among the noninoculated slices, weak fungal contamination appeared in WB at 7 days, whereas no fungal growth was evident in the other cases. The fungal growth was also visible at 21 days in the slices of WB[cp] and SB1A7, while it was lower for B1695 (Table

TABLE 5. Sensory analysis of WB, WB[cp], SB1A7, B1695, and SB1A7-1695

Sensory attribute	Score[a]				
	WB	WB[cp]	SB1A7	B1695	SB1A7-1695
Elasticity	7.5 ± 0.4^b	7.5 ± 0.4^b	7.5 ± 0.6^b	7.0 ± 0.6^a	7.5 ± 0.6^b
Color	4.5 ± 0.5^a	4.5 ± 0.8^a	6.6 ± 0.6^b	4.6 ± 0.6^a	7.6 ± 0.6^c
Acid taste	2.3 ± 0.1^a	2.5 ± 0.5^a	6.5 ± 0.6^c	4.4 ± 0.6^b	7.0 ± 0.6^d
Acid flavor	2.5 ± 0.5^a	2.3 ± 0.1^a	6.0 ± 0.8^c	3.8 ± 0.8^b	6.3 ± 0.8^c
Sweetness	6.2 ± 0.5^b	6.0 ± 0.6^b	5.4 ± 0.3^a	5.4 ± 0.3^a	5.4 ± 0.3^a
Dryness	5.8 ± 0.2^c	5.6 ± 0.2^c	3.3 ± 0.5^a	4.0 ± 0.2^b	3.5 ± 0.3^a
Taste	3.8 ± 0.3^a	4.0 ± 0.6^b	7.0 ± 0.5^d	6.1 ± 0.5^c	7.7 ± 0.5^d

[a] Sensory attributes were scored by using a scale of 0 to 10. Data are means $\pm$ standard deviations for fermentations analyzed in duplicate. Means within row with different superscript letters are significantly different ($P < 0.05$). The ingredients used for bread making are listed in Materials and Methods.

6). No fungal contamination was noticeable for SB1A7-1695 at 28 days of storage (Table 6). After 28 days, the moisture of the slices gradually decreased (to values below 29.0%), indicating that no growth of molds was probably due to the decrease of the water activity.

DISCUSSION

Yearly, the growth of fungi destroys large amounts of vegetables, fruits, cereal grains, ingredients, foods, and feed (35). Beyond economic losses, the growth of fungi in foods causes a decrease of the nutritional value and the synthesis of allergenic spores and hazardous mycotoxins (23). Traditionally, fungicides and chemical preservatives have been used to prevent fungal growth, but concerns about environmental pollution and consumer health, along with problems of microbial resistance, favor the demand for alternative methods of control (16).

Besides the synthesis of other inhibitory compounds, lactic acid bacteria may release, through proteolysis, encrypted antimicrobial peptides from food proteins (10, 37). The purification of the antifungal compounds contained in the water/salt-soluble extract of sourdough fermented by *L. plantarum* 1A7 allowed the identification of 9 novel antifungal peptides. Several of the identified peptides show homology with known antifungal peptide sequences (47). The sequence SAFEFAD EHKGAYS was commonly found in two partially purified fractions. It is encrypted in the cereal enzyme endoglucanase 23 (NCBI accession no. Q69NF5). The antifungal activity of glucanases has already been shown by *in vitro* assays as well as by using transgenic plants (41). As shown in the Antimicrobial Peptide Database (47), using prediction and design tools, the

TABLE 6. Fungal contamination of slices of WB, WB^{cp}, SB1A7, B1695, and SB1A7-1695 packed in polyethylene bags and incubated at room temperature for 28 days

Day	Contamination[a]									
	Inoculated slices					Noninoculated slices				
	WB	WB^{cp}	SB1A7	B1695	SB1A7-1695	WB	WB^{cp}	SB1A7	B1695	SB1A7-1695
7	++	−	−	−	−	+	−	−	−	−
14	+++	+	+	−	−	++	−	−	−	−
21	++++	++	++	+	+/−	+++	+	+	+/−	−
28	++++	+++	++	++	+/−	++++	++	++	+	−

[a] Contamination was scored as follows: −, 0% contamination of the slice surface; +/−, 10% contamination; +, 20% contamination; ++, 40% contamination; +++, 80% contamination; ++++, 100% contamination. For each bread, a slice was inoculated by nebulization with a suspension of 10^2 conidia/ml of *Penicillium roqueforti* DPPMAF1, and the other slice was not inoculated. Fermentations were carried out in triplicate, and each bread was analyzed twice.

AAIIFGSIFWNVGMKR peptide may have a helix conformation with at least 6 residues on the same hydrophobic surface. This allows us to hypothesize an antimicrobial activity due to interactions with microbial membranes. The sequence ALGF EMTPEQIHQMI has 36% homology with the antimicrobial peptide alpha-basrubrin, identified in Ceylon spinach seeds (46). The peptide EGTYLDYIQNNGKTLGAEDSTNEFG WDNK has 36% homology with the lantibiotic lacticin 3147 (24). Overall, the MICs of the partially purified fractions from the water/salt-soluble extract of the sourdough fermented with *L. plantarum* 1A7 were lower than 10 mg/ml. These concentrations compared well with those of similar proteinaceous substances which also showed antifungal activities (10, 37, 40). Furthermore, the MIC of the mixture of the two chemically synthesized peptides SAFEFADEHKGAYS and AAIIFGSIF WNVGMKR was 2.05 mg/ml. The synergistic activity of antimicrobial peptides has been reported previously (17).

Yeasts also have promising features for use as alternatives to chemical fungicides (7, 35). Yeasts do not produce mycotoxins or allergenic spores, the vast majority are not pathogenic to humans or other animals, and several species grow at low values for water activity and at low partial pressures of O$_2$ (16, 34). Previously, other reports (3, 5, 16, 32) showed that *W. anomalus* (formerly known as *Hansenula anomala* or *Pichia anomala*) decreased the growth of *P. roqueforti* during storage of cereal grains. *W. anomalus* was the best among 60 different yeast species in the capacity to inhibit the growth of *P. roqueforti* in grain silo plants (16). *W. anomalus* decreased the accumulation of ochratoxin A during growth in coculture with *Penicillium verrucosum* (34). The water/salt-soluble extract of *W. anomalus* LCF1695 was not affected by either enzyme digestion or thermal treatment. This probably excluded an inhibitory activity related to protein killer toxins or cell wall-degrading enzymes, which are often synthesized by *W. anomalus* strains (45). Ethanol and ethyl acetate were also considered responsible for the antifungal activity of *W. anomalus* (4). In particular, ethyl acetate is considered the key inhibitory component (16), while a marginal but synergic role has been attributed to ethanol (16). Concentrations of 35.1 and 73.8 mM ethanol and ethyl acetate were found in the water/salt-soluble extract of the dough fermented with *W. anomalus* LCF1695. Ethyl acetate is the most significant ester synthesized by yeasts during fermentation (44). Compared to that in *Saccharomyces cerevisiae* or other yeasts, the synthesis of ethyl acetate by *W. anomalus* occurs via an inverse esterase from

acetate rather than from acetyl-coenzyme A (acetyl-CoA) via ethanol acetyltransferase (30). Considerable levels of ethyl acetate were synthesized by *W. anomalus* during *in vitro* growth or simulated biocontrol in silos with airtight-stored grain (16, 30), but no reports previously showed its synthesis and antifungal effect during bread making.

With the aim of comparing antifungal activities and technological properties, three doughs or sourdoughs fermented by *W. anomalus* LCF1695, *L. plantarum* 1A7, and their combination were manufactured according to traditional sourdough technology. As already shown for calcium propionate at a concentration of 0.3% (wt/vol) (10, 23), a weak effect against conidial germination was found for the sourdough fermented with *L. plantarum* 1A7, which suggested a fungistatic activity (10). After 24 h, the germination of *P. roqueforti* DPPMAF1 decreased ca. 90% when it was treated with the dough fermented by *W. anomalus* LCF1695. In agreement, a marked decrease of the germination of several fungi was found in the presence of strains of *W. anomalus* synthesizing ethyl acetate (32). The water/salt-soluble extract of the dough fermented by *W. anomalus* LCF1695 showed a large spectrum of activity toward fungal species which are commonly isolated from contaminated baked goods. The inhibition was higher than 45% for 7 of the 12 fungi tested. The spectrum of activity was narrower and the activity was generally lower during treatment with the water/salt-soluble extract of the sourdough fermented with *L. plantarum* 1A7. As expected, the sourdough fermented with both strains had an intermediate antifungal activity. The synthesis of ethyl acetate by *W. anomalus* LCF1695 slightly decreased during fermentation with *L. plantarum* 1A7. Compared to antifungal peptides, a predominant role of ethyl acetate was hypothesized.

Despite *W. anomalus* being found commonly in artisan bakery sourdoughs (14), to the best of our knowledge no reports have previously considered its use as a starter for sourdough fermentation (15). The bread manufactured with the combination of *L. plantarum* 1A7 and *W. anomalus* LCF1695 showed the most volume and softness, and in a sensory analysis, it was appreciated for elasticity, color, and overall taste. Although the concentration of ethyl acetate decreased in part during bread making under pilot plant conditions, this bread showed a very prolonged shelf life. Fungal growth was delayed at least until 28 days of storage at room temperature under common storage conditions. The inhibitory activity was higher than that found for calcium propionate (0.3% [wt/wt]). This work demonstrates the use of the nonconventional yeast *W. anomalus* as a mixed

starter for sourdough fermentation to extend the shelf life of baked goods while also keeping optimal taste and structure.

REFERENCES

1. **AACC.** 2003. Approved methods of the American Association of Cereal Chemistry, 10th ed. AACC, St. Paul, MN.
2. **Atanassova, M., et al.** 2003. Isolation and partial biochemical characterization of a proteinaceous anti-bacteria and anti-yeast compound produced by *Lactobacillus paracasei* subsp. *paracasei* strain M3. Int. J. Food Microbiol. **87:**63–73.
3. **Björnberg, A., and J. Schnürer.** 1993. Inhibition of the growth of grain-storage molds *in vitro* by the yeast *Pichia anomala* (Hansen) Kurtzman. Can. J. Microbiol. **39:**623–628.
4. **Borling, J.** 2010. Feed improvement by energy efficient storage using *Pichia anomala* inoculated ensiled cereal grain. M.S. thesis. Uppsala BioCenter, Department of Microbiology, Faculty of Natural Resources and Agriculture Science, Swedish University of Agricultural Science, Uppsala, Sweden.
5. **Boysen, M. E., S. Björneholm, and J. Schnürer.** 2000. Effect of the biocontrol yeast *Pichia anomala* on interactions between *Penicillium roqueforti*, *Penicillium carneum*, and *Penicillium paneum* in moist grain under restricted air supply. Postharvest Biol. Technol. **19:**173–179.
6. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
7. **Chand-Goyal, T., and R. A. Spotts.** 1996. Enumeration of bacterial and yeast colonists of apple fruits and identification of epiphytic yeasts on pear fruits in the Pacific Northwest United States. Microbiol. Res. **151:**427–432.
8. **Chen, Y., S. W. Know, S. C. Kim, and Y. Zhao.** 2005. Integrated approach for manual evaluation of peptides identified by searching protein sequence databases with tandem mass spectra. J. Proteome Res. **4:**998–1005.
9. **Church, F. C., H. E. Swaisgood, D. H. Porter, and G. L. Catignani.** 1983. Spectrophotometric assay using *o*-phthaldialdehyde for determination of proteolysis in milk and isolated milk proteins. J. Dairy Sci. **66:**1219–1227.
10. **Coda, R., et al.** 2008. Long-term fungi inhibitory activity of water-soluble extract from *Phaseolus vulgaris* cv. Pinto and sourdough lactic acid bacteria during bread storage. Appl. Environ. Microbiol. **74:**7391–7398.
11. **Coda, R., et al.** 2010. Spelt and emmer flours: characterization of the lactic acid bacteria microbiota and selection of mixed starters for bread making. J. Appl. Microbiol. **108:**925–935.
12. **Corsetti, A., M. Gobbetti, J. Rossi, and P. Damiani.** 1998. Antimould activity of sourdough lactic acid bacteria: identification of a mixture of organic acids produced by *Lactobacillus sanfrancisco* CB1. Appl. Microbiol. Biotechnol. **50:**253–256.
13. **Crowley, P., T. Schober, C. Clarke, and E. Arendt.** 2002. The effect of storage time on textural and crumb grain characteristics of sourdough wheat bread. Eur. Food Res. Technol. **214:**489–496.
14. **Daniel, H. M., M.-C. Moons, S. Huret, G. Vrancken, and L. De Vuyst.** 2010. *Wickerhamomyces anomalus* in the sourdough microbial ecosystem. Antonie Van Leeuwenhoek. doi:10.1007/s10482-010-9517-2.
15. **Dantigny, P., A. Guilmart, F. Radoi, M. Bensoussan, and M. Zwietering.** 2005. Modelling the effect of ethanol on growth rate of food spoilage moulds. Int. J. Food Microbiol. **98:**261–269.
16. **Druvefors, U. A., V. Passoth, and J. Schnürer.** 2005. Nutrient effects on biocontrol of *Penicillium roqueforti* by *Pichia anomala* J121 during airtight storage of wheat. Appl. Environ. Microbiol. **71:**1865–1869.
17. **Egorov, T. A., T. I. Odintsova, V. A. Pukhalsky, and E. V. Grishin.** 2005. Diversity of wheat anti-microbial peptides. Peptides **26:**2064–2073.
18. **European Union.** 1995. European Parliament and Council directive no. 95/2/EC of 20 February 1995 on food additives other than colours and sweeteners, p. 53. European Union, Brussels, Belgium. http://europa.eu.int /eur-lex/en/consleg/pdf/1995/en_1995L0002_do_001.pdf.
19. **Gobbetti, M., A. Corsetti, and J. Rossi.** 1994. The sourdough microflora. Interactions between lactic acid bacteria and yeasts: metabolism of carbohydrates. Appl. Microbiol. Biotechnol. **41:**456–460.
20. Reference deleted.
21. **Haglund, Å., L. Johansson, and L. Dahlstedt.** 1998. Sensory evaluation of wholemeal bread from ecologically and conventionally grown wheat. J. Cereal Sci. **27:**199–207.
22. Reference deleted.
23. **Lavermicocca, P., et al.** 2000. Purification and characterization of novel antifungal compounds from the sourdough *Lactobacillus plantarum* strain 21B. Appl. Environ. Microbiol. **66:**4084–4090.
24. **Magnusson, J., and J. Schnürer.** 2001. *Lactobacillus coryniformis* subsp. *coryniformis* Si3 produces a broad-spectrum proteinaceous antifungal compound. Appl. Environ. Microbiol. **67:**1–5.
25. **Magnusson, J., K. Ström, S. Roos, J. Sjögren, and J. Schnürer.** 2003. Broad and complex antifungal activity among environmental isolates of lactic acid bacteria. FEMS Microbiol. Lett. **219:**129–135.
26. Reference deleted.
27. **Niku-Paavola, M., L. A. Laitila, T. Mattila-Sandholm, and A. Hikara.** 1999. New types of antimicrobial compounds produced by *Lactobacillus plantarum*. J. Appl. Microbiol. **86:**29–35.
28. **Okkers, D. J., L. M. T. Dicks, M. Silvester, J. J. Joubert, and H. J. Odendaal.** 1999. Characterization of pentocin TV35b, a bacteriocin-like peptide isolated from *Lactobacillus pentosus* with a fungistatic effect on *Candida albicans*. J. Appl. Microbiol. **87:**726–734.
29. **Osborne, T. B.** 1907. The proteins of the wheat kernel. Carnegie Institute of Washington publication 84. Judd and Detweiler, Washington, DC.
30. **Passoth V., E. Fredlund, U. Ä. Druvefors, and J. Schnürer.** 2006. Biotechnology, physiology and genetics of the yeast *Pichia anomala*. FEMS Yeast Res. **6:**3–13.
31. **Pattison, T. L., D. Lindsay, and A. von Holy.** 2004. Natural antimicrobial as potential replacements for calcium propionate in bread. S. Afr. J. Sci. **100:**339–342.
32. **Petersson, S., and J. Schnurer.** 1995. Biocontrol of mold growth in high-moisture wheat stored under airtight conditions by *Pichia anomala*, *Pichia guilliermondii*, and *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. **61:**1027–1032.
33. Reference deleted.
34. **Petersson, S., N. Jonsson, and J. Schnürer.** 1999. *Pichia anomala* as a biocontrol agent during storage of high-moisture feed grain under airtight condition. Postharvest Biol. Technol. **15:**175–184.
35. **Piano, S., V. Neyrotti, Q. Migheli, and M. L. Gullino.** 1997. Biocontrol capability of *Metschnikowia pulcherrima* against *Botrytis* postharvest rot of apple. Postharvest Biol. Technol. **11:**131–140.
36. **Quiroga, E. N., A. R. Sampietro, and M. A. Vattuone.** 2001. Screening antifungal activities of selected medicinal plants. J. Ethnopharmacol. **74:**89–96.
37. **Rizzello, C. G., A. Cassone, R. Coda, and M. Gobbetti.** 2011. Antifungal activity of sourdough fermented wheat germ used as an ingredient for bread making. Food Chem. **127:**952–959.
38. **Rizzello, C. G., L. Nionelli, R. Coda, M. De Angelis, and M. Gobbetti.** 2010. Effect of sourdough fermentation on stabilization, and chemical and nutritional characteristics of wheat germ. Food Chem. **119:**1079–1089.
39. **Rizzello, C. G., L. Nionelli, R. Coda, R. Di Cagno, and M. Gobbetti.** 2010. Use of sourdough fermented wheat germ for enhancing the nutritional, texture and sensory characteristics of the white bread. Eur. Food Res. Technol. **230:**645–654.
40. **Rizzello, C. G., et al.** 2009. Long-term fungal inhibitory activity of water-soluble extract from *Amaranthus* spp. seeds during storage of gluten-free and wheat flour breads. Int. J. Food Microbiol. **131:**189–196.
41. **Selitrennikoff, C. P.** 2001. Antifungal proteins. Appl. Environ. Microbiol. **67:**2883–2894.
42. **Sjögren, J., J. Magnusson, A. Broberg, J. Schnürer, and L. Kenne.** 2003. Antifungal 3-hydroxy fatty acids from *Lactobacillus plantarum* MiLAB 14. Appl. Environ. Microbiol. **69:**7554–7557.
43. **Ström, K., J. Sjögren, A. Broberg, and J. Schnürer.** 2002. *Lactobacillus plantarum* MiLAB 393 produces the antifungal cyclic dipeptides cyclo(L-Phe-L-Pro) and cyclo(L-Phe-L-*trans*-4-OH-L-Pro) and β-phenyllactic acid. Appl. Environ. Microbiol. **68:**4322–4327.
44. **Swiegers, J. H., E. J. Bartowsky, P. A. Henschke, and I. S. Pretorius.** 2005. Yeast and bacterial modulation of wine aroma and flavour. Aust. J. Grape Wine Res. **11:**139–173.
45. **Walker, G. M., A. H. Mcleod, and V. J. Hodgson.** 1995. Interactions between killer yeasts and pathogenic fungi. FEMS Microbiol. Lett. **127:**213–222.
46. **Wang, H., and T. B. Ng.** 2001. Novel antifungal peptides from Ceylon spinach seeds. Biochem. Biophys. Res. Commun. **288:**765–770.
47. **Wang, Z., and G. Wang.** 2004. APD: the Antimicrobial Peptide Database. Nucleic Acids Res. **32:**590–592.
48. **Weiss, W., C. Vogelmeier, and A. Gorg.** 1993. Electrophoretic characterization of wheat grain allergens from different cultivars involved in bakers' asthma. Electrophoresis **14:**805–816.