Anti-aflatoxigenic activity of some bacteria related with fermentation

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Aflatoxin B_1 is a secondary fungal metabolite produced by *Aspergillus flavus* and *A. parasiticus* and is a public health hazard because it is a human carcinogen. This study was performed to investigate the inhibitory effects of four bacteria (*Leuconostoc mesenteroides*, *Lactobacillus plantarum*, *Lactobacillus casei*, and *Bacillus subtilis*) which are found in fermented foods on the growth and aflatoxin production of *A. parasiticus*. Microorganisms were grown in a modified APT broth and incubated at 28℃ for 9 days. Aflatoxin B_1 was determined by use of high performance liquid chromatography (HPLC). Reduction of mycelial growth of *A. parasiticus* as a result of co-inoculation of the four bacteria was observed to range between 20.9 to 86.2% while reduction of aflatoxin production ranged from 21.6 to 70.4%. The great reduction was found when the mold was co-inoculated with *B. subtilis*, then with *Leu. mesenteroides*, then with *L. casei*, and the least reduction with *L. plantarum*. These results indicate that mold growth and aflatoxin production can be inhibited by the bacterial growth. The results also indicate that the bacteria could have potential as anti-carcinogenic materials. More research is needed to study the inhibitory effects of the metabolites of the bacteria as well as the foods fermented with the strains.

Keywords aflatoxin B_1; *A. parasiticus*; *Leu. mesenteroides*; *L. plantarum*; *L. casei*; *B. subtilis*

1. Introduction

One family of mycotoxins, the aflatoxins produced by *Aspergillus flavus* and *A. parasiticus* are proven carcinogens, immunotoxins, and cause growth retardation in animals [1. 2]. Aflatoxins are also hazardous to public health because they are human carcinogens [1-3]. The toxins have been found in many countries, especially in tropical and subtropical regions where conditions of temperature and humidity are favorable for the growth of the molds and for production of the toxin. Aflatoxin B_1, the most toxic compound in this series, is found to contaminate a wide variety of important foods and agricultural products such as peanuts, maize, rice and cottonseed [2, 4].

Although good crop management and surveillance programs are essential for the control of aflatoxin contamination and aflatoxin levels can be lessened in food and feed by these strategies, the toxins are not eliminated completely in food chain. Furthermore the aflatoxins are heat-stable, therefore they are rarely degraded during cooking and processing. This makes us control or eliminate aflatoxins in foods to be more difficult.

Various physical, chemical, and biological methods to reduce the aflatoxin level in foods and feeds have been tried for many years. The method that has received the most attention is the treatment of aflatoxin-contaminated feeds (primarily cottonseed, corn, and peanut products) with ammonia (i.e., ammoniation) [2]. However, they can only be used with limited success and are improper for use with foods. Therefore, the detoxification and reduction of aflatoxins in foods and feed still remains a significant topic to be studied and presently no novel method exist that can be used to resolve the aflatoxin problem.

In the view that there might be several ways to solve this difficult problem, scientists have tried to find more safe methods which are applicable to feed and even to foods. Different biological control methods have been practiced in order to prevent or to delay mold spoilage of foods and feeds and to inactivate or degrade aflatoxins in foods [5, 6]. These approaches include the use of biocontrol agents, such as bacteria, yeast, and fungi [7-13]. Biological control is a promising approach for reducing both preharvest and postharvest aflatoxin contamination in foods.

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Koreans traditionally eat many kinds of fermented foods such as kimchi (Korean fermented vegetables), doen-jang (Korean fermented soybean paste), kan-jang (Korean fermented soy sauce) and sik-hye (Korean fermented rice beverage). This study was designed to investigate the inhibition of growth and aflatoxin production of aflatoxigenic fungi by biological agents that can be available in daily life. This effect was studied by the combination of *A. parasiticus* and several bacteria (*Leu. mesenteroides*, *L. plantarum*; *L. casei*, and *B. subtilis*) found in fermented foods which Koreans enjoy to eat in their daily life.

2. Materials and methods

2.1 Chemicals

All reagents used were analytical grade purity or better. Aflatoxin standards for HPLC injection were purchased from Supelco (Bellefonte, Pa). The standard solutions were diluted prior to analyses.

2.2 Fungal inoculum preparation

Fungal inoculum was prepared from single-spore cultures of *A. parasiticus* ATCC 15517. The fungus was grown on potato-dextrose agar (Difco Lab., Detroit, Mich.) in Petri plates for 10 days at 28℃. Spores were washed from the plates with sterile distilled water containing 0.1% Tween 80. The concentration of dislodged spores was determined with a hemacytometer and diluted to 10^6 conidia/ml. Spore suspensions were prepared one day before inoculation and stored at 4℃.

2.3 Bacterial strains and inoculum

Leuconostoc mesenteroides KCCM 11325, *Lactobacillus plantarum* KCCM 11322, *Lactobacillus casei* KCCM 12452, and *Bacillus subtilis* KCCM 11316 were used in this study. The bacteria were transferred to MRS broth (Difco Lab.) and incubated at 28~30ºC for 24 h. The broth culture of each bacterial strain was diluted with 0.9% NaCl-solution to yield 10^6 CFU/ml.

2.4 Cultures and media

A modified APT broth (Difco Lab.) medium was used for growth and aflatoxin production of *A. parasiticus* ATCC 15517 with the bacteria, *Leu. mesenteroides*, *L. plantarum*, *L. casei* or *B. subtilis*. A volume of 1ℓ of this liquid medium contains 7 g of glucose. The medium was sterilized at 121℃ for 15 min, cooled to room temperature. Test tubes, each containing the same volume of the prepared medium, were inoculated with spore suspension of *A. parasiticus* and inoculum of each bacterium, and then incubated at 28℃ for 9 days. An *A. parasiticus* culture grown in the absence of the bacteria was used as control.

2.5 Determination of fungal growth

After incubation mycelial mats from cultures were collected on dried, preweighed Whatman No. 1 filter paper. They were then washed with distilled water and dried at 55~60℃ overnight. The dry weight of mycelial mats was used as the measurement of fungal growth.

2.6 Extraction and analysis of aflatoxin

After incubation aflatoxin was extracted from the cultures and quantified by use of high performance liquid chromatography (HPLC). The procedure used for extracting aflatoxin was a modified AOAC method that described previously [14-17]. The extract was evaporated to dryness under a stream of nitrogen gas, and trifluoroacetic acid (TFA) was added before redissolving the residue in an appropriate volume of injection solvent. TFA treated standards and sample extracts were injected on the HPLC column.

The HPLC equipment was comprised of Nova-pak C_{18} column (15 cm by 3.9 mm [inner diameter]), an M510 pump, an M746 integrator, and an M470 fluorescence detector (excitation at 365 nm and emission at 425 nm) (Waters, Milford, Mass.). Sample injection was done on a Rheodyne injector (Rheodyne M7125, Coiati, Calif.) with a 20 $\mu\ell$ sample loop. The mobile phase was water-acetonitrile (20:80, vol/vol). The chromatograms were obtained at ambient temperature with the mobile phase at a flow-rate of 1.0 ml/min [15-17].

3. Results

3.1 Effects of bacteria on the growth of *A. parasiticus*

Growth of *A. parasiticus* ATCC 15517 on APT broth with and without the bacteria was monitored by mycelial mat. Effects of the bacteria on the growth of *A. parasiticus* are shown in Figs 1-4. The growth of *A. parasiticus* was affected by the four bacteria (*Leu. mesenteroides*, *L. plantarum*, *L. casei*, and *B. subtilis*) during the incubation period although growth of the mold increased over time on APT broth.

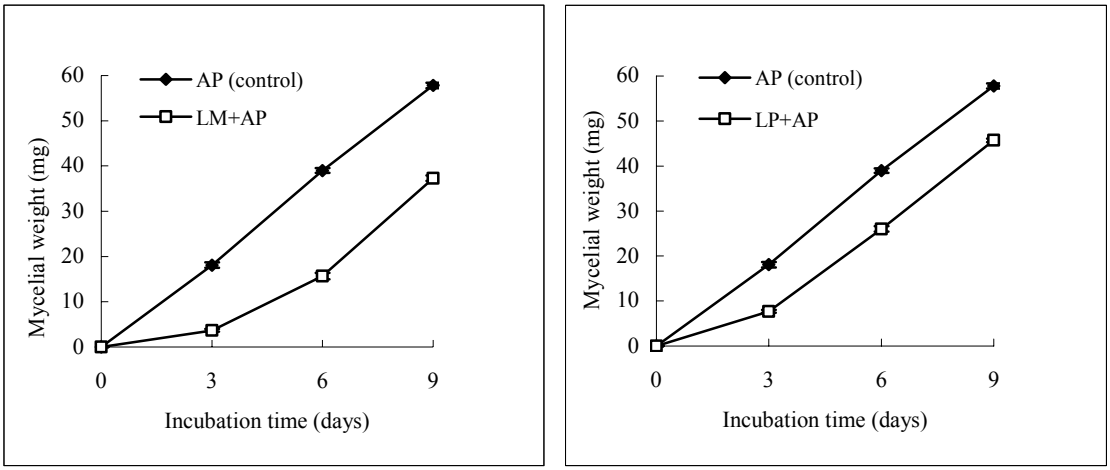
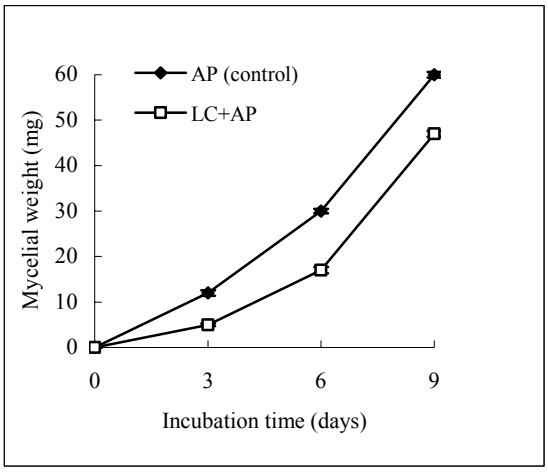


Fig. 1 Mycelial growth of *A. parasiticus* in a modified APT broth with and without *Leu. mesenteroides.* AP: *A. parasiticus* was inoculated alone. LM+AP: *Leu. Mesenteroides* and *A. parasiticus* were inoculated simultaneously (left).

Fig. 2 Mycelial growth of *A. parasiticus* in a modified APT broth with and without *L. plantarum*. AP: *A. parasiticus* was inoculated alone. LP+AP: *L. plantarum* and *A. parasiticus* were inoculated simultaneously (right).



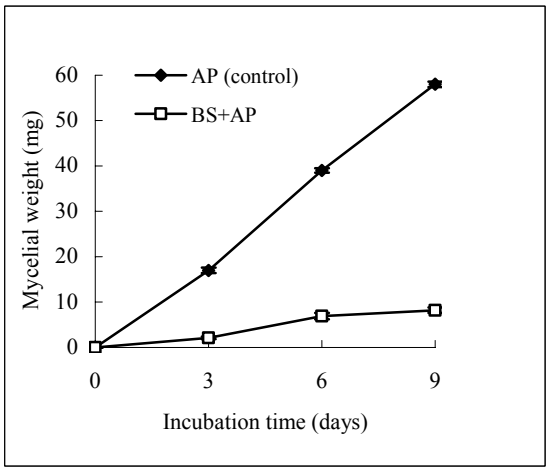


Fig. 3 Mycelial growth of *A. parasiticus* in a modified APT broth with and without *L. casei*. AP: *A. parasiticus* was inoculated alone. LC+AP: *L. casei* and *A. parasiticus* were inoculated simultaneously (left).
Fig. 4 Mycelial growth of *A. parasiticus* in a modified APT broth with and without *B. subtilis.* AP: *A. parasiticus* was inoculated alone. BS+AP: *B. subtilis* and *A. parasiticus* were inoculated simultaneously (right).

Dry mycelial weight of *A. parasiticus* in the mixed culture was reduced by 35.5%, 20.9%, 21.5%, and 86.2%, respectively, in comparison to the control at the end of the incubation period ($p<0.05$). The most inhibition of mycelial growth was exhibited when *B. subtilis* was co-inoculated to the APT broth (Fig. 4).

3.2 Effects of bacteria on the aflatoxin production of *A. parasiticus*

Aflatoxin production of *A. parasiticus* ATCC 15517 on APT broth with and without bacteria was determined by use of HPLC. Effects of the bacteria on aflatoxin B_1 production of *A. parasiticus* are given in Figs 5-8. The production of aflatoxin B_1 of *A. parasiticus* on APT broth was also inhibited by the four bacteria during incubation.

The levels of aflatoxin B_1 produced in the mixed culture were 59.7%, 21.6%, 24.0%, and 70.4% lower, respectively, than the control at the end of the incubation period ($p<0.05$). The most inhibition of aflatoxin B_1 production was obtained by co-inoculation with *B. subtilis* as observed in the inhibition of mold growth (Fig. 8).

4. Discussion

In this study, reduction of growth and aflatoxin production by an aflatoxigenic mold in the presence of bacteria which are found in fermented foods. The percentage of inhibition of growth of *A. parasiticus* ranged from 20.9 to 86.2% at the end of the incubation period. When aflatoxin production was determined, the effect of the bacteria was also remarkable. The reduction of aflatoxin B_1 production ranged from 21.6 to 70.4%. The maximum inhibition of mold growth and aflatoxin production was observed when the mold was combined with *B. subtilis*. This is the case where marked aflatoxin inhibition was concomitant with marked mycelium inhibition. The results of this study are supported by Klich et al. [18]. They reported that a *B. subtilis* strain showed antifungal properties against various fungal strains. In their report, peptidolipid compounds possess inhibitory property.

The inhibitory activity of other bacteria related with fermentation such as lactic acid bacteria species on harmful mold was studied by numerous scientists. Inhibitory effects of several lactic acid bacteria on aflatoxin production have been reported [19-26]. Also, lactic acid bacteria such as *Lactobacillus* spp.

were found to inhibit aflatoxin biosynthesis [19, 20, 25]. Specific strains of lactic acid bacteria have been shown to non-covalently bind the potent toxin, aflatoxin B_1 [27-30]. Similar results of inhibition of mold growth and aflatoxin production have been obtained by the investigators although the degree of inhibitory effect was different. The differences might be due to the nature of the strain itself, substrate and/or culture condition.

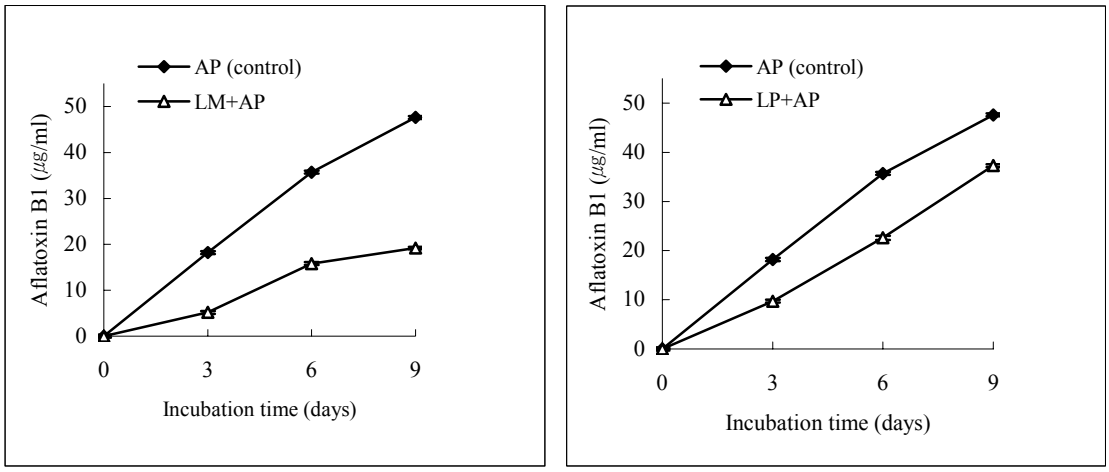


Fig. 5 Aflatoxin B_1 production by *A. parasiticus* in a modified APT broth with and without *Leu. mesenteroides*. AP: *A. parasiticus* was inoculated alone. LM+AP: *Leu. mesenteroides* and *A. parasiticus* were inoculated simultaneously (left).
Fig. 6 Aflatoxin B_1 production by *A. parasiticus* in a modified APT broth with and without *L. plantarum*. AP: *A. parasiticus* was inoculated alone. LP+AP: *L. plantarum* and *A. parasiticus* were inoculated simultaneously (right).

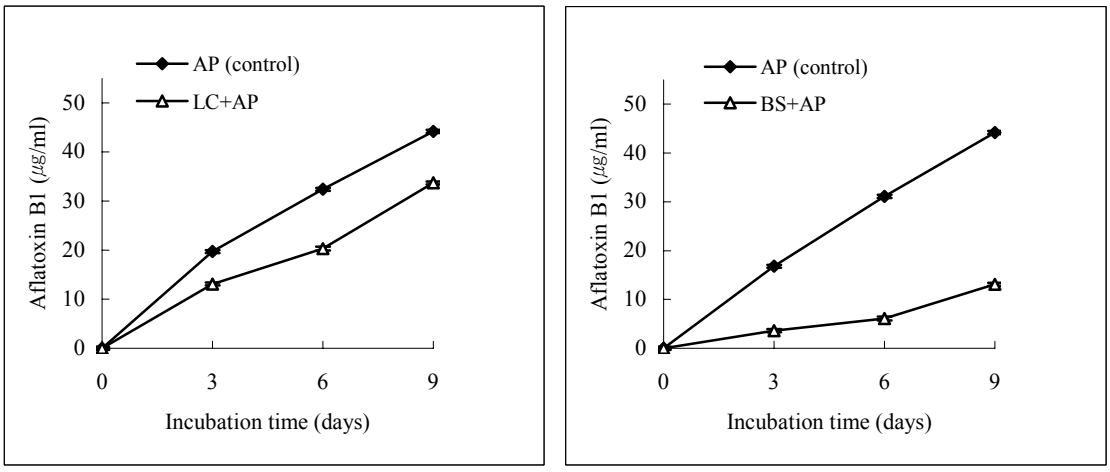


Fig. 7 Aflatoxin B_1 production by *A. parasiticus* in a modified APT broth with and without *L. casei*. AP: *A. parasiticus* was inoculated alone. LC+AP: *L. casei* and *A. parasiticus* were inoculated simultaneously (left).
Fig. 8 Aflatoxin B_1 production by *A. parasiticus* in a modified APT broth with and without *B. subtilis*. AP: *A. parasiticus* was inoculated alone. BS+AP: *B. subtilis* and *A. parasiticus* were inoculated simultaneously (right).

Koreans traditionally eat many fermented foods. The strains used in this study are commonly found in Korean feremented foods. However, the role of their benificial effects in reducing or preventing aflatoxin production has not been studied intensively. *Leu. mesenteroides* and *L. plantarum* are the

predominant species of bacteria in kimchi (Korean fermented vegetables). We can expect their co-existance might have a synergistic effect on the mold growth and aflatoxin production. *B. subtilis* is the predominant species of bacteria in doen-jang (Korean fermented soybean paste), and *L. casei* is one of the strains used in manufacturing fermented milk. The results of the above investigators and the results of this study constitute evidence that the foods which contain the bacteria are beneficial reducing both growth of harmful mold and aflatoxin production, and their effects in contaminated foods. Humans are exposed to many carcinogens in the environment, which can cause mutations that can be inherited. The use of anti-carcinogens in daily food may be the most effective and economic way to prevent cancer in humans.

We could expect more inhibition by other bacteria related with fermentation in these foods. Long-term controlled animal and human studies are needed to evaluate the effects of the fermented foods as well as the bacteria themselves. Also, further research dealing with metabolites of the bacterial strains that may be responsible for the effects and comparing the effects of bacteria and the effects of the metabolites should be conducted.

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