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Determining the suitability of Lactobacilli antifungal metabolites for inhibiting mould growth

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Summary

In recent years, public concern about indoor mould growth has increased dramatically in the United States. In this study, lactic acid bacteria (LAB), 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 inhibit mould fungi that typically colonize wood. Based on biomass measurement, cell-free supernatants from *Lactobacillus casei* subsp. *rhamnosus* and *Lactobacillus acidophilus* grown in deMan Rogosa Sharpe (MRS) broth inhibited 95–100% growth of three mould fungi and one stain fungus associated with wood-based building materials. Lactic acid and four unknown compounds $\leqslant 1$ 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% growth of all test fungi.

Introduction

Recently, indoor air quality issues and awareness of home damage due to mould infestation have increased dramatically in the United States. A number of factors can lead to high moisture conditions that are conducive to mould growth (Clausen 2000). 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 mildew-killing agents into construction materials would add another level of protection from mould establishment. Chemical fungicides that are commonly used to control the growth of mould are not appropriate for many indoor applications. Consequently, developing natural alternatives that are environmentally friendly and have low mammalian toxicity are needed. Potera (1994) suggested that using microbial fungicides might be an alternative to chemical fungicides. 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 antifungal effects of LAB have been extensively studied (Stiles 1996; Salminen & von Wright 1998). The microbiocidal 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 (Ouwehand 1998), which to date, have not been identified. Lactic acid and phenyllactic acid in combination have shown antifungal properties (Lavermicocca *et al.* 2003).

The objectives of this study were to evaluate the ability of LAB metabolites to inhibit mould growth in liquid culture and to attempt to characterize the antifungal properties of these metabolites.

Materials and methods

Bacterial and fungal propagation

Bacterial cultures of *Lactobacillus casei* subsp. *rhamnosus, Lactobacillus acidophilus,* and *Pseudomonas aeruginosa* ATCC 10145 provided by the University of Wisconsin, Department of Bacteriology (Madison, Wisconsin, USA), were maintained on nutrient agar (Difco Laboratories, Detroit, Michigan, USA) 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, Virginia, USA), *Penicillium chrysogenum* PH02, and *Aureobasidium pullulans* MDX-18 (provided by U.S. Department of Agriculture, Forest Products Laboratory, Madison, Wisconsin, USA), were maintained on 2% 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 into individual 200-ml Erlenmeyer flasks containing 100 ml MRS broth and cultivated at 32 °C and 200 rev/min for 24 h. Cell-free supernatants were collected by centrifugation at $3000 \times g$ for 40 min. Ten milliliter 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 mould fungi grown in MRS broth. *Pseudomonas aeruginosa,* a nonacid-producing bacterium, was also grown as previously described 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, Massachusetts, USA). One milliliter of each filtrate was dispensed into wells of a 24-well microtitre plate and inoculated with an agar plug from individual test fungi (n = 3). Microtitre plates were incubated at 27 °C for 3–4 days and were visually assessed for mould growth.

Thin layer Chromatography. Filtrate fractions $\leqslant 1$ 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, Missouri, USA). 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 $\leqslant 1$ kDa were acidified with 5 µl 50% (w/v) H_2SO_4, centrifuged at $10000 \times g$ for 5 min, and filtered through a 0.45-µm filter. Lactate was determined by high-performance liquid chromatography (HPLC) using a Hewlett Packard (Palo Alto, California, USA) 1050 pump and a HP u.v. monitor at 210 nm. The eluant was 2.5 mM H_2SO_4 at 0.5 ml per minute. Eighty microliters were injected into an Ion-300 organic acid column (Phenomenex, Torrance, California, USA). 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% lactic acid (pH 4 and 6), MRS broth supplemented with 1% 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 microtitre 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, 70, 90 and 121 °C for 30 min and were then placed on ice to stop heating. Heated samples were placed in wells 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 previously described. Growth (absorbance) was recorded at 550 nm after 2 days incubation at 27 °C.

Results and discussion

Fungal biomass inhibition

Based on dry weight measurements of fungal biomass, both *Lactobacilli* inhibited 90–99% fungal growth compared with MRS controls (data not shown). 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 culture 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% inhibition of all test fungi, indicating that the antifungal metabolites have $M_r \leqslant 1$ kDa.

Fractionated supernatants ($\leqslant 1$ kDa) of Lactobacilli analysed by TLC showed a distinct lactic acid band as the major component 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 (Figure 1).

The HPLC profiles of both *Lactobacilli* cell-free supernatants produced one major peak identified as lactic acid (Figure 2a) 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, California, USA) to remove lactic acid (Figure 2b). The molecular weight of the sample retained inside the dialysis bag was > 1 kDa. This finding suggests there are additional antifungal metabolites with M_r >1 kDa, which may contribute synergistically to the antifungal activity.

Metabolite characterization

Results summarized in Table 1 demonstrate that low pH alone inhibits growth of the test fungi. MRS broth adjusted to pH 4.0 with either 1% lactic acid or 1% acetic acid inhibited fungal growth. A concentration of 1% acid was selected based on HPLC analysis that indicated 0.8% lactic acid was produced in *Lactobacilli* cell-free supernatant. Conversely, growth of test fungi was not inhibited in MRS broth containing 1% 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 caused by the presence of lactic acid in cell-free supernatant.

Table 2 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 *T. viride* at pH 6 and against *A. niger,* and *A. pullulans* at all pH levels comparable with MRS controls. Culture supernatant from *L. acidophilus* inhibited 63–73% growth of *A. niger* and *A. pullulans* at pH 5.5 and 6.0, but did not retain antifungal activity against *P. chrysogenum* or *T. 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 the 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 *T. viride* and *P. chrysogenum* and at pH 4.5 against *A. niger* and *A. pullulans.*

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

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

Culture supernatant minimum inhibitory concentration

Dilutions of culture supernatant (4:1, 2:1, 1:1, 1:2 and 1:4) were prepared in MRS broth to determine the minimum concentration of metabolites that will inhibit test fungi. *L. 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). *L. acidophilus* retained partial inhibition of *T. 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 mould growth in liquid culture. Many reports have suggested that antifungal activity is a combination of organic acids such as lactic, acetic, and phenyllactic acids (Niku-Paavola *et al.* 1999, Laitila *et al.* 2002, Mag nusson *et al.* 2003, Lavermicocca *et al.* 2003) or

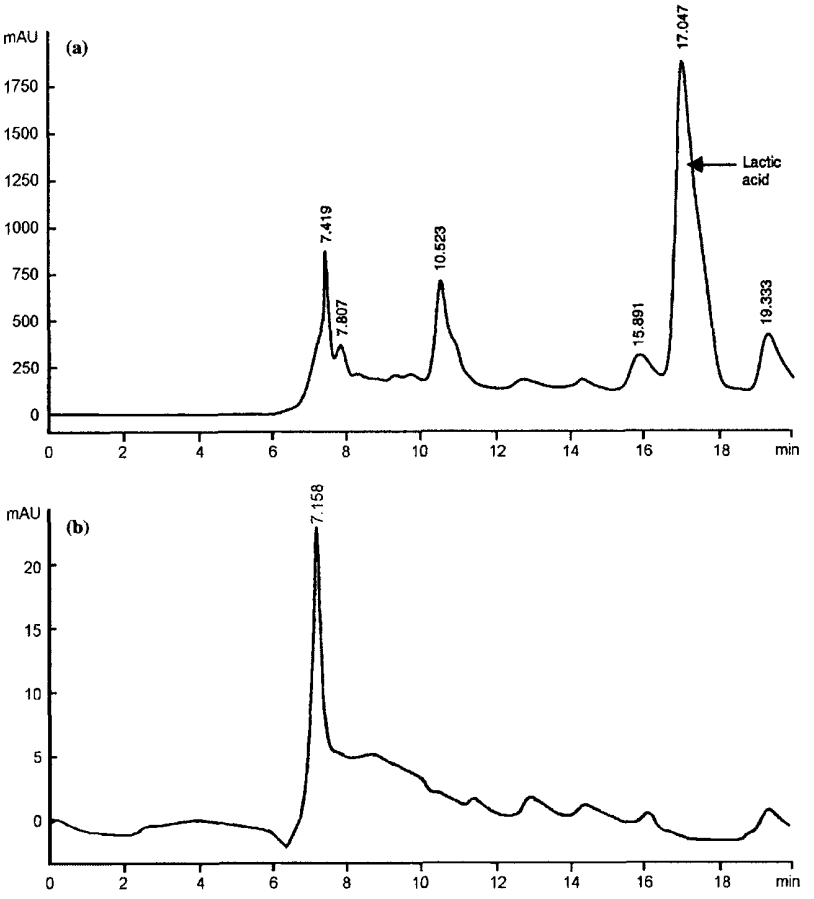

Figure 2. High-performance liquid chromatography profiles of (a) *L. casei* cell-free supernatant and (b) dialysed *L. casei* supernatant

Table 1. Acid and pH effects on the growth inhibition of test fungi.

Test fungi	Incubation (days)	MRS broth supplements and pH level			
		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	0.357/0.005[a]	0.23/0.005	0.344/0.009	0.306/0.007
	4	1.377/0.115	0.248/0.015	0.358/0.009	1.259/0.268
P. chrysogenum	0	ND[b]	0.235/0.001	0.346/0.01	0.309/0.003
	4	ND	0.261/0.007	0.365/0.001	0.923/0.33
A. niger	0	ND	0.23/0.003	0.339/0.005	0.277/0.007
	4	ND	0.255/0.001	0.359/0.005	3.90/0.91
A. pullulans	0	ND	0.245/0.008	0.346/0.001	0.306/0.001
	4	ND	0.274/0.01	0.390/0.12	3.85/0.245

[a]Values/standard deviation represent the average of three optical density readings at wavelength 550 nm
[b]ND, not determined in this experiment. Results from previous study showed active growth of *T. viride on* day 4

bacteriocins (Mørtvedt *et al.* 1991) and low molecular weight antimicrobial agents and peptides (Ouwehand 1998, Stiles *et al.* 1999, Ström *et al.* 2002). 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 mould 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% growth of test fungi.

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Table 2. Effect of neutralization on antifungal activity in culture supernatants of two ***Lactobacilli.***

Test fungus	pH	Culture supernatant		
		None (MRS control)	*L. casei*	*L. acidophilus*
T. viride	4.5	0.328/0.05[a]	0.232/0.003	0.352/0.008
	5.0	0.326/0.004	0.329/0.028	0.371/0.05
	5.5	0.433/0.006	0.553/0.009	0.674/0.128
	6.0	0.902/0.13	0.566/0.009	0.937/0.104
P. chrysogenum	4.5	0.316/0.006	0.234/0.006	0.348/0.008
	5.0	0.322/0.007	0.356/0.021	0.38/0.07
	5.5	0.39/0.005	0.587/0.06	0.501/0.147
	6.0	0.493/0.035	0.629/0.1	0.691/0.18
A. niger	4.5	0.316/0.007	0.233/0.001	0.352/0.09
	5.0	0.55/0.016	0.413/0.044	1.095/0.225
	5.5	2.317/0.28	0.618/0.011	1.506/0.249
	6.0	2.209/0.171	0.727/0.04	1.622/0.463
A. pullulans	4.5	0.318/0.005	0.241/0.009	0.2270.006
	5.0	0.658/0.272	0.421/0.021	0.997/0.288
	5.5	2.465/0.094	0.646/0.062	1.562/0.482
	6.0	2.719/0.096	0.734/0.042	1.736/0.193

[a]Values/Standard deviation represent the average of three optical density reading at wavelength 550 nm.

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