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INHIBITION OF *ASPERGILLUS FLAVUS* AND *A. PARASITICUS* FUNGAL GROWTH AND ITS AFLATOXINS (B_1, B_2, G_1 AND G_2) PRODUCTION BY *LACTOBACILLUS ACIDOPHILLUS*

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ABSTRACT

This study aimed to investigate the inhibition effect of two strains of *Lactobacillus acidophilus* ATCC4495 and *Lactobacillus acidophilus* ATCC20552 on Aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2) producing fungi in both of culture media and corn grains (*Zea maize*). Aflatoxins (AFs), the secondary metabolites produced by species of Aspergilli, specifically *Aspergillus flavus* and *Aspergillus parasiticus*, have harmful effects on humans, animals, and crops that result in illnesses and economic losses. Both *L. acidophilus* strains were in vitro tested for its antifungal activity against *A. flavus* and *A. parasiticus* individually. The results indicated that both of strains showed antifungal activity against tested fungi. The cell free supernatant of *L. acidophilus* ATCC4495 recorded stronger inhibition against *A. flavus* (11.5m) and *A. parasiticus* (13m) while *L. acidophilus* ATCC20552 was less active. In addition, the activity was decreased by diluting the supernatant, for both *L. acidophilus* strains. Corn grains treated with original supernatant (100%) recorded the lowest total damage percentage during 30 days of storage at room temperature. Strain *L. acidophilus* ATCC4495 recorded high activity against two fungal strains. Corn grains treated only with LAB supernatant (negative controls) recorded the lowest aflatoxins content followed by infected grains with either *A. flavus* or *A. parasiticus* treated with cell free supernatant of *L. acidophilus* ATCC4495, followed by *L. acidophilus* ATCC20552, while infected grains with *A. flavus* or *A. parasiticus* (positive controls) contained the highest aflatoxins content.

Keywords: *Aspergillus*, Aflatoxins, Lactic acid bacteria, *Zea maize*.

INTRODUCTION

Aflatoxins (AFs) are toxic secondary metabolites produced by species of Aspergilli, especially *Aspergillus flavus* and *Aspergillus parasiticus*. These fungi can grow on certain foods and feeds under favorable conditions of temperature and humidity and generate AFs before and/or during harvest, handling, shipment and storage (Bushby & Wogan, 1984; Peraica *et al.*, 1999). The four major naturally occurred AFs are known as aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1) and aflatoxin G_2 (AFG_2). AFs have been shown to be potent carcinogens, mutagens and teratogens in addition to serious economic losses (Peraica *et al.*, 1999; Kotsonis *et al.*, 2001; Giray *et al.*, 2007). AFB_1, the most toxic compound in this series, has been found to be one of the most potent carcinogens occurring naturally and it was classified as Group I human carcinogen by the International Agency for Research on Cancer (IARC) in 1987 (IARC, 1987). The metabolic effects of AFs include: Inhibition of DNA, RNA and protein synthesis; reduction in miscellaneous enzyme activities; depression of glucose metabolism; inhibition of lipid synthesis, including that of phospholipids, free fatty acids, triglycerides and cholesterol and its esters; and depression of clotting factor synthesis (Bushby & Wogan, 1981).

Aflatoxins occur worldwide in maize (Kpodo *et al.*, 2000), maize being a dietary staple food in many countries in the world (Thiel *et al.*, 1996). Maize is consumed in different forms in the world. Traditional African maize products were found in various forms including porridges, pastes, dumplings, cakes, fritters, and beverages (Nago *et al.*, 1997). Maize grains aflatoxins were produced regardless of type of storage container, time and climatic condition (Thompson and Henke, 2002).

Lactic acid bacteria (LAB) are of particular interest as biopreservation organisms. Their preserving effect mainly relates to the formation of lactic acid, acetic acid, and hydrogen peroxide; competition for nutrients; and the production of

bacteriocins (Stiles, 1996). Early research suggested antifungal activities from a *Lactobacillus casei* strain that inhibited both the growth and the aflatoxin production of *Aspergillus parasiticus* (El-Gendy and Marth, 1981). A mixture of *Lactobacillus* spp. was found to reduce both molds growth and spore germination, as well as aflatoxin production by *Aspergillus flavus* subsp. *parasiticus* (Gourama and Bullerman, 1995). Aflatoxin B, can be reduced or prevented to be produced by *A. flauvs* by applying lactic acid fermentation in fermented maize meal products (Mokoena *et al.*, 2006). *L. fermentum* gave the strongest degradation of Aflatoxin B_I followed by *L. delbruekii* and *L. plantarum* (Arina, 2002). The antifungal activity of *Lactobacillus sanfrancisco* CBI, was caused by formation of several short-chained fatty acids, among which caproic acid was the most important molecule (Corsetti *et al.*, 1998). While, hydroxylated fatty acids, phenyl-lactic acid and 4-hydroxy-phenyl-lactic acid from *L. plantarum*. had broad spectrum fungicidal activity against several moulds (Lavermicocca *et al.*, 2000; Magnusson, 2003).

This study aimed to investigate the inhibition of *Aspergillus flavus* and *A. parasiticus* fungal growth in vitro and their aflatoxins (B_1, B_2, G_1 and G_2) production in corn grains *(Zea maize)* during 30 days of storage at room temperature using two *L. acidophillus* strains (coded ATCC4495 and ATCC20552) which may suggest their use as food preservatives against aflatoxin producing aspergilli or as aflatoxin detoxificant in tropical foods and feeds.

MATERIALS AND METHODS

Maize grain:

Single Crosse 10 cultivar of corn grains (*Zea maize* L.) used in this study wase obtained from local market.

Lactic acid cultures and growth conditions:

Both strains of *Lactobacillus acidophilus* coded ATCC4495 and ATCC20552 obtained from American Type Culture Collection, USA. Strains were grown routinely on MRS broth medium at 30°C for 48 h.

Fungal test strains:

***Aspergillus flavus* and *Aspergillus parasiticus* fungal strains used in this investigation were isolated from infected corn grains. The isolates were purified and identified by Fungal Taxonomy Dep., Plant Pathology Inst., Agric. Res. Center (ARC). Strains were maintained on Potato Dextrose Agar (PDA, Oxoid, Hampshire, UK) plates at 25°C. Once good growth of the cultures was established, they were stored at 4°C until further use and subcultured on a monthly basis (Cassandra *et al.*, 2004).**

Cultures Supernatant obtained:

Cell free supernatant of lactic acid strains were prepared according the method described by Schillinger and Lucke, (1989) by centrifuging the LAB culture at 6000g/15min. The pH of cell free supernatant adjusted to 6.5 using 10N of NaOH, then sterilized by filtration through 0.22ml Millipore filter.

Antifungal activity assays:

Disc diffusion method described by Cassandra *et al.*, (2004) was used to determine the antifungal activity of LAB *in vitro*. Three sterile Whatmann No. 1 filter disks (+5m) placed on a Potato Dextrose Agar (PDA) plate inoculated with 100µl of a fungal spore solution. A potential antifungal substance (10µl) is then applied on these filter disks. For each fungal strain, a blank without filter disks was made. Plates were incubated aerobically at 25°C and examined for inhibition zones around the filter disks during 10days. The radius of the observed inhibition zones was measured as an average of three.

Preparation of grain samples:

Corn grains were scratched by shaking with sand for 1 min disinfested by immersing in 5% sodium hypochlorite for 2 min, washed thoroughly with sterilized water and dried in hot-air oven at 44°C for 42 hrs Osman (1982). The moisture content of grains determined by Jac 2100 in Central Lab. for Food and Feed, divided into sub-samples and transferred into sterilized plastic suck 1.0 liter (Eisa *et al.*, 1996).

Inoculation of spore suspension:

Spore suspension was prepared from pure cultures of *Aspergillus flavus* and *A. parasiticus* (21 days old) grown on PDA plates (9 cm). These plates were flooded with 15 ml of sterilized distilled water and brushed thoroughly for 1–2 min. The suspension was filtered through three layers of cheesecloth to remove the mycelia residues. Number of spores/ml was counted in the collected spore suspension by using a Spencer haemacytometer and was about 7×10^7 spores/ml. Spore suspension was inoculated to tested grains to give a final density of approximately $\times10^3$ spore/gram of corn grains as described by (Eisa *et al.*, 1996).

Corn grains were divided into three major portions, (1): was not inoculated (not infected) (negative controls), (2): inoculated with *A. flavus* spores, while the (3^{rd}) inoculated with *A. parasiticus* spores. The moisture content of the major groups was adjusted to 20% by adding calculated volumes according to American Association of Cereal Chemists (1962) of sterilized I: distilled water only

(positive control without addition LAB supernatant) or II: distilled water 75%:25% lactic acid bacteria filtrate, III: distilled water 50%: 50% lactic acid bacteria filtrate and IV: 100% lactic acid bacteria filtrate. These treatments were carried out once for *L. acidophilus* ATCC4495 and other for *L. acidophilus* ATCC20552. All treatments were stored at room temperature for 30 days. Total damage percentage and Aflatoxins content was determined.

Total damage percentage:

From three replicates, (each replicate was 100 kernel) the infected kernels were counted, and then total damage percentage of grain was calculated.

Determination of aflatoxins:

Aflatoxins were determined according to Roos *et al.*, (1997) using HPLC apparatus as follow: The mobile phase was consisted of water: methanol: acetonitrile (54:29:17, v/v/v) at flow rate of 1ml/min. the extraction and emission wavelengths for all aflatoxins were 362 and 360nm, respectively. The quantity of each aflatoxin (B_1, B_2, G_1 and G_2) was measured as ppb.

RESULTS AND DISCUSSION

Effect of *L. acidophilus* supernatants on *A. flavus* and *A. parasiticus in vitro*:

Table (1): antifungal activity of *L. acidophilus* strains against aflatoxin producing molds.

	L. acidophilus ATCC4495			*L. acidophilus ATCC20552*		
	Inhibition zone diameter* by mm					
	25%	**50%**	**100%**	**25%**	**50%**	**100%**
A. flavus	6.0	8.5	11.5	6.0	7.5	10.5
A. parasiticus	6.5	9.0	13.0	6.0	8.0	10.0

* paper disc diameter is 5m

The antifungal activity of both *L. acidophilus* strains using disc diffusion methods showed in table (1). The inhibition zones were observed against both of *A. flavus* and *A. parasiticus*.

The original cell free supernatant (100%) of *L. acidophilus ATCC4495* showed the highest inhibition zones (11.5mm and 13.0mm) than that of *L. acidophilus* ATCC20552 (10.5 and 10.0mm) against two aflatoxin-producing *A. flavus* and *A. parasiticus*, respectively. The inhibition zones diameter gradually decreasing by decreasing the cell free supernatant concentration. By dilution the cell free supernatant to 50% the inhibition zones diameter gradually decreased. The least inhibition zone was observed with 25% supernatant. These results are similar to those reported by Batish *et al.*, (1990) and by Plockova´ *et al.*, (1997a, b) who reported that dilution of *L. acidophilus* LMG 9433 supernatant due to reduction the activity against tested fungi. The inhibition action of lactic acid strains may be due to reduced permitting sporulation to go ahead as reported by Onilude *et al.*, (2005).

***L. acidophilus* ATCC4495 had higher antifungal activity against aflatoxin producing *A. flavus* and *A. parasiticus* than other strain. *A. parasiticus* fungal strain was more sensitive than *A. flavus* to the cell free supernatant and its dilution. The antifungal activity of lactic acid may be due its ability to produce fungistatic bacteriocin-like substance, phenyllactic acid and 4hydroxyphenyllactic acid, short-chain fatty acids and low-molecular-weight substances, such as benzoic acid, methylhydantoin, mevalonolactone, and cyclo(Gly-L-Leu) (Corsetti *et al.*, 1998; Niku *et al.*, 1999;Okkers *et al.*, 1999 and Lavermicocca *et al.*, 2003).**

Effect of *L. acidophilus* on total damage percentage of corn grains:

Data presented in tables (2 and 3) showed the effect of both *L. acidophilus* strains on the corn grain (total damage percentage during 30 days of storage at room temperature).

Effect of *L. acidophilus* ATCC4495:

The results in the table (2) indicated that total damage percentage gradually increased by increasing the storage period till the end. Treated non infected corn grains with original supernatant (100%) of *L. acidophilus* ATCC4495 (negative control) recorded the lowest infection percentage than other treatments.

Table (2): Effect of *L. acidophilus* ATCC4495 strain of total damage maize grain (20% moisture) during 30 days of storage.

Infected fungi	**Treatments**	**Total damage (%)**		
		Zero	**15 days**	**30 days**
Non infected (Negative controls)	**100%**	3	4	6
	50%	3	8	20
	25%	3	14	41
A. flavus	**Post. control**	**3**	**75**	**100**
	100%	3	7	10
	50%	3	14	21
	25%	3	21	60
A. parasiticus	**Post. control**	**3**	**90**	**100**
	100%	3	10	17
	50%	3	21	30
	25%	3	28	50

Post. control= without supernatant **100%=** original supernatant
50%= diluted supernatant to 50% **25%=** diluted supernatant to 25%

Infected corn grains with either *A. flavus* or *A. parasiticus* only (positive controls) showed the highest total damage percentage during all storage period

(except at zerotime) reached the maximum (100%) at the end of storage. *A. parasiticus* showed more total damage percentage than that of *A. flavus*. Treating corn grains with *L. acidophilus* ATCC4495 supernatant (100%, 50% and 25%) with either *A. flavus* or *A. parasiticus* sharply reduced the total damage percentage comparing with positive controls but not than negative control.

Effect of *L. acidophilus* ATCC20552:

Modification of non infected corn grains (negative controls) moisture content to 20% using *L. acidophilus* ATCC20552 supernatant 100%, 50% and 25% recorded the highest total damage percentage reduction than all other treatments. In addition, infected corn grains treated with *L. acidophilus* ATCC20552 supernatant by 100%, 50 and 25% concentration reduced the total damage percentage than that of positive control but not than negative control. While infection the grains with *A. flavus* or *A. parasiticus* without supernatant treatment (positive controls) recorded the highest total damage percentage during storage period, reached the maximum (100%) at 30 days of storage. In addition, infected corn grains treated with *L. acidophilus* ATCC20552 supernatant dilutions 50 and 25% reduced the total damage percentage than that of positive control but not than 100% supernatant or negative control.

Table (3): Effect of *L. acidophilus* ATCC20552 strain on total damage maize grain (20% moisture) during 30 days of storage.

Infected fungi	Treatments	Total damage percentage (%)		
		Zero	15 days	30 days
Non infected (Negative controls)	100%	3	5	7
	50%	3	11	13
	25%	3	13	28
A. flavus	Post. control	3	75	100
	100%	3	8	16
	50%	3	18	22
	25%	3	25	55
A. parasiticus	Post. control	3	90	100
	100%	3	11	20
	50%	3	21	30
	25%	3	31	65

Post. control= without supernatant **100%=** original supernatant

50%= diluted supernatant to 50% **25%=** diluted supernatant to 25%

Generally, both of two *L. acidophilus* strains strongly reduced the infected percentage of corn grains inoculated with aflatoxins-producing *A. flavus* or *A. parasiticus*. In addition, *L. acidophilus* ATCC4495 showed stronger activity than that of *L. acidophilus* ATCC20552 against tested fungi. These results confirmed those of antifungal activity described in table (1). The total damage percentage of both *L. acidophilus* strains gradually increased by decreasing the supernatant concentration, sine the original supernatant (100%) treatment showed the lowest total damage percentage than both of 50 and 25% dilutions treatments. The reduction of total damage percentage of treated of infected corn grains with lactic acid bacteria may be due to its effects on inhibition the different *Aspergillus* species mycelial development prior to the sporulation as mentioned by Onilude *et al.*, (2005).

Effect of *L. acidophilus* strains on aflatoxin production of maize grain:

The concentrations of individual AFs and total AFs detected in all samples are given in tables 4 and 5. Both *L. acidophilus* strains reduced the AFs production during 30 days of storage at room temperature. Moisture modification of uninfected corn grains to 20% using 100, 50 and 25% of cell free supernatant (negative controls) recorded the lowest AFs concentration.

Effect of *L. acidophilus ATCC4495*:

Effect of *L. acidophilus ATCC4495* on AFs production in infected corn grains was presented in table (4). Infected corn grains with *A. flavus* (positive control) recorded the highest values for produced AFs (B_1, B_2, G_1 and G_2), followed by *A. parasiticus* (positive control). While, corn grains treated with 100% *L. acidophilus* ATCC4495 supernatant (control negative) showed lowest AFs concentration than all other treatments. Furthermore, infected corn grains with *A. flavus* contained higher total AFs content than the same treatments infected with *A. parasiticus*. AFB_1 recorded the highest content than that of all other AFs for all treatments. Also, grain samples contained high AFG_1 than AFG_2.

The AFs production increased by decreasing the supernatant concentration, since the 100% treatment reduced the production by 99.8% and 99.77, followed by 50% supernatant (69.93% and 64.04%), then 25% (73.08% and 61.40%) for infected grains with *A. flavus* and *A. parasiticus*, respectively. Aflatoxins G_1 and G_2 production by *A. parasiticus* was more sensitive for *L. acidophilus ATCC4495* supernatant than *A. flavus*.

Effect of *L. acidophilus* ATCC20552:

Table (5) showed the effect of *L. acidophilus* ATCC20552 on AFs production in infected corn grains. Both of positive control treatments (infected with *A. flavus* or *A. parasiticus*) recorded the highest values for produced AFs (B_1, B_2, G_1 and G_2) than other treatments. Moisture modification of corn grains with 100% *L. acidophilus ATCC20552* supernatant showed lowest AFs concentration than all other treatments.

Table (4): Effect of *L. acidophilus* ATCC4495 strain on aflatoxins production of maize grain (20% moisture) during 30 days of storage.

Infected fungi	Treatments	AFs concentration (ppb)				Total AFs	% Red.
		B_1	B_2	G_1	G_2		
Non infected (Negative controls)	**100%**	21	12	9.0	7	49.0	--
	50%	1083	174	447	72	1776	--
	25%	1238	234	433	84	1989	--
A. flavus	**Post. control**	**40000**	**20000**	**8000**	**6250**	**74250**	--
	100%	68	7	30	10	115	100
	50%	17412	1076	2323	1510	22321	70
	25%	15480	2109	2175	221.6	19985.6	73
A. parasiticus	**Post. control**	**21031**	**6342**	**4454**	**2993**	**44820**	--
	100%	58.4	6.06	29.2	6.6	100.26	100
	50%	9717	1160	4850	388	16115	64
	25%	10000	2921	3113	1270	17304	61

Post. control= without supernatant **100%= original supernatant**

50%= diluted supernatant to 50% **25%= diluted supernatant to 25%**

Table (5): effect of *L. acidophilus* ATCC20552 strain on aflatoxin production of maize grain (20% moisture) during 30 days of storage.

Infected fungi	Treatments	AFs concentration (ppb)				Total AFs	% Red.
		B_1	B_2	G_1	G_2		
Non infected (Negative controls)	**100%**	20	10	8	0	38	--
	50%	50	20	15	10	95	--
	25%	200	28	100	30	358	--
A. flavus	**Post. control**	**40000**	**20000**	**8000**	**6250**	**74250**	--
	100%	88	9.0	30	10	137	100
	50%	361	56	115	27	559	99
	25%	3490	459	5013	543	9505	87
A. parasiticus	**Post. control**	**21031**	**6342**	**4454**	**2993**	**44820**	--
	100%	90	15	32	5	142	100
	50%	8700	1950	502	403	11555	74
	25%	19485	5173	488	639	25785	43

Post. control= without supernatant **100%=** original supernatant

50%= diluted supernatant to 50% **25%=** diluted supernatant to 25%

Addition of supernatant 100, 50 and 25% reduced the total AFs production by 99.8, 99.2 and 87.19% for *A. flavus* treatments, and by 99.7, 74.2 and 42.5% for *A. parasiticus* treatments. Generally, infected corn grains with *A. parasiticus* treated with cell free supernatant (100, 50 and 25%) showed higher total AFs content than the same treatments infected with *A. flavus*. AFB_1 recorded the highest AFs content than that of all other AFs for all treatments, also, grain samples contained high AFG_1 than AFG_2.

In the same trend of negative controls and the *L. acidophilus* ATCC4495 treatments, AFs production increased by decreasing the supernatant concentration. This may be due to decreasing the antifungal activity of cell free supernatant by dilution as reported by Batish *et al.* (1990) and by Plockova´ *et al.* (1997a, b).

The obtained results revealed that all treatments included negative controls contained low AFs that may be due to its humidity content (20%) and storage temperature (room temperature) as reported by (Smith and Moss, 1985) who found that the production of AFs and the growth of the responsible fungi are dependent upon temperature and humidity during storage. High moisture levels in the samples and high temperature are favorable for the growth of AF-producing fungi. Optimum conditions are 16–24% moisture at 20–38°C. However, it is reported that AF production can also takes place at

temperatures as low as 7–12°C (Steyn and Stander, 2000).

Reduction of aflatoxins production by *A. flavus* or *A. parasiticus* by both of *L. acidohpillus* strains may be due to the antifungal activity of strains against aflatoxins producing fungi. *L. acidohpillus* inhibited the fungal growth and mycelial development as mentioned by Onilude *et al.*, (2005). Many investigators isolated the antimicrobial compounds from cell free supernatants identified lactic acid, PLA and the two cyclic dipeptides cyclo (L-Leu-L-Pro) and cyclo (L-Phe- L-Pro) as the major components responsible for this activity (Dal Belloa *et al.*, 2006). In addition, cyclic dipeptide at low concentrations is responsible for inhibition of aflatoxin production, although higher concentrations are needed to inhibit the growth of *Aspergillus parasiticus* (Yan *et al.*, 2004). Other compounds produced by different LAB shown to be active against moulds (Magnusson and Schnu¨rer, 2001; Lavermicocca *et al.*, 2000, 2003; Stro¨m *et al.*, 2002). Numerous studies have described the isolation and characterization of antifungal components from LAB (Lavermicocca *et al.*, 2000, 2003; Messens and De Vuyst, 2002; Stro¨m *et al.*, 2002; Nes and Johnsborg, 2004).

Generally, inhibition activity of two lactic acid strains (*L. acidophilus* ATCC4495 and *L. acidophilus* ATCC20552) against aflatoxins producing *A. flavus* and *A. parasiticus* growth and their aflatoxin production revealed that these strains can be used to protect the stored corn grains from both of fungal growth and aflatoxins production. The total damage percentage was highly reduced by treating the corn grains with LAB supernatant (100%). Aflatoxins analysis revealed that the treated corn grains with cell free supernatant of lactic acid bacteria contained the minimum aflatoxins concentration even in the occurrence of either *A. flavus* or *A. parasiticus*. The antifungal activities of lactic acid strains inhibited both the growth and the aflatoxins production of *A. parasiticus* as reported (El-Gendy and Marth,1981; Vanne *et al.*, 2000). In addition, Vanne *et al.*, (2000) showed that the growth of toxigenic storage fungi could be restricted by LAB *in vitro*. From the results arrived at, it could be safely concluded that the action of the lactic acid bacteria supernatant used in this work is being active against both of *A. flavus* and *A. parasiticus*. Similar result has been reported by Lavermicocca *et al.*, (2000) and Onilude *et al.*, (2005).

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