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Inhibition of Aflatoxin Production of *Aspergillus flavus* by *Lactobacillus casei*

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***Lactobacillus casei* KC-324 was tested for its ability to inhibit aflatoxin production and mycelial growth of *Aspergillus flavus* ATCC 15517 in liquid culture. Aflatoxin B_1 biosynthesis and mycelial growth were inhibited in both simultaneous culture and individual antagonism assays, suggesting that the inhibitory activity was due to extracellular metabolites produced in cell-free supernatant fluids of the cultured broth of *L. casei* KC-324. In cell-free supernatant fluids of all media tested, deMan, Rogosa and Sharpe broth, potato dextrose broth, and Czapek-Dox broth + 1% yeast extract showed higher anti-aflatoxigenic activity. In these case, fungal growths, however, was not affected as measured by mycelial dry weight. The anti-aflatoxigenic metabolites from *L. casei* KC-324 were produced over wide range of temperatures between 25°C and 37°C. However, these metabolites were not thermostable since the inhibitory activity of the supernatant was inactivated within 30 minutes at 100°C and 121°C. The inhibitory activity was not influenced by changing pH of supernatant between 4 and 10. However, the antiaflatoxigenic activity was slightly reduced at pH 10.**

KEYWORDS: Aflatoxin, Antiaflatoxigenic metabolites, *Aspergillus flavus*, *Lactobacillus casei*

Aspergillus flavus and *A. parasiticus* are post-harvest pathogens of several important food crops including maize, peanuts, and several tree nut crops (Farr *et al.*, 1989). Many strains of *A. flavus* and nearly all strains of *A. parasiticus* are the major species of fungi producing aflatoxins (Klich and Pitt, 1988), which are known to be potent carcinogens (Squire, 1981) and hepatotoxinogenic chemicals (Campbell and Stoloff, 1974) and possess a severe carcinogen to animal and human health (Eaton and Callagher, 1994). Therefore, international regulatory limits have been established for aflatoxins in food and animal feed (van Egmond, 1995). In Korea, most agricultural products used as the feeds have come from other countries; they have a great possibility to be contaminated by aflatoxins. Therefore, the regulation in Korean limits Aflatoxin B_1 (AFB_1) and total aflatoxins including AFB_1, B_2, G_2 to 10 ppb for young animals and 20 ppb in mature animals, respectively (Han *et al.*, 2006). (AFB_1) is the most potential carcinogen among the four naturally occurring aflatoxins namely aflatoxins B_1, B_2, G_1 and G_2. These toxins are linked to fungal growth and the environment in which the grains/cereals are stored. Fungal growth and subsequent mycotoxin production in stored grains can be inhibited by physical methods such as aeration, cooling, modified atmospheres, *etc.* or by fungistats of which the propionic, acetic and sorbic acids are the most commonly used (Paster *et al.*, 1988).

In the storage agroecosystem, complex interactions occur between biotic and abiotic factors that have an impact on growth, toxin production and biological behavior. Then three main different factors such as physical, biological and nutritional factors, affect aflatoxin production (Gourama. and Bullerman, 1995). In nature aflatoxin-producing fungi share the same habitat with other microorganisms which can influence aflatoxin production. It has been reported that aflatoxin production is inhibited by lactic acid bacteria (Gourama and Bullerman, 1995), *Bacillus subtilis* (Kimura and Hiram, 1988) and many fungi (Choudhary, 1992). Many lactic acid bacteria such as *Lactobacillus* spp. were found to inhibit aflatoxin biosynthesis (Coallier-Ascah and Idziak, 1985; Karunaratne *et al.*, 1990). A silage inoculant mixture of *Lactobucillus* species was inhibitory to mycotoxin biosynthesis of *Penicillium* and *Aspergillus* species (Gourama and Bullerman, 1995; Karunaratne *et al.*, 1990). In previous study (Kim, 2005), several *Lactobacillus* isolates were obtained from the mixture and screened for their inhibitory effect on mycelial production. The strain, which was identified as *L. casei* KC-324, showed the strongest antifungal activity on fungal growth and spore germination (Kim, 2005).

In this paper, inhibition of aflatoxin production of *A. flavus* ATCC 15517 by *L. casei* KC-324, the effect of growth conditions of the bacterium on its antiaflatoxigenic activity and thermal stability of the inhibitory activity are presented.

Materials and Methods

Chemicals. Aflatoxin B_1 (AFB_1) standard was purchased from of Sigma Chemical Co. St. Louis, MO, USA.

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Thin layer chromatography (TLC) Silica gel 60F254 plates were purchased from E. Merck, Germany. All other solvents and reagents were of analytical grade supported by Sigma Chemical Co, USA.

Bacterial and fungal strain. Bacterial culture of *Lactobacillus casei* KC-324 isolated from Kimchi by our laboratory (Kim, 2005) was maintained on malt extract agar slants at 4°C and was transferred to 10 *ml* sterile Modified Rogosa (*Lactobacillus* MRS, Difco Laboratories, Detroit, MI, USA) broth to regenerate before used. *Escherichia coli* was grown at 37°C in nutrient broth (Difco Laboratories, Detroit, MI, USA) for using positive control. Fully grown cell suspensions were stored at –80°C in the presence of 90% glycerol as cryoprotectant, for long-term conservation.

Aspergillus flavus ATCC 15517 producing aflatoxin B_1 (AFB_1) was purchased from KCCM (Korean Culture Center of Microorganisms; Suwon, Korea), and aflatoxin B_1 (AFB_1) production during the growth of the fungal strain was closely observed. The fungus was grown on potato dextrose agar (PDA, Difco Laboratories, Detroit, MI, USA) slants at 25°C and stored at 4°C until further use and subcultured on a monthly basis.

Preparation of inoculum and growth media. *Lactobacillus casei* KC-324 was transferred to deMan, Rogosa and Sharpe (MRS) broth (Difco Laboratories, Detroit, MI, USA) and was incubated at 37°C for 48 h. The supernatant was prepared by centrifuging the *Lactobacillus* culture at 3000 ×*g* for 15 min and sterilized by filtration with 0.45 μm pore size filter (Millipore Corp., Bedford, MA, USA). The fungal spore suspension was prepared as described by Fan and Chen (1999). The fungus was grown on PDA petridishes for 5~7 days at 25°C until sporulation occurred. Spores of *A. flavus* incubated at 25°C were collected from 10 day old PDA solid cultures in a sterile phosphate buffer solution containing 0.05% of Tween 80. The spores were loosened using a flamed wire loop. Mycelial debris was removed by filtration through sterile cheese cloth. This was further diluted to obtain a final spore suspension containing approximately 1.5×10^4 spores/*ml* determined using a Petroff-Hauser counting chamber (hemocytometer), spread plate technique on PDA plates and was used for all experiments.

Fungal growth determination. Fungal dry weight calculated according to Rasooli and Razzaghi-Abyaneh (2005) was considered as growth index. The fungal mycelia were separated from culture media using a separatory funnel and then washed thoroughly with distilled water. The mycelial mats were collected by filtration through Whatman no. 4 filter papers, washed twice thoroughly with distilled water and dried in an oven at 95°C until constant weight. Mycelial dry weights were then determined. Fungal growth inhibition (%) was calculated according to the following formula: Fungal growth inhibition (%) = [(Total control weight – Total sample weight)/ Total control weight] × 100.

Inhibition assays. Both simultaneous and deferred antagonism assays were used. In the simultaneous antagonism assay, each isolate of *L. casei* was grown with *A. flavus* in 250 *ml*-Erlenmeyer flasks containing 100 *ml* of sterilized MRS broth. The medium was inoculated with 2.5 *ml* of a spore suspension of *A. flavus* containing ca. 1.5×10^4 spores/*ml*, and 2.5 *ml* of bacterial inoculum containing ca. 1×10^7 cfu (colony forming units)/*ml*. Cultures were incubated at 25°C and 100 rpm in an orbital shaker incubator for 10 days and then analyzed for aflatoxin production. Cell-free supernatant fluids were used in the deferred antagonism assay. Each bacterial isolate was grown at 30°C and 100 rpm for 48 h in 100 *ml* of MRS broth inoculated with 2.5 *ml* of bacterial inoculum containing ca. 1×10^7 cfu/*ml*. Then cell-free supernatant fluids were prepared by centrifuging the culture at 20,000 ×*g* and 4°C for 15 minutes. To compensate for possible depletion of sucrose, 2.5 g of sucrose were added to 100 *ml* of cell-free supernatant fluids. In addition, the pH of supernatant fluids was adjusted to 7.0 (pH of control MRS) by 0.1 N NaOH in order to rule out possible inhibitory effect due to lowered pH in the growth medium. Supernatant fluids were sterilized by filtration through a 0.45 μm pore size filter (Millipore Corp., Bedford, MA, USA) and 2.5 *ml* were aseptically dispensed in 250 *ml*-Erlenmeyer flasks before being inoculated with 2.5 *ml* of a spore suspension of *A. flavus* containing ca. 1.5×10^4 spores/*ml*. Cultures were incubated at 25°C for 10 days and analyzed for aflatoxin production.

Determination of AFB_1 production. AFB_1 concentration was measured in both mycelia and culture media using a TLC method. A known amount of fresh mycelia from each flask was processed for AFB_1 estimation. After freezing of the fungal mat by liquid nitrogen, mycelia were homogenized by mortar and pestle in presence of chloroform as the extraction solvent. The culture media were extracted by chloroform after shaking in a separatory funnel. The chloroform extracts of both mycelia and media from each flask were separately collected and then concentrated near to dryness using an rotary evaporator. The sample extracts were spotted on Silica gel 60F precoated TLC plates along with the AFB_1 standards and developed in a TLC chamber with chloroform:methanol (98 : 2, v/v) as mobile phase. The amount of AFB_1 was calculated at 365 nm using CAMAG TLC Scanner 3 (CAMAG, Switzerland) by comparison of under-curved areas of samples and standards.

Composition of the growth medium. Three media were compared: MRS broth (pH 7.0), potato dextrose broth (PDB, Difco Laboratories, Detroit, MI, USA; pH 4.9), and Czapek-Dox broth (Difco Laboratories, Detroit, MI, USA) + 1% yeast extract (CZDY, pH 7.3). One hundred *ml* of each sterile medium in 250-m*l* Erlenmeyer flasks were inoculated with 2.5 *ml* of bacterial inoculum containing ca. 1×10^7 cfu/m*l* and incubated at 30°C and 100 rpm for 48 h. Cell-free supernatant fluids were prepared and tested for antiaflatoxigenic activity as previously described.

Incubation temperature and time. Three incubation temperatures, i.e., 4, 25, and 37°C, were tested for their effect on production of antiaflatoxigenic activity. Potato dextrose broth (PDB) was used as the growth medium. The incubation was conducted at 100 rpm for a total duration period of 4 days. Supernatant fluids were prepared daily and tested for antiaflatoxigenic activity.

Thermal stability of antiaflatoxigenic activity. The thermal stability of the antiaflatoxigenic activity was tested by exposing supernatant fluids to 50, 70, and 100 for 30 minutes in a water bath, and 121°C for 30 minutes in an autoclave and quickly cooling with tap water. The control medium was non-heated, filter-sterilized supernatant fluids.

Stability of antiaflatoxigenic activity at different pHs. The effect of pH on the stability of antiaflatoxigenic activity was evaluated at pH 4, 6, 8, and 10 adjusted with 1 N HCl/NaOH. And it was placed at room temperature for 1 h. Cell-free supernatant fluids were sterilized by filtration through a 0.45 μm pore size filter (Millipore Corp., Bedford, MA, USA) before using.

Results and Discussion

Inhibition of aflatoxin and mycelium production of *A. flavus*. The antifungal and antiaflatoxigenic activities of *L. casei* KC-324 were analyzed and the results are summarized in Table 1. *L. casei* KC-324 inhibited aflatoxin production and mycelial growth of *A. flavus* when both microorganisms were cultivated simultaneously in MRS broth. The aflatoxin production was reduced to 99.2%, but the mycelium production was less inhibited with percentage inhibition of 34.4%. *A. parasiticus* and *A. flavus* were in inhibited their production of aflatoxin to 95% by *Bacillus subtilis* (Kimura and Hirano, 1988) and *B. pumilus*, (Munimbazi and Bullerman, 1998), respectively. Also, the elimination of aflatoxin is a rapid process involving the removal of approximately 80% AFB_1 in cases *L. rhamnosus* (El-Nezami *et al.*, 1998). Data shown in Table 2 indicated that aflatoxin production and mycelium growth were inhibited in supernatant fluids of cultured MRS broth. The levels of AFB_1 produced in the *Lactobacillus* cell-free supernatant were 92% lower than the control (Table 2). When the supernatant was diluted two-fold with distilled water, higher amounts of AFB_1 were obtained, however, the mycelial dry weight decreased (Table 2). Dilution of *Lactobacillus* cell-free supernatant with MRS increased the amounts of AFB_1 to 44%. In this case fungal growth, as measured by mycelial dry weight, was not affected.

Table 1. Inhibition of aflatoxin and mycelium production from *A. flavus* ATCC 15517 by *L. casei* KC-324 in simultaneous culture assay

Isolate	AFB_1 ($\mu g/ml$)	Mycelium dry weight (mg/*l*)
Control (only *A. flavus* ATCC 15517)	24.2 ± 1.2[a]	27.2 ± 1.7
L. casei KC-324 + *A. flavus* ATCC 15517	0.2 ± 0.0	19.4 ± 0.7
E. coli + *A. flavus* ATCC 15517	23.2 ± 1.6	20.1 ± 1.1

[a]Standard deviations.

Table 2. Effect of *Lactobacillus* cell-free supernatant on aflatoxin and mycelial production from *A. flavus* ATCC 15517

Medium	AFB_1 ($\mu g/ml$)	Mycelium dry weight (mg/*l*)
Control	18.9 ± 1.1[a]	36.4 ± 1.7
Cell-free supernatant	1.5 ± 0.1	24.6 ± 1.5
Cell-free supernatant + water (1 : 1, v/v)	15.4 ± 1.0	16.9 ± 0.7
Cell-free supernatant + MRS (1 : 1, v/v)	8.3 ± 0.3	25.2 ± 1.7

[a]Standard deviations.

Influence of growth media on production of antiaflatoxigenic activity. In Table 3, all media tested for production of antiaflatoxigenic metabolites were related to growth of *L. casei* and production of inhibitory metabolites. The inhibitory effects of culture broth on aflatoxin production were 94.7% for MRS broth, 98.9% for PDB,

Table 3. Effect of growth media on antiaflatoxigenic activity by *L. casei*

Medium	AFB_1 ($\mu g/ml$)	Mycelium dry weight (mg/*l*)
MRS-CFS[a]	1.4 ± 0.1[c]	19.1 ± 2.1
MRS-CON[b]	26.3 ± 2.3	28.8 ± 1.3
PDB-CFS	0.1 ± 0.0	1.1 ± 0.1
PDB-CON	9.3 ± 0.5	29.1 ± 0.9
CZDY-CFS	1.3 ± 0.1	1.9 ± 0.2
CZDY-CON	21.5 ± 1.8	19.7 ± 0.7

[a]MRS-CFS: cell-free supernatant of MRS broth culture.
[b]MRS-CON: control of MRS broth culture. PDB-CFS: cell-free supernatant of PDB culture. PDB-CON: control of PDB culture. CZDY-CFS: cell-free supernatant of CZDY culture. CZDY-CON: control of CZDY culture.
[c]Standard deviations.

and 94.0% for CZDY. Kimura and Hirano (1988) reported the inhibitory activity of *B. subtilis* against production of aflatoxin was due to inhibitory compounds other than organic acids produced in supernatant culture broth of PDB. Leifert *et al.* (1990) also tested that the effect of various media including PDB and nutrient broth on production of antifungal metabolites by *B. subtilis* and *B. pumilus*. When the bacteria were cultured on nutrient broth and PDB, very low or no antifungal activity was detected. However, other investigators (Kimura and Hirano, 1988; Munimbazi and Bullerman, 1998) did not find PDB/PDA to inhibit production of antifungal metabolites by *Bacillus* species.

Incubation temperature and time on production of antiaflatoxigenic metabolites. The effect of incubation temperature and time on the production of antiaflatoxigenic metabolites was tested at three different temperatures, 4, 25 and 37°C, for 1 to 4 days of incubation time (Table 4). Antiaflatoxigenic metabolites were not produced when the bacterium was incubated at 4°C since there was no evident bacterial growth in these cultures. The minimum temperature for growth of lactic acid bacteria is 5°C (Dantigny and Molin, 2000; Gourama, 1997). Inhibitory activity was obviously detected in the supernatant fluids of cultures cultivated in 25 and 37°C. AFB_1 production was completely inhibited in supernatant fluids obtained from 3- and 4-days-old bacterial cultures. Gournama (1997) reported that temperature had a considerable effect on production of antifungal activity by *L. casei* with the great inhibitory effect against *Penicilluium* and *Aspergillus* species occurring at 37°C.

Thermostability of antiaflatoxigenic metabolites. The thermostability of the inhibitory substance was tested at four different temperatures, namely, 60, 80, 100 and 121°C (Table 5). The *Lactobacillus* cell-free supernatant lost their inhibitory activity when heated to 100 and 121°C, however heating the supernatant at 50 and 70°C did not affect their antiaflatoxigenic activity. The maximum aflatoxin level was obtained when the supernatant was heated to 100°C in all media tested. At 100°C, the antifungal and antiaflatoxigenic activity were affected. At 121°C, aflatoxin production was not as high as 100°C. This could be due to some changes in the chemical composition of the media at the higher temperature, which may interfere with the biosynthesis of aflatoxins. Hence, the effect of heating on the inhibitory activity suggested the presence of protein compounds in the supernatant, thus the loss of the inhibitory activity upon heating at 100°C was probably due to denaturation of the protein(s) or cofactors.

Table 5. Effect of temperature on the antiaflatoxigenic activity by *Lactobacillus* cell-free supernatant fluids

Temperature (°C)	AFB_1 (*μg/ml*)	Mycelium dry weight (mg/*l*)
Control[a]	17.4 ± 1.4	31.0 ± 2.1
50°C	0.0 ± 0.0	26.7 ± 1.5
70°C	0.0 ± 0.0	21.1 ± 1.6
100°C	22.5 ± 2.2	26.0 ± 2.2
121°C	15.4 ± 1.1	27.4 ± 1.9

[a]Control: non-heated, filter-sterilized cell-free supernatant fluids.

Effect of pH on the stability of antiaflatoxigenic metabolites. The inhibitory activity did not influenced by changing pH of supernatant fluids to pHs between 4 and 10 (Table 6). However, the antiaflatoxigenic activity was slightly reduced at pH 10. Since lactic acid bacteria produce organic acids, these might also activate other antifungal compounds like peptides by lowering the pH and are, therefore, also eliminated by neutralization (Muyncka *et al.*, 2004). Magnusson and Schnürer (2001) found such a low pH-activated compound possessing a weak inhibition of *Penicillium paneum* caused by a peptide produced by *L. coryniformis* subsp. *coryniformis* Si3 strain. The activity of this peptide was stable at pH-values between 3.0 and 4.5 but rapidly decreased between 4.5 and 6.0. No inhibitory activity was detected at a pH

Table 4. Effect of incubation time and temperature on antiaflatoxigenic activity by *L. casei*

Incubation time	Incubation temperature					
	4°C		25°C		37°C	
	AFB_1 (*μg/ml*)	Mycelium dry weight (mg/*l*)	AFB_1 (*μg/ml*)	Mycelium dry weight (mg/*l*)	AFB_1 (*μg/ml*)	Mycelium dry weight (mg/*l*)
Control[a]	15.0 ± 0.6	7.5 ± 0.3	17.8 ± 1.7	8.5 ± 0.9	18.0 ± 1.2	8.6 ± 0.7
1 day	13.4 ± 0.3	6.3 ± 0.2	2.3 ± 0.1	7.2 ± 0.4	0.5 ± 0.0	5.6 ± 0.3
2 day	14.2 ± 0.7	5.9 ± 0.3	0.1 ± 0.0	4.0 ± 0.1	nd	2.8 ± 0.1
3 day	14.8 ± 0.9	6.1 ± 0.1	nd[b]	3.1 ± 0.2	nd	2.4 ± 0.1
4 day	15.1 ± 0.5	5.2 ± 0.2	nd	2.3 ± 0.1	nd	1.8 ± 0.1

[a]Control: potato dextrose broth (PDB) without bacterial inoculation.
[b]nd: none detected.
[c]Standard deviations.

Table 6. Effect of pH on antiaflatoxigenic activity by *Lactobacillus* cell-free supernatant fluids

pH	AFB_1 ($\mu g/ml$)	Mycelium dry weight (mg/*l*)
Control[a]	17.9 ± 1.5	32.1 ± 2.3
4	0.6 ± 0.1	30.7 ± 1.9
6	0.4 ± 0.0	32.1 ± 1.8
8	0.7 ± 0.1	36.0 ± 2.7
10	11.6 ± 1.2	33.7 ± 0.8

[a]Control: PDB was adjusted to pH 4.9 before inoculation of the fungus.

above 6.0. The antifungal activity of *L. acidophilus* R described by Batish *et al.* (1990) was also caused by a proteinaceous compound, but there were no results on the pH-dependency of this peptide available. Therefore, it suggests that our lactic acid bacterium *L. casei* KC-324 produce a wide spectrum of compounds that might act synergistically towards filamentous fungi.

The presence of antifungal and antiaflatoxigenic activities in cell free supernatants of lactic acid bacteria have been reported by various investigators (El-Nezami, 1998; Gournama and Bullerman, 1997; Karunaratne *et al.*, 1990; Munimbazi and Bullerman, 1998). *Lactobacillus* species have been reported to inhibit aflatoxin production and retard fungal growth (Gournama and Bullerman, 1997; Karunaratne *et al.*, 1990). Gournama and Bullerman (1995) suggested the inhibition of aflatoxins by *L. casei* in the inoculants mixture was suspected to be due to low molecular weight bacterial metabolites(s) that diffused through a dialysis membrane. Although, the sensitivity of the inhibitory compound to heating and its diffusion though a dialysis sack of low molecular weight suggests that the compound(s) could be small peptides.

Finally, we conclude that antiaflatoxigenic substances are either acidic, proteinaceous compounds with wide pH range for activity or other pH-dependent organic metabolites. Further studies on the purification, identification, chemical nature, and biological characteristics of these antiaflatoxigenic metabolites will be required.

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