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THE EFFECT OF *LACTOBACILLUS* AND *BIFIDOBACTERIUM* STRAINS ON THE GROWTH AND AFB_1 PRODUCTION OF *ASPERGILLUS FLAVUS*

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The effect of three individual strains of *Lactobacillus* (*L. acidophilus* NCC 12*, L. acidophilus* NCC 36*, L. acidophilus* NCC 68) and two *Bifidobacterium* strains (*B. bifidum* Bb13, *B. bifidum* NCC 381) on the growth and AFB_1 production of *Aspergillus flavus* NRRL 2999 was determined. The commercial MRS broth and skim milk medium were used to determine the effect of tested bacteria on the growth and AFB_1 production of *A. flavus* NRRL 2999. The bacteria were not effective in preventing the growth of *A. flavus* NRRL 2999 both in MRS broth and skim milk. However, higher levels of AFB_1 were obtained in the presence of individual strains of *Lactobacillus* and *Bifidobacterium*. The greatest difference was observed in the AFB_1 production of *A. flavus* NRRL 2999 in skim milk medium compared with MRS broth.

Keywords: antifungal, lactobacilli, bifidobacteria, aflatoxin B_1

The growth of mould is a common cause of decomposition of products and deterioration of organoleptic properties (FLORIANOWICZ, 2001). In addition to food deterioration, almost 50 species of *Aspergillus* are known to produce aflatoxins, ochratoxin A and cyclopiazonic acid, etc. that exhibit different range of toxicities (DOYLE et al., 1997). Aflatoxins are potent toxic and carcinogenic secondary metabolites produced by toxigenic strains of *A. flavus*, *A. parasiticus* and *A. nomius* (MOSS, 1992). In recent years, some biological control agents have been studied to reduce aflatoxin contamination in various food materials (HORN et al., 2000). Lactic acid bacteria are of special interest as preservation organisms, since they have a long history of use in food and are "generally regarded as safe" organisms (MAGNUSSON et al., 2003). There are some contradictory results in the literature about the antifungal effect of lactic acid bacteria. The main objective of this study was to determine the effect of lactic acid bacteria on the growth and AFB_1 production of *A. flavus* NRRL 2999.

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1. Materials and methods

1.1. Bacteria and mould strains

Bacteria used were *Lactobacillus acidophilus* strains NCC 12, NCC 36, NCC 68, *Bifidobacterium bifidum* strains Bb 13 and Bl 24. The bacterial strains were obtained from Nestle-Switzerland as a freeze-dried powder. Bacteria were activated in de Man Rogosa and Sharpe broth (MRS, Merck, Germany) containing 0.05% Cysteine for 48 h at 37 °C under anaerobic conditions (Gas Paks System, Oxoid, UK). *A. flavus* NRRL 2999 were obtained from the Culture Collections of The Scientific and Technical Research Council (TUBITAK) and grown on potato dextrose agar (PDA, Merck, Germany) slants at 25 °C for 7–10 days.

1.2. Preparation of spore suspension of A. flavus *NRRL 2999*

After 7–10 days (or until sporulation) of incubation at 25 °C, spores were harvested using 5 ml of sterile phosphate-buffered saline (PBS, pH 7.2, 0.8% NaCl, 0.12% K_2HPO_4, 0.03% KH_2PO_4) containing 0.05% Tween-80 by brushing the slant surface with a sterile inoculating loop. Mycelia was removed by filtration through sterile cheese cloth. Both mould spores and bacteria cell concentration was determined using traditional spread plate technique.

1.3. Microbial interaction

One ml of 10^8 CFU ml^{-1} of the bacteria culture and 1 ml of *A. flavus* NRRL 2999 (10^4 CFU ml^{-1}) spore suspension were added in a 250 ml Erlenmeyer flask containing 100 ml of either MRS broth or skim milk. The Erlenmeyer flasks were incubated at 30 °C for 10 days. The single culture inoculation assay served as negative control.

1.4. pH determinations

The pH values of mono culture (each test bacteria) and mixed culture (*A. flavus* + each lactic culture) were measured by using WTW (Inolab, Germany) digital pH-meter.

1.5. Microbiological analysis

At the end of the incubation period, 10 ml samples containing *A. flavus* mono- or mixed-culture were diluted with 90 ml of sterile 0.1% (w/v) peptone-water. After the incubation period, the number of *A. flavus* NRRL 2999 colonies was counted on two serial PDA plates.

1.6. Determination of AFB_1 production

AFB_1 both in MRS broth and skim milk was analyzed with thin layer chromatography (TLC) according to the modified A.O.A.C. method (1990). The AFB_1 extracts and AFB_1 standard (1 μg ml^{-1}) were spotted on TLC plates (20×20 cm. Merck, Germany).

The TLC plates were developed in 100 ml of chloroform/acetone/methanol (87:10:3, v/v/v). After development, the TLC plates were dried in the dark and exposed to long-wave-ultra-violet (365 nm, Camag) for visual estimation and comparison of sample spots to AFB_1 standard. All assays were performed in triplicate. The quantification of AFB_1 was calculated using the formula: (S×Y×V)/(X×W), where S: AFB_1 standard equal to unknown; Y: concentration of AFB_1 standard, μg ml^{-1}; V: μl of spotted sample; X: μl sample spotted giving fluorescent intensity equal to S (AFB_1 standard); W: quantification of sample.

2. Results and discussion

2.1. Changes in pH value

The pH value of the mixed culture at the end of the incubation period ranged from 6.05 to 6.58 and 4.95 to 5.35 for MRS broth and skim milk medium, respectively (Table 1). Using the lactic acid bacteria mono culture, the pH of MRS broth and skim milk dropped as expected following a 10-day incubation and ranged from 3.72 to 3.83 and 3.70 to 3.80, respectively. However, the pH value of *A. flavus* NRRL 2999 mono culture in MRS medium and skim milk was 5.40 and 5.24, respectively.

Table 1. The pH values of mono culture and mixed culture after 10 days of incubation at 25 °C

Microorganisms	pH	
	MRS broth	Skim milk
L. acidophilus NCC 12 + *A. flavus* NRRL 2999	6.43	5.30
L. acidophilus NCC 36 + *A. flavus* NRRL 2999	6.20	5.18
L. acidophilus NCC 68 + *A. flavus* NRRL 2999	6.05	4.95
B. bifidum Bb 13 + *A. flavus* NRRL 2999	6.58	5.35
B. bifidum NCC 381 + *A. flavus* NRRL 2999	6.15	5.18
L. acidophilus NCC 12	3.73	3.70
L. acidophilus NCC 36	3.75	3.77
L. acidophilus NCC 68	3.72	3.75
B. bifidum Bb 13	3.83	3.80
B. bifidum NCC 381	3.77	3.80
A. flavus NRRL 2999	5.40	5.24
MRS broth control	6.50	–
Skim milk control	–	6.45

The bifidobacteria fermentation pathway results in 3 mol of acetic acid and 2 mol of lactic acid per 2 mol of glucose in an ideal synthetic medium (CHICK et al., 2001). However, *Lactobacillus* uses the Embden-Meyerhof-Parnas (EMP) pathway and produces 2 mol of lactic acid per 1 mol of glucose (REES, 1997). CABO and co-workers (2002) reported that the levels of lactic acid produced by the various strains of lactic acid bacteria ranged from 5.32 to 18.3 g l^{-1} within 72 h of culturing. *Lactobacillus* and *Bifidobacterium* mono culture utilized available carbon source in the medium and the

concentration of lactic acid and acetic acid increased during incubation. Therefore, the pH value of medium decreased to 3.70 after 10-day incubation. However, the pH of mixed culture medium gradually increased toward neutral pH. It is believed that the increasing of the pH in the mixed culture system is due to the utilizing of organic acid such as lactic acid as a source of carbon and energy by *A. flavus*. Similarly, EL-GENDY and MARTH (1981) reported the initial pH value (7.10) clearly decreased to 3.70 in 3 days and later it increased to 7.20 at the end of the incubation period in the presence of *L. casei* and *A. parasiticus* mixed culture.

2.2. The effect of lactic acid bacteria on A. flavus *growth*

The counts of *A. flavus* NRRL 2999 in mixed cultures at the end of the incubation period are shown in Table 2. The numbers of *A. flavus* NRRL 2999 in control samples at the end of the incubation period were 3.2×10^4 and 6.0×10^4 CFU ml^{-1} for MRS broth and skim milk, respectively. When the mould grew with *L. acidophilus* strains NCC 12, NCC 36 and NCC 68, the mould counts were 1.2×10^5, 6.2×10^4 and 3.8×10^4 for MRS broth and 1×10^5, 7.1×10^4 and 2.7×10^4 CFU ml^{-1} for skim milk, respectively. However, the mycelium development of *A. flavus* NRRL 2999 was slightly affected in the presence of *L. acidophilus* NCC68. As can be seen in Table 2, the numbers of *A. flavus* were 1.9×10^5 and 2.0×10^5 for MRS broth, and 5.0×10^4 CFU ml^{-1} and 6.0×10^4 CFU ml^{-1} for skim milk in the presence of *B. bifidum* Bb13 and *B. bifidum* NCC 381, respectively.

Table 2. The growth and AFB_1 production of *A. flavus* NRRL 2999 in the presence of *Lactobacillus* and *Bifidobacterium* strains within 10 days

Microorganisms	MRS broth		Skim milk	
	A. flavus (CFU ml^{-1})	AFB_1 (μg l^{-1})	*A. flavus* (CFU ml^{-1})	AFB_1 (mg l^{-1})
L. acidophilus NCC12 + *A. flavus*	1.2×10^5	125	1.0×10^5	1200
L. acidophilus NCC36 + *A. flavus*	6.2×10^4	100	7.1×10^4	1050
L. acidophilus NCC68 + *A. flavus*	3.8×10^4	106	2.7×10^4	1000
B. bifidum Bb13 + *A. flavus*	1.9×10^5	130	5.0×10^4	1150
B. bifidum NCC381 + *A. flavus*	2.0×10^5	115	6.0×10^4	1200
A. flavus control	3.2×10^4	37	6.0×10^4	750

There are some contradictory results in the literature about the antifungal effect of lactic acid bacteria. This may be due to different lactic acid bacteria used against various mould strains by different investigators. GOURAMA (1997) pointed out that only four of 420 lactic acid bacteria strains exhibited antifungal activity against *Penicillium* species. In another study, EL-GENDY and MARTH (1981) reported that *A. flavus* did not remain viable beyond 4 days in the presence of cells of *L. plantarum*, *L. acidophilus* and the silage inoculant. The antifungal activity of lactic acid bacteria has been reported to be associated with the low pH of a culture and competitive growth in the scientific

literature (CABO et al., 2002). BATISH and co-workers (1997) reported that lactic acid was responsible for the observed antifungal activity. In contrast, lactic acid has no effect in the inhibition of mould growth as has previously been reported (GOURAMA, 1997; CABO et al., 2002). The results clearly indicate that lactic acid bacteria used in this study have no antifungal activity against *A. flavus* NRRL 2999.

2.3. The effect of lactic acid bacteria on AFB_1 production

In the presence of *Lactobacillus* and *Bifidobacterium* strains, more AFB_1 was detected at the end of the incubation period. While 37 μg l^{-1} AFB_1 was produced by *A. flavus* mono culture in MRS broth within 10 days, 125, 100 and 106 μg l^{-1} AFB_1 was produced by *A. flavus* in the presence of *L. acidophilus* strains NCC 12, NCC 36 and NCC 68, respectively. Similarly, 130 and 115 μg l^{-1} AFB_1 was detected in MRS broth in the presence of individual *B. bifidum* strains Bb13 and NCC 381, respectively. However, considerably lower amount of AFB_1 was detected in MRS broth compared with skim milk. These changes in AFB_1 production between in MRS broth and skim milk showed that substrate is one of the important factors in aflatoxin production. The levels of AFB_1 produced by *A. flavus* NRRL 2999 in the presence of tested strains in MRS broth were approximately ten fold lower than in skim milk. As can be seen in Table 2, up to 1050 to 1200 μg l^{-1} AFB_1 can be produced by *A. flavus* in the presence of *Lactobacillus* and *Bifidobacterium* strains. This increase in AFB_1 production in the presence of lactic acid bacteria may have been the result of microbial competition for the nutrients. If the mould is under stress, significant levels of AFB_1 may be produced in the medium by *A. flavus* NRRL 2999.

3. Conclusions

Even though some contradictory results have been found in the literature about the antiaflatoxigenic activity of lactic acid bacteria, our studies have clearly shown that *L. acidophilus* and *B. bifidum* strains have stimulated AFB_1 production of *A. flavus* NRRL 2999. Greater levels of AFB_1 were produced by *A. flavus* in skim milk compared with commercial MRS broth medium. However, more lactobacilli and bifidobacteria should be tested for their antifungal and antiaflatoxigenic activity.

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