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Inhibition of Growth and Mycotoxin Production of *Penicillium* by *Lactobacillus* Species

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Lactic acid bacteria isolated from dairy products, vegetables and fruits were screened for their antifungal and antimycotoxigenic activities. From 420 isolates tested, four isolates were found to be inhibitory to four Penicillium *species. Two of the lactic acid bacteria isolates showed the largest inhibitory activity against* Penicillum citrinum *and* Penicillium expansum. *The two isolates were identified as* Lactobacillus casei *species. Production of patulin and citrinin was also inhibited in the presence of the* Lactobacilli *supernatants. The inhibitory activity in the two* Lactobacilli *cell-free supernatants was shown to be unrelated to the production of lactic acid or hydrogen peroxide, and was found to be sensitive to proteolytic enzymes and to high temperature (100 °C). The antimycotoxigenic activity was not affected by the addition of glucose used by* Lactobacilli *species during incubation.*

Keywords: *Lactobacilli*; *Penicillium*; mycotoxin; patulin; citrinin

Introduction

The genus *Penicillium* is an important contaminant of foods and agricultural commodities. Food spoilage by *Penicillium* species is common, especially at lower temperatures. In addition to food deterioration, many *Penicillium* species are known to produce a number of mycotoxins (1). Cole and Cox (2) reported that there are 85 *Penicillium* species producing 42 different toxic compounds. Pitt (1) stated that such a list is incomplete and many of the *Penicillium* species reported in the literature are misidentified. Pitt and Leistner (3) reported that the majority of the investigations in the literature focused on the chemistry and toxicology of *Penicillium* toxins, while the mycology of *Penicillium* species, especially identification, needs further research. The following mycotoxins are known to be produced by *Penicillium* species: citrinin, cyclopiazonic acid, ochratoxins, patulin, penicillic acid, penitrem A, PR toxin, griseofulvin and viridicatumtoxin (1). Many of these toxins are produced by more than one *Penicillium* species.

Penicillium species are common contaminants of fermented dairy products such as cheese during ripening and storage. Many investigations have shown that *Penicillium* spp. are the predominant contaminants of cheese, followed by *Aspergillus* species (4). The *Penicillium* species isolated from cheese are known to produce various mycotoxins. Other fungal species isolated from fermented dairy products include *Cladosporium, Alternaria, Fusarium, Geotrichum Candidum*, and *Mucor*, in addition to other potentially mycotoxigenic fungal species (4). There are many conflicting reports in the literature with regard to the production of mycotoxins on cheese and other dairy products. Overall, cheese was found to be a better medium for mold growth than for mycotoxin production (4). However, numerous investigations have shown the production of *Penicillium* toxins on cheese under laboratory conditions (4). In addition, milk, butter, cream, milk powder and condensed milk were also found to support mold growth.

It is known that lactic acid bacteria (LAB) possess antimicrobial activity (5, 6). Lactic acid bacteria are used in many fermented dairy products because of their preservative potential and their effects on the organoleptic properties (7). The preservative effect is generally attributed to acid production and low pH. Although most of the spoilage and pathogenic bacteria cannot tolerate low pHs, molds can grow in fermented products, causing significant economic losses. Some of these molds are also potential mycotoxin producers. Numerous investigations reported the antimycotic and antimycotoxigenic activity of lactic acid bacteria (5). The inhibition of fungi by these types of fermenting lactic acid bacteria could improve the shelf-life of fermented products and reduce the potential health hazard of mycotoxins.

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This study was performed in order to investigate the ability of lactic acid bacteria to inhibit the growth and mycotoxin production of *Penicillium* species.

Materials and Methods

Cultures

Four *Penicillium* species (*Penicillium italicum WDC 16, Penicillium cyclopium NRRL 3471, Penicillium citrinum NRRL 1843* and *Penicillium expansum NRRL 23-04*) were used. Two *Aspergillus* species were also used for comparison (*A. flavus* subsp. *parasiticus* NRRL 2999 and *A. ochraceus* NRRL 3174). The mold cultures were grown on potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI) slants and stored at 5 °C and transferred to fresh PDA slants every 2 months. Lactic acid bacteria were isolated from various food products such as dairy products, vegetables and fruits. The foods were purchased from supermarkets in the area of Reading, Pennsylvania. Each food sample was suspended in butterfield phosphate buffer and macerated in a Stomacher 400 (Tekmar, Cincinnati, OH), serially diluted and surface plated on deMan Rogosa Sharpe (MRS) agar (Difco Laboratories, Detroit, MI). The plates were incubated at 37 °C for 36–40 h. Colonies from each food sample (420 colonies in total) were selected. Microscopic morphology and Gram stain reaction of purified isolates were determined. Lactic acid bacteria were grown on MRS agar slants and stored at 5 °C. The identity of some of the lactic acid bacteria isolates was determined using the API Rapid CH strip (bio Merieux Vitek Inc, Hazelwood, MO).

Inocula

Spores of *Penicillium* and *Aspergillus* species were harvested from 10-d PDA slant cultures in sterile phosphate buffer solution containing 0.05% Tween 80. The spores were loosened using a flamed wireloop. Mycelical debris were removed by filtration through sterile cheese cloth. The spore count of the mold spore suspension was determined using a Petroff-Houser Counting chamber and the spread plate technique on PDA plates that were incubated at 25 °C for 3–5 d. Lactic acid bacteria isolates were transferred to MRS broth and incubated at 37 °C for 24 h. The supernatant of each isolate was prepared by centrifuging the LAB culture at $3000 \times g$ for 15 min and sterilized by filtration through a 45 μm pore size filter (Millipore Corp., Bedford, MA).

Antimycotic activity test

Fifteen millilitres of sterile molten PDA were transferred to Petri dishes and allowed to solidify. The PDA plates were surface inoculated with 0.1 mL of mold spore suspension (10^4 spores). Sterilized paper discs (5 mm diameter) were saturated with the cell-free supernatant and plated on inoculated PDA plates. The plates were incubated at 25 °C for 3–4 d. Zones of inhibition around the discs were measured in mm. The possibility of an inhibitory effect due to hydrogen peroxide was tested by adding catalase (Sigma Chemical Co., St. Louis, MO) to the supernatants (45 μg/mL). Controls consisted of sterilized MRS broth and MRS acidified with lactic acid (pH 4.2).

Effect on mold spore germination and germ tube elongation

A known number of mold spores were mixed with 20 g/L malt extract broth (Difco) and LAB supernatant. After homogenization, 0.15 mL of the mixture were pipetted on to a sterile glass microscope slide. The suspension was retained within a rectangle delineated on the slide with molten paraffin wax. The slides were incubated in moist Petri dishes at 25 °C for 24 h. The slides were examined under a microscope every 3 h for up to 12 h and then at 24 h. A direct count of germinated spores was made. Control consisted of 20 g/L malt extract broth inoculated with mold spores (8).

Effect of proteolytic enzymes

Solutions of trypsin and pepsin (Sigma) were prepared in phosphate buffer at optimal pH values of 7.6 and 2.0, respectively. Five millilitre sets of LAB cell-free supernatants were adjusted to the above pH values with 0.1 mol/L HCL and 2 mol/L NaOH. After adjustment of the pH, the supernatants were treated with enzymes (1 mg/mL) and incubated at 37 °C for 1 h. The pH was then adjusted to pH 4.2 (initial pH of the supernatant) and the supernatants in the vials were then sterilized by filtration (0.45 μm). A mold spore suspension (10^4 spores) was added to the supernatants and incubated at 25 °C for 5 d. The vials were supplemented with filter sterilized glucose solution (10 g/L) at the time of mold inoculation. Patulin and citrinin, and mycelial dry weights were determined at the end of the incubation period.

Effect of temperature of the antimycotic activity of LAB supernatants

The supernatants were heated at three temperatures: 50, 70 and 100 °C for a period of 10 min. The supernatants were inoculated with mold spore suspension (10^4) and incubated at 25 °C for 5 d. The supernatants were supplemented with filter sterilized glucose solution (10 g/L) at the time of mold inoculation. Patulin, citrinin and mycelial dry weight were determined at the end of the incubation period.

Analyses

All the experiments were performed in triplicate and averages were considered.

Mycelial dry weight. The mycelial mats were collected

Table 1 Antifungal activity of lactic acid bacteria culture isolates against *Penicillium* species

	Diameter of zone of inhibition (mm)			
Mold culture	A1	A2	B3	B4
P. italicum	14.0	29.0	15.0	14.0
P. cyclopium	9.0	12.0	21.0	12.0
P. citrinum	13.0	21.0	28.0	10.0
P. expansum	12.0	25.0	27.0	13.0
A. flavus	–	–	–	–
A. ochraceus	–	–	–	–

–=No inhibition.

Table 2 Antifungal activity of lactic acid bacteria culture isolates with the addition of catalase

	Diameter of zone of inhibition (mm)			
Mold culture	A1	A2	B3	B4
P. italicum	5.0	27.0	18.0	6.0
P. cyclopium	2.0	15.0	19.0	–
P. citrinum	–	22.0	22.0	4.0
P. expansum	7.0	23.0	25.0	–
A. flavus	–	–	–	–
A. ochraceus	–	–	–	–

–=No inhibition.

by filtration through Whatman No. 4 filter paper, washed twice with water, dried in an oven at 95 °C until constant weight (24–48 h), and weighed.

Mycotoxin determination. Patulin and citrinin (Sigma Chemical Co., St. Louis, MO) standard solutions were prepared in chloroform at various concentrations (1, 5, 10, 20 and 50 μg/mL) and stored at 4 °C. Analysis of patulin and citrinin was performed according to the AOAC method (9). The residues were dissolved in ethyl acetate and subjected to thin layer chromatography (TLC) for quantification. The extracts were spotted on TLC plates (20 × 20 cm coated with a 0.25 nm thick layer of silica gel HPK; Whatman, Clifton, NJ). The TLC plates were developed in toluene–EtOAc–90% formic acid (6 + 3 + 1). After development, the TLC plates were exposed to ammonia fumes, dried and exposed to long-wavelength ultraviolet light for visual estimation. Samples spots were visually compared to patulin and citrinin standard spots and the quantities of these mycotoxins were estimated.

Results

Among 420 isolates that were examined in the present study, four isolates exhibited antifungal activity against the four *Penicillium* tested, namely *Penicillium italicum*, *Penicillium cyclopium*, *Penicillium citrinum* and *Penicillium expansum*. Two strains of *Aspergillus flavus* and *Aspergillus ochraceus* were also tested for comparison. Results of the antimycotic activity test are summarized in **Table 1**. The four LAB isolates (A1, A2, B3, B4) exhibited various degrees of inhibition against the *Penicillium* species tested. LAB isolates A2 and B3 exhibited the most inhibition. LAB A2 was identified as *Lactobacillus casei* DSM 20312, while LAB B3 was identified as *Lactobacillus casei* CCM 1825 according to API Rapid CH strip tests. None of the lactic acid bacteria isolates were found to inhibit *A. flavus* and *A. ochraceus*. Results for the antifungal activity of LAB isolates with the addition of catalase are summarized in **Table 2**. Addition of catalase to the supernatants of LAB isolates showed that the antimycotic activity of LAB isolates A1 and B4 was probably due to the production of hydrogen peroxide. Addition of catalase reduced totally or partially the antimycotic activity (**Table 2**). However, the antimycotic activity of LAB isolates A2 and B3 was found to be unrelated to the production of hydrogen peroxide (**Table 2**). Acidification of nonfermented MRS broth to pH 4.2 with lactic acid (85%) did not cause any inhibition. The effect of LAB supernatants of the isolates A2 and B3 on spore germination was also investigated (data not shown). The *Penicillium* mold spores in the control started to germinate between 6–9 h of incubation. The mean percentage of germination in the control reached 97% after 24 h of incubation. Spore germination in the presence of the lactic acid bacteria supernatants was greatly affected. Germination in both supernatants starts after 9 h of incubation, and the maximum mean percentage germination reached 15–16% after 24 h for both LAB isolates A2 and B3. The effect of treatment of supernatant LAB isolate B3 with proteolytic enzymes trypsin and pepsin is shown in **Table 3**. The antimycotic and antimycotoxigenic activity of the supernatant was found to be sensitive to both trypsin and pepsin. Addition of trypsin to the LAB B3 supernatant (1 mg/mL) restored the citrinin production of *P. citrinum* by 76%. In the presence of the supernatant the mycelial dry weight of *P. citrinum* was reduced by 48%. Addition of trypsin and pepsin restored mycelial weight by 70 and 86%, respectively. In the case of *P. expansum* the mycelial dry weight and patulin production were reduced by 59% and 27%, respectively, in the supernatant (**Table 3**). Addition of trypsin to the supernatant restored both mycelial growth and patulin production by 70% and 86%, respectively (**Table 3**).

Table 3 Effect of proteolytic enzymes on the antimycotic activity of the lactic acid bacteria isolate B3

	P. citrinum		*P. expansum*	
	Mycelia (mg)	Citrinin (μg/ml)	Mycelia (mg)	Patulin (μg/ml)
Control	25.1	4.1	27.1	3.5
Supernatant	12.1	1.1	16.1	0.9
Trypsin	19.1	3.1	19.1	3.0
Pepsin	21.2	1.8	17.6	1.9

The thermostability of the inhibitory activity in the LAB B3 supernatant at three different temperatures

Table 4 Effect of heat on the antimycotic and antimycotoxigenic activity of LAB isolate B3

		P. citrinum		*P. expansum*	
	Temp (°C)	Mycelia (mg)	Citrinin (µg/mL)	Mycelia (mg)	Patulin (µg/mL)
Control	50	26.1	4.5	25.4	3.1
	70	25.3	4.7	25.1	3.1
	100	24.9	4.1	24.1	3.2
Supernatant	50	11.5	0.9	15.2	1.1
	70	10.5	0.2	7.1	1.2
	100	15.1	2.7	17.8	2.6

(50, 70 and 100 °C) was tested. The results are shown in **Table 4.** Heating the supernatant at 50 and 70 °C did not affect the inhibitory activity of the supernatant. However, at 100 °C there was a significant loss of the antimycotic and antimycotoxigenic activity of the supernatant.

Discussion

This study shows that lactic acid bacteria affected *Penicillium* growth and mycotoxin production. The antimycotic and antimycotoxigenic activity of some of the lactic acid bacteria isolates was found to be unrelated to the production of lactic acid or hydrogen peroxide. Addition of proteases to the LAB supernatants affected the inhibitory activity. This indicates that the antimycotic and antimycotoxigenic activity is partially or totally due to LAB compounds that are proteinaceous in nature. These findings are consistent with the findings of other investigators. Numerous workers reported the inhibition of mold growth and mycotoxin production by various bacterial species (5, 6, 10–13). Batish *et al.* (11) screened numerous lactic acid bacteria strains for their antifungal activity against various mold species. *Aspergillus fumigatus* was found the most sensitive. The inhibitory compound was found to be polypeptide in nature. Gourama and Bullerman (6) reported that *Lactobacilli* species isolated from a commercial silage inoculum reduced mold growth and aflatoxin production by *Aspergillus flavus* subsp. *parasiticus*. The *Lactobacillus* cell-free supernatant inhibited aflatoxin production without greatly reducing mold growth. The inhibitory principle(s) was suspected to be low molecular weight bacterial metabolite. The inhibitory activity in the *Lactobacillus* cell-free supernatant was found to be sensitive to proteolytic enzymes. Other workers have shown the opposite where lactic acid bacteria stimulated the production of aflatoxin (14). These differences in findings may be due to the different lactic cultures used by different investigators, and to the different assay techniques used. Collins and Hardt (15) reported that *L. acidophilus* inhibited *Candida albicans* and other pathogenic yeasts. However, other workers could not demonstrate the inhibition of yeast by lactic cultures. Apparently hydrogen peroxide was found to be the cause of the inhibition and the toxin production for the isolates A1 and B4. El-Gazzar and Marth (16) found that 3 to 5 g/L hydrogen peroxide completely prevented growth and toxin production by *A. parasiticus*.

The results of this study have shown that LAB cell-free supernatants greatly affected the germination of *Penicillium* spores, which could be the cause of the inhibition of mold growth as expressed by the reduction of mycelial dry weight. These findings are similar to the those of Gourama and Bullerman (5). Reduction in the germination of mold spores could be the result of various factors. Mold spore germination is a physiological reaction that is controlled by nutrient penetration barriers, metabolic blocks, low water content, pH and fungal inhibitors (17). From the results on the effect of temperature on the production of antifungal and antimycotoxigenic activities (**Table 4**), it is clear that the antimycotic and antimycotoxigenic activity is relatively thermostable and could survive pasteurization. Since the use of proteolytic enzymes suggested the presence of proteinic compounds in the supernatants, the loss of inhibitory activity at 100 °C may suggest that it was due to denaturation of the protein(s) or cofactors. Similar results were found by other workers (6, 10).

Kimura and Hirano (18) reported that the strain of *Bacillus subtilis* isolated from soil inhibited the growth and aflatoxin production of *Aspergillus parasiticus* NRRL 2999 in laboratory media and in peanuts and corn. Klich *et al.* (19) reported that *Bacillus subtilis* inhibited mycotoxigenic fungi by producing a peptidolipid, iturin A. *P. italicum, P. viridicatum, A. ochraceus* and *A. versicolor* were found to be the most sensitive to iturin A.

Although there are still some contradictory results in the literature about the antimycotic effect of LAB, it appears from this study and the work of other investigators that lactic acid bacteria and other bacteria and their metabolites have a great potential to be used in foods as natural biological control agents to prevent growth and mycotoxin production by molds. Many of the investigators have shown that this inhibition is due to compounds other than hydrogen compounds and acidity. There are strong indications that some of these inhibitory compounds are proteinaceous in nature. The nature of the antifungal and antimycotoxigenic activity is the subject of another investigation.

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