Antifungal Metabolites of *Lactobacilli*

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Abstract

In recent years, public concern about indoor mold growth has increased dramatically in the United States. In this study, lactic acid bacteria, which are known to produce antimicrobial compounds important in the biopreservation of food, were evaluated to determine if the same antimicrobial properties can be used to protect wood from mold establishment. Based on biomass measurement, cell-free supernatants from *Lactobacillus casei* subsp. *rhamnosus* and *Lactobacillus* acidophilus grown in deMan Rofosa Sharpe (MRS) broth inhibited 95 to 100 percent growth of three mold - and one stain fungus associated with wood-based building materials. Lactic acid and four unknown compounds £ 1 kDa molecular weight were fractionated from the culture supernatant by thin layer chromatography and high-performance liquid chromatography. Antifungal activity, which was attributed to one or more unknown metabolites, was retained during heating and neutralization. A 1:2 dilution of *L. casei* supernatant inhibited 100 percent growth of all test fungi.

Introduction

Recently, indoor air quality issues and awareness of home damage due to mold infestation have increased dramatically in the United States. A number of factors can lead to high moisture conditions that are conducive to mold growth (1). Moisture management needs to be addressed from the standpoint of construction practices, construction materials, and homeowner maintenance. In the absence of proper moisture management, incorporating mildewcides into construction materials would add another level of protection from mold establishment. Chemical fungicides that are commonly used to control the growth of mold are not appropriate for many indoor applications. Consequently, developing natural alternatives that are environmentally friendly and have low mammalian toxicity are needed. Potera (8) suggested that using microbial fungicides might be an alternative to chemical fungicides.

Research in biopreservation has led to the development of using naturally occurring antimicrobial compounds. Several bacterial species produce various antibiotics, some of which demonstrate antifungal activity. Lactic acid bacteria (LAB) have been widely used in food and feed fermentation applications. The antibacterial effects of LAB contribute to the safety, stability, flavor, and structure of many products. The antifungal effects of LAB have been extensively studied (9, 10). The microbiocidic action of LAB is based on both competition for nutrients and the production of various compounds, such as organic acids, hydrogen peroxide, bacteriocins, and low molecular weight antimicrobial agents (7), which to date, have not been identified. Lactic acid and phenyllactic acid in combination have shown antifungal properties (3).

The objectives of this study were to evaluate the ability of LAB metabolites to inhibit mold growth in liquid culture and characterize the antifungal properties of these metabolites to determine their suitability to protect wood from mold growth.

Materials and Methods

Bacterial and Fungal Propagation

Bacterial cultures of *Lactobacillus casei* subsp. *rhamnosus, Lactobacillus acidophilus,* and *Pseudomo-*

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nas aeruginosa ATCC 10145 provided by the University of Wisconsin, Department of Bacteriology (Madison, WI), were maintained on nutrient agar (Difco Laboratories, Detroit, MI) and stored at 5°C.

Fungal cultures, *Trichoderma viride* ATCC 20476, *Aspergillus niger* 2.242 (provided by the University of Virginia, School of Medicine, Charlottesville, VA), *Penicillium chrysogenum* PH02, and *Aureobasidium pullulans* MDX-18 (provided by USDA FPL, Madison, WI), were maintained on 2 percent malt extract agar (Difco Laboratories) and stored at 5°C.

deMan Rogosa Sharpe (MRS) broth (Difco Laboratories) was selected as a test medium capable of supporting growth of all bacterial and fungal test organisms.

Fungal Biomass Inhibition

Lactobacillus acidophilus and *L. casei* were inoculated in individual 200-ml Erlenmeyer flasks containing 100 ml MRS broth and cultivated at 32°C and 200 rpm for 24 hours. Cell-free supernatants were collected by centrifugation at 3,000 × *g* for 40 minutes. Ten-ml aliquots of supernatant were placed in 50-ml flasks and inoculated in triplicate with each test fungus. Stationary cultures were incubated at 27°C for 7 days. Fungal growth was harvested on preweighed Whatman # 1 filter paper (Whatman International, Maidstone, England) and air-dried at 25°C for 2 days. The average fungal biomass was calculated for each test fungus and compared with the fungal biomass of positive controls consisting of mold fungi grown in MRS broth. Negative controls consisted of uninoculated cell-free bacterial supernatants. *Pseudomonas aeruginosa*, a nonacid producing bacterium, was also grown as described above and served as an indicator organism to ensure that fungal inhibition was not simply due to nutrient exhaustion of the growth medium, or acid production.

Supernatant Fractionation

Cell-free supernatants from *L. acidophilus* and *L. casei* were fractionated consecutively with 10-, 3-, and 1-kDa molecular weight cutoff filters in a Diaflo Ultrafiltration apparatus (Amicon Corporation, Danvers, MA). One milliliter of each filtrate was aliquotted into wells of a 24-well microtiter plate and inoculated with an agar plug from individual test fungi (n = 3). Microtiter plates were incubated at 27°C for 3 to 4 days and were visually assessed for mold growth.

Thin Layer Chromatography

Filtrate fractions £ 1 kDa were applied to a 0.25-mm precoated silica gel with fluorescent indicator UV254 (Polygram SilG/UV254, Macherey-Nagel, Duren, Germany). Fractionated MRS broth served as a negative control. Lactic acid and phenyllactic acid standards were obtained from Sigma Chemical Co. (St. Louis, MO). The plate was developed with chloroform-methanol-acetic acid (9:1:0.1) and observed at 254 nm.

High-Performance Liquid Chromatography

One-milliliter samples of filtrate fractions £ 1 kDa were acidified with 5 µl 50% w/w H_2SO_4 and centrifuged at 10,000 × g for 5 minutes. The samples were then filtered through a 0.45-µm filter into sample vials. Lactate was determined by high-performance liquid chromatography (HPLC) using a Hewlett Packard (Palo Alto, CA) 1050 pump and a HP UV monitor at 2 10 nm. The eluant was 0.005 N H_2SO_4 at 0.5 ml per minute. Eighty microliters were injected into an ion 300 organic acid column (Phenomenex, Torrance, CA). Lactate was quantified with calibrated standards.

Metabolite Characterization

To determine if lactic acid was solely responsible for the inhibition of test fungi, MRS broth supplemented with 1 percent lactic acid (pH 4 and 6), MRS broth supplemented with 1 percent acetic acid (PH 4), and MRS broth (PH 6.4) were inoculated with each of the test fungi and incubated for 4 days at 27°C in a 96-well microtiter plate. Growth (absorbance) was recorded at 550 nm.

To determine the effect of pH on the retention of antifungal activity, cell-free culture supernatants were adjusted to 4.5, 5.0, 5.5, and 6.0. Neutralized samples were placed in wells of a 96-well microtiter plate, inoculated with each of the test fungi, and incubated for 3 days at 27°C. Growth (absorbance) was recorded at 550 nm. Test fungi inoculated into MRS broth served as a comparative control.

To determine the effect of heat stability, cell-free culture supernatants were treated at 50°C, 70°C, 90°C, and 121°C for 30 minutes and were then placed on ice to stop heating. Heated samples were placed in wells of a 96-well microtiter plate and inoculated with test fungi as described above. Growth (absorbance) was recorded at 550 nm.

Culture Supernatant Minimum Inhibitory Concentration

Cell-free supernatants of *L. acidophilus* and *L. casei* were diluted 4:1, 2:1, 1:1, 1:2, and 1:4 in MRS broth and inoculated with test fungi as described above. Growth (absorbance) was recorded at 550 nm.

Results and Discussion

Fungal Biomass Inhibition

Figure 1 depicts normal fungal growth in control flasks of MRS broth (right) compared with no visible growth in a matching set of flasks containing cell-free *Lactobacillus casei* supernatant (left).

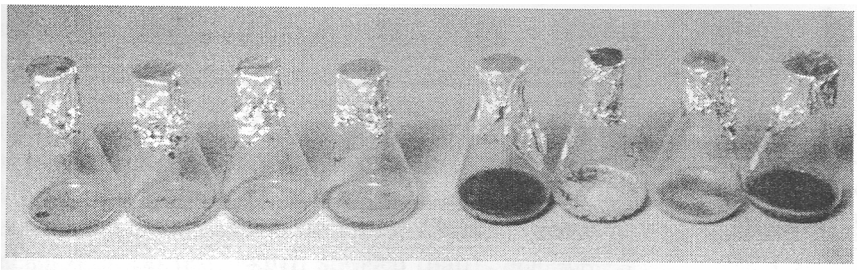

Figure 1. ~ *On the left, cell-free supernatant from Lactobacillus casei susp. rhamnosus inhibits visible growth of A. niger, P. chrysogenum, T. viride, and A. pullulans. The right side shows luxurious growth of the same test fungi in MRS broth.*

Table 1. ~ *Percentage inhibition of fungal biomass by cell-free bacterial supernatants.*

	Test fungi		
Test fungi	*L. casei*	*L. acidophilus*	*P. aeruginosa*
	----------(%)----------		
T. viride	90	92	0
P. chrysogenum	96	97	61
A. niger	94	99	0
A. pullulans	93	90	57

Based on dry weight measurements of fungal biomass, both Lactobacilli inhibited 90 to 99 percent of the fungal growth compared with controls grown in MRS **(Table 1).** Two of four test fungi (*T. viride* and *A. niger*) showed no inhibition by cell free culture from *P. aeruginosa*, a nonacid producing bacterium, suggesting that the fungal inhibition seen with *Lactobacillus* cell free supernatant was not simply due to nutrient exhaustion.

Supernatant Fractionation

Cell-free supernatants from *L. acidophilus* and *L. casei*, fractionated consecutively with 10-, 3-, and 1-kDa molecular weight cutoff filters and inoculated with *A. niger*, *T. viride*, *P. chrysogenum*, and *A. pullulans* resulted in 100 percent inhibition of all test fungi, indicating that the antifungal metabolites are £ 1 kDa.

Fractionated supernatants (£ 1 kDa) of *Lactobacilli* analyzed with thin layer chromatography (TLC) showed a distinct lactic acid band as the major component in this fraction together with several minor components. No phenyllactic acid was observed for either *L. acidophilus* or *L. casei*. Likewise, no lactic acid was present in the MRS control **(Fig. 2).**

The HPLC profiles of both *Lactobacilli* cell-free supernatants produced one major peak identified as lactic acid **(Fig. 3a)** and four minor peaks, which corresponded to the number of unidentified bands in TLC. No lactic acid was detected in the MRS control.

It was determined that lactic acid was not responsible for the antifungal properties of the *Lactobacillus* supernatant since antifungal activity was retained following dialysis with a 1-kDa DispoDialyzer (Spectrum Laboratories, Inc., Rancho Dominguez, CA) to remove lactic acid **(Fig. 3b).** The MW of the sample retained inside the dialysis bag is larger than 1 kDa. This finding suggests there are additional antifungal metabolites > 1 kDa which may contribute synergistically to the antifungal activity.

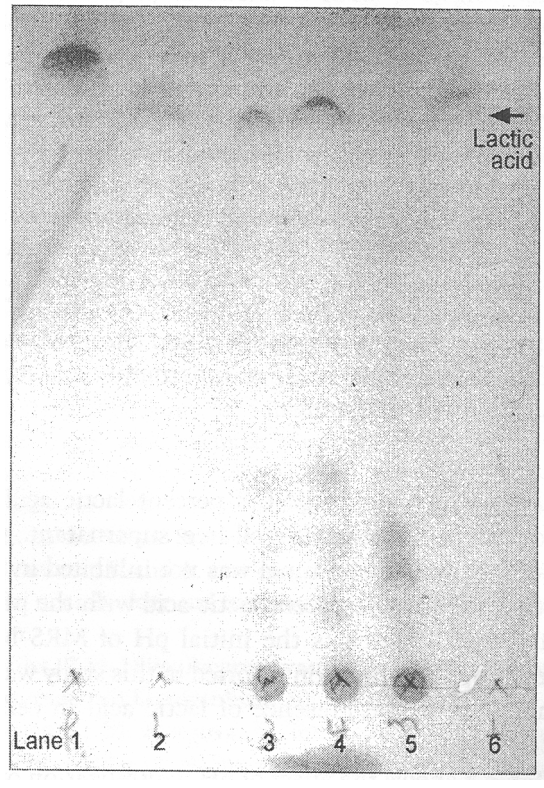

Figure 2. ~ *Thin layer chromatograph of <1 kDa filtrates from L. casei and L. acidophilus. Fractionated MRS served as control, and phenyllactic acid and lactic acid served as standard. Lane 1, phenyllactic acid; Lane 2 and 6, lactic acid; Lane 3, L. casei; Lane 4, L. acidophilus; Lane 5, MRS control.*

Metabolite Characterization

Results summarized in **Table 2** demonstrate that low pH alone inhibits growth of test fungi. MRS broth adjusted to pH 4.0 with either 1 percent lactic acid or 1 percent acetic acid inhibited fungal growth. A concentration of 1 percent acid was selected based on HPLC

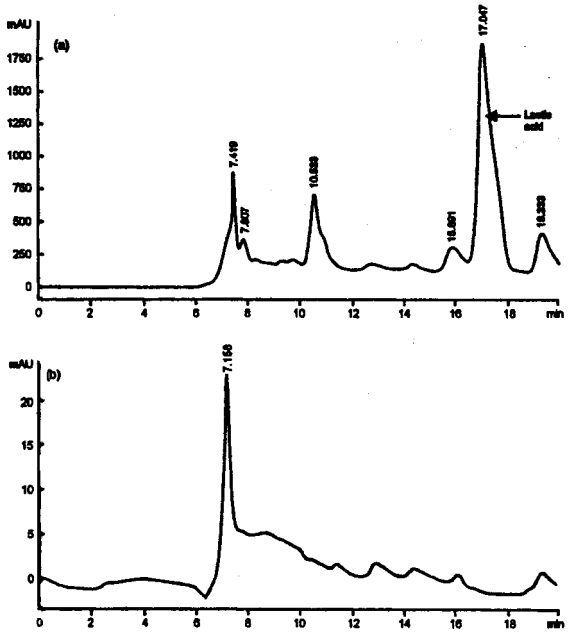

Figure 3. ~ *High-performance liquid chromatography profiles of (a) L. casei cell-free supernatant and (b) dialyzed L. casei supernatant.*

analysis which indicated 0.8 percent lactic acid was produced in *Lactobacilli* cell-free supernatant. Conversely, growth of test fungi was not inhibited in MRS broth containing 1 percent lactic acid with the pH adjusted to 6.4 which is the initial pH of MRS broth. Therefore, the inhibition reported in this study was not simply due to the presence of lactic acid in cell free supernatant.

Table 3 shows the effect of pH neutralization on the antifungal activity of the cell-free supernatant for *L. casei* and *L. acidophilus* against test fungi after 3 days incubation. Adjustments to pH (0.5 increments) were made to the supernatant between the starting pH of MRS broth (6.4) and the final pH of the bacterial culture (4.0) before inoculation with the test fungi. *L. casei* supernatant retained antifungal activity against 7: viride at pH 6 level, and *A. niger,* and *A. pullulans* at all pH levels compared to MRS controls. Culture supernatant from *L. acidophilus* inhibited 63 to 73 percent growth of *A. niger* and *A. pullulans* at pH 5.5 and 6.0 compared with control values but did not retain antifungal activity against *P. chrysogenum* or 7: viride at the same pH. At pH 4.5 and 5.0, growth rates of all four test fungi in *L. casei* supernatant are similar to controls, probably due to the inhibitory effect of low pH described earlier. A similar pH-induced inhibitory effect was seen for *L. acidophilus* cell free culture at pH 4.5 and 5.0 against 7: viride and *P. chrysogenum* and at pH 4.5 against *A. niger* and *A. pullulans.*

Table 2. ~ *Acid and pH effects on the growth inhibition of test fungi.*

		MRS broth supplements and pH level			
Test fungi	Incubation (days)	No supplement pH 6.4	1% lactic acid pH 4.0	1% acetic acid pH 4.0	1% lactic acid pH 6.4
T. viride	0	0.357[a]	0.23	0.344	0.306
	4	1.377	0.248	0.358	0.125
P. chrysogenum	0	ND[b]	0.235	0.346	0.309
	4	ND	0.261	0.365	0.923
A. niger	0	ND	0.23	0.339	0.277
	4	ND	0.255	0.359	3.90
A. pullulans	0	ND	0.245	0.346	0.306
	4	ND	0.274	0.390	3.85

[a] Values represent the average of three optical density readings at wavelength 550 nm.
[b] ND, not determined in this experiment.

Table 3. ~ *Effect of neutralization on antifungal activity in culture supernatants of two Lactobacilli.*

		Culture supernatant		
Test fungi	pH	None (MSR control)	*L. casei*	*L. acidophilus*
T. viride	4.5	0.328[a]	0.232	0.352
	5.0	0.326	0.329	0.371
	5.5	0.433	0.553	0.674
	6.0	0.902	0.566	0.937
P. chrysogenum	4.5	0.316	0.234	0.348
	5.0	0.322	0.356	0.38
	5.5	0.39	0.587	0.501
	6.0	0.493	0.629	0.691
A. niger	4.5	0.316	0.233	0.352
	5.0	0.55	0.413	1.095
	5.5	2.317	0.618	1.506
	6.0	2.209	0.727	1.622
A. pullulans	4.5	0.318	0.241	0.227
	5.0	0.658	0.421	0.997
	5.5	2.465	0.646	1.562
	6.0	2.719	0.734	1.736

[a] Values represent the average of three optical density readings at wave length 550 nm.

From the results of heat treatment at various temperatures, cell-free supernatants from *L. casei* and *L. acidophilus* retained their antifungal activity- against four test fungi (data not shown).

Culture Supernatant Minimum Inhibitory Concentration

Dilutions of culture supernatant (4: 1, 2:1, 1:1, 12, and 1:4) were prepared in MRS broth to determine the

minimum concentration of metabolites that will inhibit test fungi. *Lactobacillus casei* supernatant maintained inhibition of all test fungi at a 1:2 dilution but failed to inhibit at a 1:4 dilution with MRS (data not shown). *Lactobacillus acidophilius* retained partial inhibition of 1 : viride (80%) and *P. chrysogenum* (91%) even at the 1:4 dilution. This contradicted results of the neutralization test, which showed that *L. acidophilus* was not as effective overall at fungal inhibition and, in particular, at neutral pH. Of course, higher dilutions of supernatant in MRS resulted in greater neutralization.

Conclusions

We have determined that *L. casei* and *L. acidophilus* produce several metabolites that may act together to inhibit mold growth in liquid culture. Many reports have suggested that antifungal activity is a combination of organic acids such as lactic, acetic, and phenyllactic acids (2-4, 6) or bacteriocins (5) and low molecularweight antimicrobial agents and peptides (7, 11, 12). However, we have shown that culture supernatants retained antifungal activity in the absence of lactic acid. The LAB isolates in this study also produced no phenyllactic acid. Rather, cell-free culture supernatants of *L. casei* and *L. acidophilus* produced at least four unknown compounds that exhibited antifungal activity against three mold fungi and one stain fungus. Metabolites were heat resistant (to 121°C) and retained activity after neutralization. A 1:2 dilution of *L. casei* supernatant inhibited 100 percent growth of test fungi.

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Literature Cited

1. Clausen, C.A. 2000. Recognize, remove, and remediate mold and mildew. *In*: Proc. of the 2nd Annual Conf. on Durability and Disaster Mitigation in Wood-Frame Housing, Nov. 6-8, Madison, WI, pp. 231-234.
2. Laitila, A., H.L. Alakomi, L. Raaska, T. Mattila-Sandholm, and A. Haikara. 2002. Antifungal activities of two *Lactobacillus plantarum* strains against Fusarium moulds in vitro and in malting of barley. J. of Applied Microbiology. 93:566-576.
3. Lavennicocca, P., F. Valerio, and A. Visconti. 2003. Antifungal activity of phenyllactic acid against molds isolated from bakery products. Applied Environmental Microbiology. 69:634-640.
4. Magnusson, J., K. Strom, S. Rooa, J. Sjogren, and J. Schniirer. 2003. Broad and complex antifungal activity among environmental isolates of lactic acid bacteria. FEMS Microbiology Letters. 219:129-135.
5. Mortvedt, C.I., J. Nissen-Meyer, K. Sletten, and I.F. Nes. 1991. Purification and amino acid sequence of lactocin S, a bacteriocin produced by Lactobacillus sake L45. Applied Environmental Microbiology. 57:1829-1834.
6. Niku-Paavola M-L., A. Laitila, T. Mattila-Sandholm, and A. Haikara. 1999. New types of antimicrobial compounds produced by *Lactobacillus plantarum*. J. of Applied Microbiology. 86:29-35.
7. Ouwehand, A.C. 1998. Antimicrobial components from lactic acid bacteria. *In*: Salminen S. and J. von Wright, Eds. Lactic Acid Bacteria: Microbiology and Functional Aspects. New York: Marcel Kekker, Inc., pp. 139-159.
8. Potera, O. 1994. From bacteria: A new weapon against fungal infection. Science. 265:605.
9. Salminen, S. and A. von Wright. 1998. Lactic acid bacterial microbiology and functional aspects. Marcel Dekker, NY.
10. Stiles, E.M. 1996. Biopreservation by lactic acid bacteria. Antonie van Leeuwenhoek 70: 331-345.
11. Stiles, J., M. Plockova, V. Toth, and J. Chumchalova. 1999. Inhibition of Fusarium sp. DMF 0101 by Lactobacillus strains grown in MRS and Elliker broths. Advances in Food. Science (CMTL).2 1: 117-121.
12. Strom, K., J. Sjogren, A. Broberg, and J. Schnfuer. 2002. Lactobacillusplantarum MiLAB 393 produces the antifungal cyclic dipeptides cyclo (L-Phe-L-Pro) and cyclo (1-Phe-trans-4-OH-L-Pro) and 3-phenyllactic acid. Applied Environmental Microbiology. 68:4322-4327.

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