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Antifungal Activity of Phenyllactic Acid against Molds Isolated from Bakery Products

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Phenyllactic acid (PLA) has recently been found in cultures of *Lactobacillus plantarum* that show antifungal activity in sourdough breads. The fungicidal activity of PLA and growth inhibition by PLA were evaluated by using a microdilution test and 23 fungal strains belonging to 14 species of *Aspergillus*, *Penicillium*, and *Fusarium* that were isolated from bakery products, flours, or cereals. Less than 7.5 mg of PLA ml^{-1} was required to obtain 90% growth inhibition for all strains, while fungicidal activity against 19 strains was shown by PLA at levels of ≤10 mg ml^{-1}. Levels of growth inhibition of 50 to 92.4% were observed for all fungal strains after incubation for 3 days in the presence of 7.5 mg of PLA ml^{-1} in buffered medium at pH 4, which is a condition more similar to those in real food systems. Under these experimental conditions PLA caused an unpredictable delaying effect that was more than 2 days long for 12 strains, including some mycotoxigenic strains of *Penicillium verrucosum* and *Penicillium citrinum* and a strain of *Penicillium roqueforti* (the most widespread contaminant of bakery products); a growth delay of about 2 days was observed for seven other strains. The effect of pH on the inhibitory activity of PLA and the combined effects of the major organic acids produced by lactic acid bacteria isolated from sourdough bread (PLA, lactic acid, and acetic acid) were also investigated. The ability of PLA to act as a fungicide and delay the growth of a variety of fungal contaminants provides new perspectives for possibly using this natural antimicrobial compound to control fungal contaminants and extend the shelf lives of foods and/or feedstuffs.

The increased interest in biopreservation of food systems has recently led to the development of new natural antimicrobial compounds having different origins. A variety of systems to prevent food spoilage have been investigated; these include animal-derived systems (lysozyme, lactiferrin, magainins, etc.), plant-derived products (phytoalexins, herbs, spices), and microbial metabolites, including bacteriocins, hydrogen peroxide, and organic acids (12). Few of the major food preservation techniques (e.g., low temperature, low water activity, acidification, etc.) act by inactivating the spoilers, while most of the newer or emerging techniques (irradiation, electroporation, high hydrostatic pressure, etc.) act by directly inactivating microorganisms. Nowadays, in the case of bread and bakery products, which can be contaminated by a variety of molds (mainly *Aspergillus* and *Penicillium* species), contamination can be prevented by irradiating a product with infrared rays or microwaves, by using a modified atmosphere, or by adding fungal inhibitors, such as ethanol, propionic acid, sorbic acid, and acetic acid (17). In particular, propionic acid and its salts are commonly used to extend the shelf lives of bakery products. Recently, the levels of chemical preservatives permitted in bakery products in Europe have been reduced due to application of EU Directive 95/2/CE (1), which allows concentrations of propionate and sorbate salts of 0.3 and 0.2% (wt/wt), respectively, for packaged sliced breads, although the latter is rarely used because of its secondary effects on bread volume (17). When 0.3% (wt/vol) calcium propionate was tested in a conidial germination assay with several molds contaminating bakery products, fungal growth still occurred, suggesting that the use of suboptimal salt concentrations might not assure preservation (16).

Among the natural preserving systems, sourdough has long been known to improve the shelf lives of bread and bakery products. Rocken (25) observed that sourdough antifungal activity was strictly related to acetic acid production. More recently, the use of sourdough lactic acid bacteria to inhibit mold growth was studied, which led to the identification of a strain of *Lactobacillus plantarum* 21B whose culture filtrate showed an important antifungal activity. Phenyllactic acid (PLA) was shown to be one of the major compounds occurring in the culture, together with lactic acid and acetic acid (16). Dieleveux et al. (8) isolated PLA from a culture filtrate of *Geotrichum candidum* and characterized it as the main compound responsible for the anti-*Listeria* activity shown by the fungal culture. These authors obtained relevant inhibition of pathogen growth in an agar diffusion well assay by using DL-PLA, while D-3-PLA inhibited the growth of *Listeria monocytogenes* cultured in liquid medium or in ultrahigh-temperature whole milk and the growth of several strains of *Staphylococcus aureus*, *Escherichia coli*, and *Aeromonas hydrophila* on solid medium (6, 7). PLA has been reported to be one of the most abundant aromatic acids to which antibacterial properties have been attributed and to occur in several honeys with different geographical origins (28, 31).

In this study the antifungal activity of PLA against a variety of fungal species isolated from bakery products and flours and two ochratoxin A-producing strains isolated from cereals was evaluated. For each strain, the minimal fungicidal or inhibitory PLA concentration was determined together with the behavior at pH conditions more similar to those in real food systems

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TABLE 1. MIC_{90} and MFC of fungal strains

Taxon	Culture collection no.	Source	MIC_{90} (mg ml^{-1})	MFC (mg ml^{-1})
Fusarium sp.	ITEM5153	Bread	3.75	3.75
Penicillium verrucosum	FR22625	Grain	3.75	5
Penicillium chrysogenum	ITEM5151	Bakery product	3.75	5
Penicillium chrysogenum	ITEM5152	Bread	5	5
Penicillium solitum	ITEM5149	Bread	5	5
Penicillium roqueforti	IBT18687	Bread	5	5
Penicillium commune	ITEM5150	Bread	5	5
Penicillium polonicum	ITEM5142	Bread	5	7.5
Penicillium polonicum	ITEM5143	Bread	5	7.5
Penicillium polonicum	ITEM5141	Bakery product	7.5	7.5
Aspergillus ochraceus	FR21991	Cereal	7.5	7.5
Penicillium sp.	ITEM5147	Wheat flour	5	7.5
Penicillium sp.	ITEM5148	Bakery product	7.5	7.5
Aspergillus niger	FTDC3227	Bread	7.5	>10
Aspergillus niger	ITEM5132	Bread	7.5	>10
Aspergillus flavus	FTDC3226	Bread	7.5	10
Aspergillus flavus	ITEM5134	Wheat flour	7.5	10
Aspergillus flavus	ITEM5135	Wheat flour	7.5	7.5
Aspergillus terreus	ITEM5136	Wheat flour	7.5	10
Penicillium brevicompactum	ITEM5140	Bread	7.5	10
Penicillium citrinum	ITEM5144	Wheat flour	7.5	>10
Penicillium citrinum	ITEM5145	Wheat flour	7.5	10
Penicillium citrinum	ITEM5146	Wheat flour	7.5	>10

with respect to the ability to inhibit and delay mold growth. The effect of PLA in combination with the main organic acids produced in culture by *L. plantarum* 21B was also investigated.

MATERIALS AND METHODS

Antifungal substances. DL-β-PLA was supplied by Sigma-Aldrich Division (Milan, Italy), L-(−)-β-PLA and D-(−)-β-PLA were supplied by Fluka (Sigma-Aldrich Division, Milan, Italy), and lactic and acetic acids were supplied by Carlo Erba (Milan, Italy).

Fungal cultures. All fungal strains used in this study were isolated from bakery products, wheat flour, or cereals (Table 1). In particular, *Aspergillus flavus* ITEM5134 and ITEM5135, *Aspergillus niger* ITEM5132, *Aspergillus terreus* ITEM5136, *Penicillium brevicompactum* ITEM5140, *Penicillium* sp. strains ITEM5147 and ITEM5148 (species morphologically related to *P. brevicompactum* but not yet characterized), *Penicillium chrysogenum* ITEM5151 and ITEM5152, *Penicillium citrinum* ITEM5144, ITEM5145, and ITEM5146, *Penicillium commune* ITEM5150, *Penicillium polonicum* ITEM5142, ITEM5143, and ITEM5141, *Penicillium solitum* ITEM5149, and *Fusarium* sp. strain ITEM5153 were isolated in Apulia, Italy, identified and confirmed by different morphological procedures (14, 22, 26), and deposited in the ITEM Culture Collection of the CNR Institute of Sciences of Food Production, Bari, Italy. *A. flavus* FTDC3226 and *A. niger* FTDC3227 were obtained from the Culture Collection of the Food Technology Department, University of Lleida, Lleida, Spain; *Penicillium roqueforti* IBT18687 and two strains of ochratoxin A producers, *Aspergillus ochraceus* FR21991 and *Penicillium verrucosum* FR22625, were obtained from the Culture Collection of the Technical University of Denmark, Lyngby, Denmark. For some strains, a high-performance liquid chromatography analysis of culture extracts obtained by microscale extraction (27) was performed to determine the chromatographic metabolite profiles. In particular, production of citrinin was ascertained for the three strains of *P. citrinum*, while the three *A. flavus* strains did not produce aflatoxins when they were grown on the solid media yeast extract sucrose agar (26) and Czapek yeast extract agar (23).

Preparation of inoculum suspension. Fungal conidia were collected from 7-day-old cultures on potato dextrose agar (PDA) (Difco Laboratories, Detroit, Mich.) and washed twice with distilled water, and a 50-μl aliquot of each conidial suspension was spread on PDA plates and incubated at 26°C for 72 h. Conidia were collected by using 0.05% (vol/vol) Triton X-100, and 10 μl of the conidial suspension, containing about 5×10^4 conidia, was used as the inoculum.

PLA solutions. Preliminary experiments showed that there were not significant differences ($P > 0.05$) in the inhibitory activities against *A. niger* FTDC3227 between the racemic and D and L isomers of PLA at a concentration of 20 mg ml^{-1}; therefore, DL-β-PLA was used in all experiments.

(i) MIC and MFC evaluation. To determine the 90% MIC (MIC_{90}) and the minimal fungicidal concentration (MFC), a PLA stock solution (pH 2.6) containing 20 mg of PLA ml^{-1} in wheat flour hydrolysate (WFH) broth (11) was serially diluted to obtain concentrations of 15, 10, 7.5, 5, 3.75, 2.5, and 1.87 mg ml^{-1}, and the preparations were filter sterilized. A WFH solution was used as a control.

(ii) Influence of pH on PLA activity. In order to test PLA at pH values closer to those in real food systems, the influence of pH on PLA activity was assayed by using *A. niger* FTDC3227. pH values of 2.6, 4.0, 4.5, 5.0, and 5.5 were obtained by dissolving PLA (20 mg ml^{-1}) in WFH diluted 1:1 (vol/vol) with water and phosphate buffer containing 0.18, 0.22, 0.25, and 0.3 mol of KH_2PO_4-K_2HPO_4 $liter^{-1}$, respectively. WFH diluted 1:1 with water was used as a control solution.

(iii) Antifungal activity of PLA at pH 4. Antifungal activity at pH 4 was evaluated by using all of the fungal strains and PLA solutions containing 20, 15, 10, 7.5, 5, and 3.75 mg of PLA ml^{-1} in WFH diluted 1:1 (vol/vol) with phosphate buffer at different concentrations (0.18, 0.12, 0.08, 0.06, 0.04, and 0.03 mol of KH_2PO_4-K_2HPO_4 $liter^{-1}$, respectively). WFH diluted 1:1 with water was used as a control solution.

(iv) Inhibitory effect of PLA in the presence of other organic acids. PLA was also tested at a concentration of 5 mg ml^{-1} and pH 4 in the presence of lactic and/or acetic acid at the concentrations found in the culture filtrate of *L. plantarum* 21B (16) (0.79 and 0.017 mg ml^{-1}, respectively) and at the following higher concentrations: 7.9 and 15.8 mg of lactic acid ml^{-1}; 0.17, 0.34, and 0.67 mg of acetic acid ml^{-1}; and 15.8 mg of lactic acid ml^{-1} and 0.67 mg of acetic acid ml^{-1}.

Microdilution tests. Microdilution tests were performed with sterile, disposable, multiwell microdilution plates (96 wells; IWAKI; Scitech Div., Asashi Techno Glass, Tokyo, Japan). Test solutions were dispensed into the wells in 190-μl portions inoculated with 10 μl of a conidial suspension containing about 5×10^4 conidia. Inoculated wells were prepared in quintuplicate, and blanks were prepared in triplicate. All microdilution plates were incubated in a humid chamber at 26°C for 120 h. Fungal growth was observed with a reverse microscope and was measured by determining the optical density at 580 nm every 24 h with a spectrophotometer (Labsystem Multiskan MS, version 3.0, type 352). In each experiment, an uninoculated control (WFH containing antifungal compounds) and an untreated inoculated control were included. The MIC_{90} was defined as the lowest concentration of PLA that resulted in at least a 90% reduction in growth, as measured by optical density, compared to the growth of an untreated control after 72 h of incubation at 26°C. To quantify the MFC, plate counts of the fungi were determined on PDA by using 10-μl portions from wells containing PLA concentrations higher than the MIC_{90}, the PLA concentration equal to the MIC_{90}, and the PLA concentration that was just below the MIC_{90}. The MFC was defined as the lowest concentration in which no conidial germi-

nation was observed after 72 h of incubation at 26°C. Triplicate determinations were performed.

Data analysis. Optical density measurements recorded every 24 h from zero time to 120 h were used to generate growth curves for each fungal strain. The Gompertz model was used as a mathematical means of fitting growth curves to estimate microbial growth kinetics (2). Three points (optical densities at 72, 96, and 120 h) of the control growth curve were used to calculate with the Gompertz model the additional time (growth delay, expressed in hours) required by PLA-treated suspensions to reach the optical density of the control at that time. The growth delays with respect to the control at these three times (GD_{72}, GD_{96}, and GD_{120}, respectively) were predicted by the Gompertz model in the cases where the optical density of the control was not reached within the experimental period. The Sigma Plot program (SPSS Science Software Gmb, Erkrath, Germany) was used for graphics and data elaboration.

RESULTS

MIC and MFC evaluation. PLA showed a broad spectrum of activity by inhibiting all fungal strains, with MIC_{90} ranging from 3.75 to 7.5 mg ml^{-1} (Table 1). No relevant variability in susceptibility among species or among strains was observed. Moreover, PLA acted with a fungicidal mechanism since no conidial germination was observed after 3 days for most strains at concentrations ranging from 3.75 to 10 mg ml^{-1} according to the fungal strain (Table 1). In particular, PLA showed fungicidal activity at levels of ≤10 mg ml^{-1} against 19 strains (of the 23 strains tested) belonging to 13 different species. For *A. ochraceus*, *A. flavus* (one strain), *P. roqueforti*, *P. chrysogenum* (one strain), *P. solitum*, *P. commune*, *P. polonicum* (one strain), *Penicillium* sp. (one strain), and *Fusarium* sp., PLA amounts corresponding to the MIC_{90} also caused the death of all conidia; i.e., the MIC_{90} and MFC values were identical. For the remaining strains, only the solution next most concentrated after the MIC_{90} solution was required to determine the MFC. The inhibition of conidial germination of *P. roqueforti* IBT18687 is shown in Fig. 1 as an example of the PLA effect in relation to the concentration tested. In the absence of the antifungal compound (Fig. 1A), a branched and spreading mycelium was observed, while treatment with 3.75 mg of PLA ml^{-1} (Fig. 1B) resulted in germination of a small number of conidia which showed limited germ tube development. Complete inhibition of conidial germination was observed when a slightly more concentrated PLA solution was applied (5 mg ml^{-1}) (Fig. 1C). Moreover, no conidial germination occurred when an aliquot of the suspension containing 5 mg of PLA ml^{-1} was subcultured on PDA plates incubated for 72 h, confirming the MFC for this fungus.

Influence of pH on PLA activity. The evaluation of the influence of pH on the inhibitory activity of PLA (20 mg ml^{-1}) against *A. niger* FTDC3227 showed that the greatest antifungal effect was obtained in unbuffered medium (pH 2.6), while a relevant decrease in PLA activity occurred at the highest pH tested (50.2% ± 2.2% at pH 5.5) (Fig. 2). At pH 4, a value close to the pH values in several real food systems and commonly reached in the culture filtrate of lactic acid bacteria, a slight reduction (15.5% ± 0.8%) in PLA activity was observed.

Antifungal activity of PLA at pH 4. When the inhibitory activity of PLA against all strains was tested at pH 4, a concentration of 5 or 7.5 mg ml^{-1} was found to cause more than 50% growth inhibition compared with the growth of the controls after 72 h (Table 2). For all fungal strains the inhibitory effect of PLA persisted until the end of the observation time (5 days). After 72 h of incubation a concentration of 5 mg ml^{-1} was sufficient to reduce the growth of 13 strains by 50 to 82%, while the growth of the other 10 strains was reduced by 50 to 78% when the organisms were incubated in the presence of 7.5 mg of PLA ml^{-1} (Table 2).

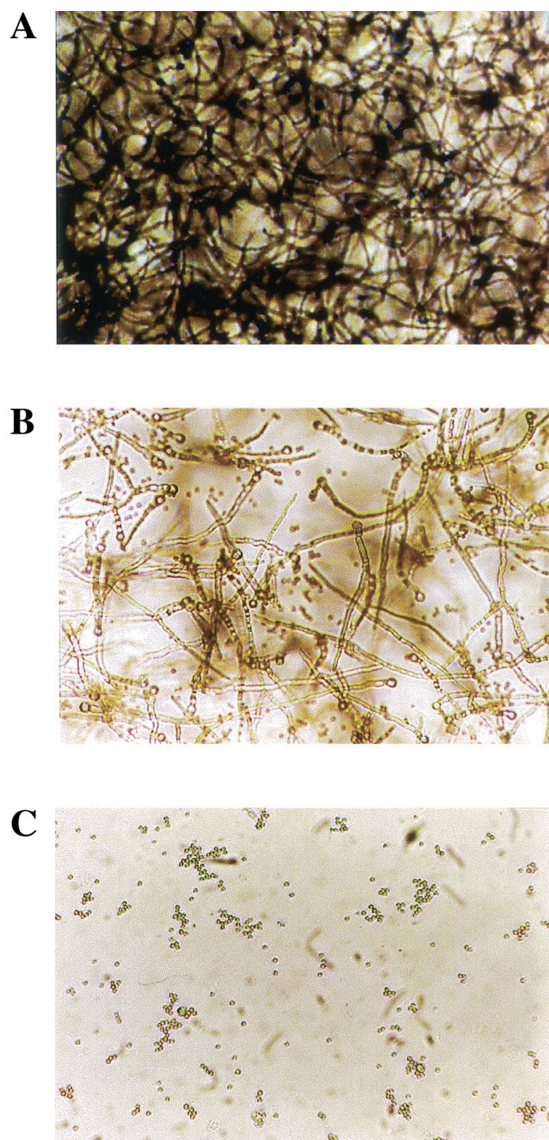

FIG. 1. Reverse microscope photographs of *P. roqueforti* IBT18687 cultures after 72 h in the absence of PLA (A) or in the presence of PLA at concentrations of 3.75 mg ml^{-1}(B) and 5 mg ml^{-1} (C).

Figure 3 shows the growth of selected fungal strains in the presence of 5 and 7.5 mg of PLA ml^{-1} compared to the growth of the controls. In particular, *P. roqueforti*, *A. niger*, and *A. flavus* were selected because they are major fungal contaminants of bakery products; three strains proven to produce mycotoxins (namely, *A. ochraceus* and *P. verrucosum* strains producing ochratoxin A and a *P. citrinum* strain producing citrinin) were also used in this analysis. Some inhibition of *A. ochraceus* FR21991 by PLA was observed, mainly after 72 h; there was a growth delay of about 24 h, and no difference was found between 5 and 7.5 mg of PLA ml^{-1}. In addition, no

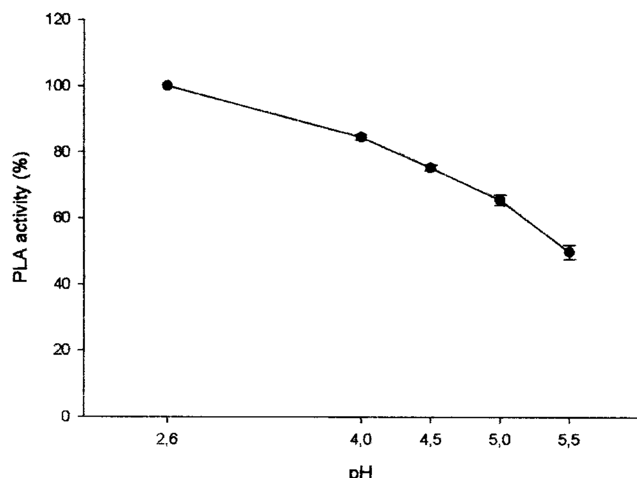

FIG. 2. Influence of pH on PLA activity against *A. niger* FTDC3227. The standard errors were less than or equal to 2.2%.

substantial difference between these PLA concentrations was observed for *A. niger* ITEM5132 and *A. flavus* ITEM5134, although both of these strains were sensitive to the compound and the *A. flavus* strain was strongly inhibited (>50%) during the entire experimental period. The effect of PLA on *P. citrinum* ITEM5144 was evident after 72 h and was greater at the higher concentration. *P. roqueforti* IBT18687 and *P. verrucosum* FR22625 behaved similarly, showing the effect of PLA after 48 to 72 h at both concentrations, while a strong difference between 5 and 7.5 mg ml^{-1} was observed at 120 h, with *P. roqueforti* maintaining a level of inhibition higher than 50%.

By fitting the growth curves in Fig. 3 with Gompertz parameters referring to the control growth at 72 h, unpredictable growth delays were found for *P. roqueforti* IBT18687 and *P. verrucosum* FR22625 with 7.5 mg of PLA ml^{-1} and for *A. flavus* ITEM5134 with both PLA concentrations. Results of the statistical analysis obtained with the Gompertz model giving growth delays for all fungal strains after 3, 4, and 5 days (GD_{72}, GD_{96}, and GD_{120}) are shown in Table 2. With *A. flavus* ITEM5134 and FTDC3226, *P. citrinum* ITEM5145 and ITEM5144, *P. commune* ITEM5150, and *Penicillium* sp. strain ITEM5147, optical densities corresponding to those of control cultures were never reached by the treated fungal suspensions and were defined as not quantifiable (Table 2). For the other strains, growth delays ranging from 20 h (*P. citrinum* ITEM5146) to 155 h (*P. polonicum* ITEM5141), from 22 h (*A. ochraceus* FR21991) to 190 h (*P. polonicum* ITEM5141), and from 7.4 h (*A. niger* FTDC3227) to 225 h (*P. polonicum* ITEM5141) were observed after 3, 4, and 5 days, respectively (Table 2). When fungal strains treated with 5 mg of PLA ml^{-1} were tested with a more concentrated solution (7.5 mg ml^{-1}), higher percentages of inhibition (50 to 92.4%) were obtained, and growth delays also became not quantifiable for the following strains: *P. roqueforti* IBT18687, *P. verrucosum* FR22625, *P. brevicompactum* ITEM5140, *Fusarium* sp. strain ITEM5153, and *P. chrysogenum* ITEM5151 and ITEM5152. For most of the other strains, increases in the growth delays were observed, leading to the conclusion that application of 7.5 mg of PLA ml^{-1} could cause an unpredictable delaying effect for 12 strains, including the most common contaminants and two mycotoxigenic strains, and may have delayed the growth of seven other strains for about 2 days. Finally, Gompertz analysis

TABLE 2. Fungal growth inhibition caused by PLA at pH 4 and fungal growth delay determined by the Gompertz model after 3 days (GD_{72}), 4 days (GD_{96}), and 5 days (GD_{120})

PLA concn (mg ml^{-1})[a]	Strain	% Growth inhibition	GD_{72} (h)	GD_{96} (h)	GD_{120} (h)
5	*Penicillium verrucosum* FR22625	50 ± 0.5	23.6	25.0	12.6
	Penicillium roqueforti IBT18687	50 ± 4.0	36	33.6	20.2
	Penicillium solitum ITEM5149	64 ± 1.0	63.5	45.0	119.4
	Penicillium polonicum ITEM5143	60 ± 4.2	57.1	68.4	57.2
	Penicillium polonicum ITEM5142	67 ± 0.8	80.0	110.3	121.6
	Penicillium brevicompactum ITEM5140	82 ± 4.6	106.5	98.2	90.0
	Fusarium sp. strain ITEM5153	66 ± 5.3	78.1	79.5	19.1
	Aspergillus niger ITEM5132	72 ± 3.4	45.6	42.3	28.3
	Aspergillus niger FTDC3227	53 ± 1.4	26.6	25.3	7.4
	Aspergillus flavus ITEM5134	61 ± 2.2	NQ[b]	NQ	NQ
	Aspergillus flavus FTDC3226	65 ± 1.2	25.3	32.0	NQ
	Penicillium chrysogenum ITEM5151	50 ± 0.2	21.3	39.6	86.7
	Penicillium chrysogenum ITEM5152	53 ± 0.4	34.5	39.7	48.3
7.5	*Penicillium polonicum* ITEM5141	78 ± 0.5	155.2	190.0	225.0
	Penicillium citrinum ITEM5144	50 ± 4.1	32.3	NQ	NQ
	Penicillium citrinum ITEM5145	56 ± 4.6	NQ	NQ	NQ
	Penicillium citrinum ITEM5146	50 ± 1.5	20.0	22.2	28.4
	Penicillium commune ITEM5150	70 ± 0.3	NQ	NQ	NQ
	Penicillium sp. strain ITEM5148	51 ± 0.8	41.1	70.3	90.6
	Penicillium sp. strain ITEM5147	53 ± 0.3	NQ	NQ	NQ
	Aspergillus flavus ITEM5135	50 ± 5.8	44.5	46.8	39.8
	Aspergillus ochraceus FR21991	50 ± 3.2	23.0	22.0	20.0
	Aspergillus terreus ITEM5136	50 ± 1.3	27.0	23.8	26.0

[a] The PLA concentrations used for the different species (5 and 7.5 mg ml^{-1}) are the concentrations tested that caused more than 50% growth inhibition.
[b] NQ, not quantifiable.

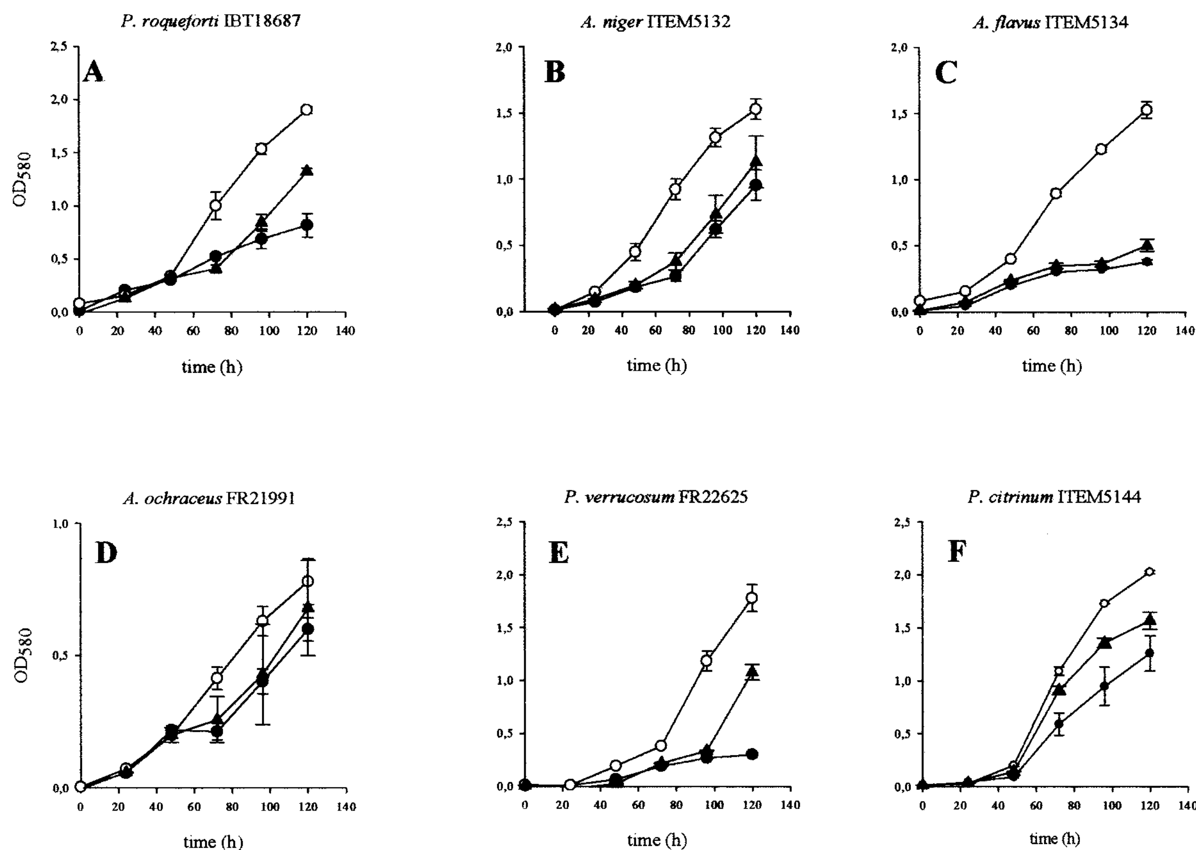

FIG. 3. Fungal growth of six species in the absence of PLA (○) or in the presence of PLA at concentrations 5 mg ml^{-1}(▲) and 7.5 mg ml^{-1}(●). The error bars indicate standard errors. OD_{580}, optical density at 580 nm.

of optical densities obtained for *A. niger* FTDC3227, *P. citrinunm* ITEM5146, *A. ochraceus* FR21991, and *A. terreus* ITEM5136 revealed growth delays of about 1 day.

Inhibitory effect of PLA in the presence of other organic acids. To evaluate the combined effect of PLA and organic acids produced by lactic acid bacteria during fermentation processes (16), the inhibitory activity of PLA (5 mg ml^{-1}) against *A. niger* FTDC3227 was compared with the inhibitory activity in the presence of lactic and/or acetic acid. Addition of lactic acid (15.8 mg ml^{-1}) caused an increase of about 30% in the PLA inhibitory activity (from 53 to 82%); when lactic acid was tested alone, a smaller inhibitory effect (33%) was observed (Fig. 4). Neither the lactic acid concentration detected in *L. plantarum* 21B culture filtrate (0.79 mg ml^{-1}) nor a more concentrated lactic acid solution (7.9 mg ml^{-1}) increased the inhibitory effect of PLA. Acetic acid (0.67 mg ml^{-1}) increased the antifungal activity of PLA by 18%, and when this acid was tested alone, a minor (7%) inhibitory effect was observed. Acetic acid did not result in relevant increases in the inhibitory activity of PLA when it was tested at lower concentrations (0.017, 0.17, and 0.34 mg ml^{-1}); addition of acetic acid (0.67 mg ml^{-1}) did not increase the activity of the mixture of PLA and lactic acid (0.2% difference) (Fig. 4). When PLA was tested in the presence of lactic acid, the delay in growth of *A. niger* after 72 h was doubled (from 26.6 to 54 h) compared with the delay in growth in the culture exposed to PLA, and an increase in the percentage of inhibition (30%) was observed. Addition of acetic acid to PLA also increased the delay in fungal growth from 26.6 to 64 h, although the percentage of inhibition was increased by only 18%. Use of a mixture of PLA, lactic acid, and acetic acid did not increase the inhibitory activity of PLA observed in the presence of lactic acid.

DISCUSSION

The findings of the present study indicate that PLA has interesting potential for practical application as an antimicrobial agent in the food industry due to its broad inhibitory activity against a variety of food-borne fungi. The fungal species investigated in this study have been found previously in flours, bakery products, and/or cereals and have the potential to produce bioactive secondary metabolites, including mycotoxins. In particular, *A. flavus* has been recovered from flour, bread, and bakery products [9, 23; F. Valerio, P. Lavermicocca, and A. Visconti, Book Abstr. 5th Congr. Naz. FIMUA (Fed. Naz. Micopatol. Umana Anim.), p. 25, 2000], and the carcinogenic aflatoxins have also been found in bakery products (24) and rye bread (10). Even though this fungus is known to produce aflatoxins, the strains evaluated in this study did not show aflatoxin production. Both *A. ochraceus*, isolated from flour (3, 23) and bread (30), and *P. verrucosum*, reported to be a contaminant of cakes (32) and cereals (19), produce several mycotoxins, including ochratoxin A. Recently, it has also been established that ochratoxin A is produced by other fungal spe-

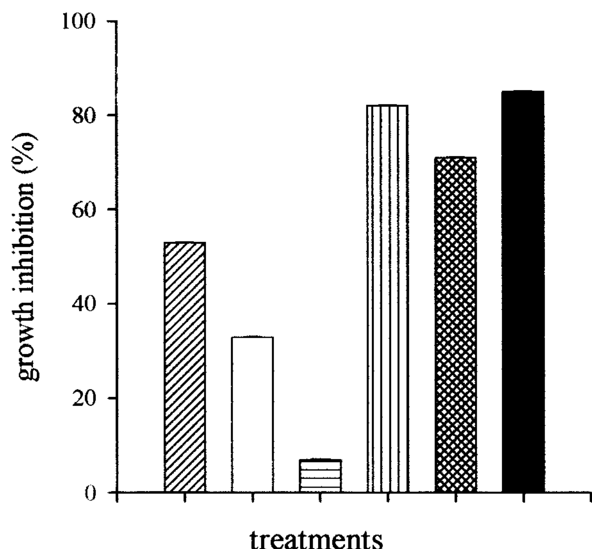

FIG. 4. Growth inhibition of *A. niger* FTDC3227 caused by 5 mg of PLA ml^{-1} (▨); 15.8 mg of lactic acid ml^{-1} (open bar); 0.67 mg of acetic acid ml^{-1} (▤); 5 mg of PLA ml^{-1} and 15.8 mg of lactic acid ml^{-1}(▥); 5 mg of PLA ml^{-1} and 0.67 mg of acetic acid ml^{-1} (▩); and 5 mg of PLA ml^{-1}, 15.8 mg of lactic acid ml^{-1}, and 0.67 mg of acetic acid ml^{-1} (solid bar). The standard errors were less than or equal to 5%.

cies, such as *A. niger* (13), which has also been found as a contaminant of bread (32; Valerio et al., Book Abstr. 5th Congr. Naz. FIMUA).

In our experiments the MFC of PLA for *A. ochraceus* FR21991 and *P. verrucosum* FR22625, both ochratoxin A producers, were 7.5 and 5 mg ml^{-1}, respectively, while only growth-inhibiting activity against *A. niger* ITEM5132 and FTDC3227 was observed. *P. citrinum* is known to produce the nephrotoxic mycotoxin citrinin, which has previously been found in bakery products, moldy bread, and rye bread (4, 5, 10, 18, 21, 24). The three citrinin-producing strains that were tested here were also inhibited by PLA (MIC_{90}, 7.5 mg ml^{-1}). PLA showed fungicidal activity against 13 of the 14 species tested, and these 13 species included potential toxigenic organisms, such as *A. ochraceus*, *A. flavus*, *P. roqueforti*, *P. verrucosum*, and *P. citrinum*. This indicates that application of PLA to reduce fungal mass in food systems has a clear advantage compared with the preservatives now commonly used in bakery products, such as propionic acid and its salts, which act by a fungistatic mechanism (15) that causes only temporary inhibition of microbial growth. Similar to the activities of other weak acid preservatives (propionic acid, benzoic acid, sorbic acid, etc.) and organic acid acidulants (lactic acid, malic acid, citric acid, acetic acid, etc.), the activity of PLA (pK 3.46) was shown to be pH dependent, indicating that its mode of action is somewhat related to the lipophilic properties which enable the undissociated form to cross microbial membranes (12). The PLA concentrations showing antifungal activity against molds isolated from bakery products are generally lower than those required for antibacterial activity. In fact, antimicrobial activity of PLA has been reported for *Listeria monocytogenes* at a concentration of 13 mg ml^{-1} and for other human pathogens (*S. aureus*, *E. coli*, and *A. hydrophila*) at a concentration of 20 mg ml^{-1} (7, 8).

A considerable effect of PLA was also observed at pH 4, a pH explored because of its broader application to real food systems; at this pH concentrations lower than 7.5 mg ml^{-1} were enough to cause both inhibition of more than 50% of fungal growth and a relevant growth delay for all strains tested. In the case of *A. flavus* (two strains), *P. citrinum* (two strains), *P. commune*, and *Penicillium* sp. (one strain), optical densities equivalent to those of control cultures were never reached by treated fungal suspensions. In conclusion, by using the Gompertz parameters, a PLA concentration of 7.5 mg ml^{-1} gave unpredictable growth delays (however, the growth delays were always longer than 2 days) for 12 strains, including the common contaminants *P. roqueforti* and *A. flavus* and the mycotoxigenic strains of *P. verrucosum* and *P. citrinum*.

The growth delays observed in these experiments are of great relevance for extending the shelf lives of food products. In particular, in the case of the acid-tolerant organism *P. roqueforti*, application of 5 mg of PLA ml^{-1} (the MFC) resulted in complete inhibition of the strain even at a low pH (pH 3.0). When used at pH 4, PLA (7.5 mg ml^{-1}) inhibited fungal development by 52% and delayed growth by an unpredictable time (i.e., the level of contamination observed in the control was not reached during the 5-day experiment). These findings support previous data which showed that the PLA-producing strain *L. plantarum* 21B, used as a starter in sourdough bread, delayed the growth of *A. niger* FTDC3227 (16) and *P. roqueforti* IBT18687 (A. Corsetti and P. Lavermicocca, unpublished data) for up to 7 days at room temperature. This delaying effect was not observed in bread started with *Lactobacillus brevis* 1D, a sourdough strain which in culture produced about the same amounts of lactic acid and acetic acid as *L. plantarum* 21B (ca. 0.8 and 0.02 mg ml^{-1}, respectively, for both strains) (16). This led to the conclusion that PLA was the major factor responsible for the antifungal activity and prolonged shelf life produced by *L. plantarum* 21B in sourdough bread and the conclusion that these effects were improved by the presence of lactic and acetic acids.

PLA production by food-related bacteria has been reported for a strain of *Propionibacterium freudenreichii* and two strains of *L. plantarum* (16, 29). It has been shown that PLA is a product of phenylalanine metabolism; in particular, phenylalanine can be transaminated to phenylpyruvic acid, which is further metabolized to PLA by hydroxy acid dehydrogenase (29). Therefore, modification of bacterial growth conditions to improve metabolite production may lead to isolation of strains with enhanced PLA production. In addition, screening a relevant number of lactic acid bacteria from several microbiotas, as well as investigations of the inhibitory activities against other microbial contaminants, could widen the potential application of PLA to other food systems. The natural occurrence of relevant amounts of PLA in several honeys from different geographical areas (28, 31) and the apparent lack of toxicity of PLA for human and animal cell lines (20) might allow its safe use in foods, even though information about its effect on rheology and flavor should be acquired for each food system. Therefore, PLA, like other antimicrobial substances produced by lactic acid bacteria, represents a promising natural device for controlling contaminants in food systems. An additional advantage compared with some of the other compounds, such

as acetic acid, may be the apparent lack of odor of PLA solutions.

Since molds are responsible for contamination of a variety of products destined for both human and animal consumption, such as dairy and meat products, fruit, vegetables, and silages, in fermented foods and feedstuffs PLA-producing strains of lactic acid bacteria may be used for in situ production of the antimicrobial compound, while the conditions for direct application of PLA should be established for other food systems.

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