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ANTIFUNGAL ACTIVITY OF SOME LACTIC ACID BACTERIA AGAINST AFLATOXIN –PRODUCING ASPERGILLI

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ABSTRACT

This study was performed to investigate the antifungal activity of lactic acid bacterial (LAB) strains. Seven bacterial strains were tested against four fungal isolates that were isolated from infected corn grains and identified as *Aspergillus flavus*, *Aspergillus parasiticus, Aspergillus niger* and *Rhizopus nigricans*. The fungal biomass inhibition method was used as a pre-screening test. The aflatoxin-producing *Aspergilli* were detected by the black-light method. *Aspergillus parasiticus* produced both B and G aflatoxins while *Aspergillus flavus* produced only B aflatoxins. Twelve LAB strains were screened for antifungal activity against two aflatoxin-producing *Aspergilli*. The tested LAB strains were *Lactobacillus acidophilus* ATCC 20552*, Lactobacillus acidophilus* NRRL-B-4495*, Lactobacillus acidophilus* LA3*, Lactobacillus acidophilus, Lactobacillus plantarum* ATCC 14917*, Lactobacillus casei* DSM 20011*, Lactobacillus rhamnosus, Bifidobacterium bifidium, Bifidobacterium longum* 15708*, Bifidobacterium lactis* (Bb12)*, Streptococcus thermophiles, Streptococcus thermophiles* ATCC-3. The antifungal activity was performed by three methods; in the Overlay method, the maximum inhibitions of *A. flavus* and *A. parasiticus* were obtained by *S. thermophiles* ATCC-3. While, in the well diffusion method, the maximum inhibitions of *A. flavus* were obtained by *B. lactis* (38.0 mm) and *B. longum* (37.0 mm) and in case of *A. parasiticus*, the maximum inhibition was obtained by *B. lactis* (35.0 mm). Whereas, in the agar dilution method; the maximum inhibitions of *A. flavus* were shown by *B. lactis* (70.3%), *L. casei* DSM 20011 (57.77 %) and *S. thermophiles* ATCC-3 (57.46 %), respectively and in case of *A. parasiticus* the maximum inhibitions were obtained by *B. bifidum* (84.59%)*, B. lactis* (82.66%) and *S. thermophiles* ATCC-3 (68.69%). This inhibition may be due to the production of antifungal compounds like organic acids, hydrogen peroxide, diacetyl and proteinaceous substances. HPLC analysis indicated that lactic and acetic acids represented the main organic acids in the cell-free extract of lactic acid bacteria.

Key words: *Inhibition, Lactic acid bacteria, Aflatoxin-producing Aspergilli.*

1. INTRODUCTION

Certain molds and yeasts represent significant spoilage contaminants of food and feed. In addition, the potential production of toxic and carcinogenic mycotoxins by moulds is of particular concern. Also, fungal spoilage of food causes a significant economic loss. Worldwide, about 5-10 % of the food production is estimated to be spoiled by these organisms (Pitt and Hocking, 2009; Aunsbjerg, 2015).

Lactic acid bacteria are among the most powerful prokaryotes when it comes to antimicrobial potential. These bacteria not only produce several antimicrobials during carbon source metabolism, but also compete with other species by acidifying their environment and by rapidly depleting the nutrients. Besides these relatively simple antagonistic mechanisms, some lactic acid bacteria also produce potent antibiotic compounds *via* complex secondary metabolism pathways. Oranusi *et al.*, (2013) indicated that the LAB isolates optimized and improved could be used as a natural, food-grade biopreservative agent for management of fungal contamination and food spoilage. Thus preventing problems associated with mycotoxins in food and feed products.

The antifungal compounds of lactic acid bacteria have potential for being effective in combating food borne yeasts and moulds. There

have been several reports on antifungal properties of lactobacilli; e.g. *Lactobacillus acidophilus* (Batish *et al*., 1989, 1990b; Plockova´ *et al*., 1997a, b), *L. casei* (Suzuki *et al*., 1991; Gourama, 1997), *L. coryniformis subsp. coryniformis* (Magnusson and Schnürer, 2001), *L. pentosus* (Okkers *et al*., 1999), *L. plantarum* (Niku-Paavola *et al*., 1999; Lavermicocca *et al*., 2000; Laitila *et al*., 2002; Ström *et al*., 2002; Lavermicocca *et al*., 2003), *L. rhamnosus* (Suzuki *et al*., 1991; Stiles *et al*., 2002), *L. salivarius* (Stiles *et al*., 1999), *L. sanfrancisco* (Gobetti and Corsetti, 1997; Corsetti *et al*., 1998), *L. lactis* subsp. *lactis* (Roy *et al*., 1996; Roy *et al*., 2001) and *L. lactis* subsp. *lactis* var. *diacetylactis* (Batish *et al*., 1989, 1990a).

Furthermore, it has been shown that certain LAB, such as a dairy strain *Lactococcus lactis*, as well as *Lactobacillus* and *Pediococcus* meat starters, silage mixtures containing *L. acidophilus, L. bulgaricus* and *L. plantarum* species and probiotic *L. rhamnosus* strains either suppress mycotoxin biosynthesis or effectively remove preformed mycotoxins (Coallier-Ascah and Idziak , 1985; Luchese *et al.* 1992; Gourama and Bullerman 1995b, 1997; El-Nezami and Ahokas 1998; El-Nezami *et al*., 1998a, 1998b; Haskard *et al*. 2001).

El-Gendy and Marth (1981) reported that *Lactobacillus casei* inhibited the growth and the aflatoxin production of *Aspergillus parasiticus*. Also, Lavermicocca *et al*. (2000) demonstrated that the antifungal compounds such as phenyl lactic acid and 4-hydrophenyllactic acid were produced by *Lactobacillus plantarum*.

Most of the antifungal capacity of the lactic acid bacteria e.g. *Lactobacillus acidophilus* NCIM5426 and *Lactobacillus amylovorus* DCE 471 is due to the production of an antifungal protein or proteinaceous compound. Others like *L. plantarum* and *L. sanfrancisco* produce special organic acids (3-phenyl-L-lactic acid and caproic acid, respectively) that have antifungal properties (Corsetti *et al*., 1998; Ström *et al*., 2002; Lavermicocca *et al*., 2003).

The aim of the present research was to screen and evaluate the antifungal activity of lactic acid bacterial strains against aflatoxin-producing Aspergilli. As the inhibition of toxigenic fungi by LAB could be of great public health significance.

2. MATERIALS AND METHOD

2.1. Lactic acid bacterial cultures

Lactobacillus acidophilus ATCC 20552, *Lactobacillus acidophilus* and *Streptococcus thermophilus* were originally obtained from Chr. Hansen's Lab (Denmark). *Lactobacillus rhamnosus*, *Bifidobacterium bifidum* and *Bifidobacterium longum* 15708 were collected from a dairy research lab in the Agriculture Research Center (ARC). *Lactobacillus acidophilus* NRRL-B-4495, *Lactobacillus acidophilus, Lactobacillus plantarum* ATCC 14917, *Streptococcus thermophilus* ATCC-3 and *Bifidobacterium lactis* (Bb12) were obtained from the laboratory of microbiology in the Agricultural Research Center. *Lactobacillus casei* DSM 20011 was obtained from MERCIN, while *Lactobacillus acidophilus* LA3 was obtained from the Dept. of Food Nutrition Sciences, College of Agriculture, King Faisal University. All the *Lactobacillus* strains were cultured according to their specific requirements of temperature and aeration level as shown in

Table (1): Culture conditions and specific requirements of temperature and aeration level of lactic acid bacteria.

Species	Medium	Temperature	Condition	Incubation time
***Lactobacillus acidophilus* ATCC 20552**	**MRS**	**37 °C**	**Anaerobic**	**24–48 h**
***Lactobacillus acidophilus* NRRL-B-4495**	**MRS**	**37 °C**	**Anaerobic**	**24–48 h**
***Lactobacillus acidophilus* LA3**	**MRS**	**37 °C**	**Anaerobic**	**24–48 h**
Lactobacillus acidophilus	**MRS**	**37 °C**	**Anaerobic**	**24–48 h**
***Lactobacillus casei* DSM 20011**	**MRS**	**30 °C**	**Aerobic**	**24–48 h**
***Lactobacillus plantarum* ATCC 14917**	**MRS**	**30 °C**	**Aerobic**	**24–48 h**
Lactobacillus rhamnosus	**MRS**	**37 °C**	**Aerobic**	**24–48 h**
***Bifidobacterium lactis* (Bb12)**	**MRS-cys**	**37 °C**	**Anaerobic**	**48–72 h**
***Bifidobacterium longum* 15708**	**MRS-cys**	**37 °C**	**Anaerobic**	**48–72 h**
Bifidobacterium bifidum	**MRS-cys**	**37 °C**	**Anaerobic**	**48–72 h**
Streptococcus thermophilus ATCC-3	**Eliker**	**42 °C**	**Anaerobic**	**24–48 h**
Streptococcus thermophilus	**Eliker**	**42 °C**	**Anaerobic**	**24–48 h**

(Table 1). Anaerobic strains were kept in an anaerobic jar (Gas Generating Kit, Oxoid England). Again, fully grown colonies were stored on plates at 4 °C until further use.

2.2. The fungal isolates and culture condition

In this study the fungal isolates were isolated from infected corn grains on Potato Dextrose Agar (PDA), and their identification was based on macroscopically and microscopically features (Domsch *et al*., 1980; Von Arx ,1981; Hanlin, 1990; Kiffer and Morelet,1997). All isolates were cultured on (Difco Laboratories, Detroit, Mich USA.) plates at 25°C. Once good growth of the cultures was established, they were stored at 4°C until further use.

2.3. Preparation of spore suspension

The fungal cultures were grown on PDA slants for 7 to 10 days at 25°C until well sporulated. The fungal spores were harvested by adding 10 ml of sterile water and aseptically dislodging the spores with a sterile inoculating loop. The spore suspensions were further adjusted with sterile water to give a final spore concentration of approximately 10^4 spores / ml by a haemocytometer. The spore concentration was determined on PDA plates using standard pour plate technique at 25 °C for 2 to 3 days. The PDA used in this study was not acidified, and the pH of this medium after sterilization was 5.6 ± 0.2 (Siriporn *et al*., 2003).

2.4. Preparation of culture supernatants of lactic acid bacteria

Cell-free culture supernatants (CFCS) of lactic acid bacteria were obtained by centrifugation (10,000 × g, 4 ◦C, 10 min) of *lactobacilli* cells grown in 20 ml MRS broth (Difco Laboratories, Detroit, MichUSA.) at 37 ◦C for 24 h. The supernatant was filtered through a 0.22 µm filter to remove residual cells (Ogunbanwo, 2005).

2.5. Pre-screening test of the antifungal activity

2.5.1. Fungal biomass inhibition method

A pre-screening test was performed by using seven strains of lactic acid bacteria against four fungal isolates. This method was based on dry weight measurements of fungal biomass. The percentage of inhibition of fungal growth was calculated by comparing the fungal growth of the control with the treated one. After five days of incubation, the dry weight of the mycelia was obtained on apre-weight filter paper in an oven at 60 °C for 48°h. The means were calculated and the percentage of inhibition was calculated using the formula:

$$\frac{R1-R2}{R1} * 100$$

Where, R1 is the dry weight of the control and R2 the dry weight of the treated fungus (Siriporn *et al*., 2003).

2.6. Detection of aflatoxin- producing *Aspergilli*

The aflatoxin- producing Aspergilli can be detected by the black-light method which correctly identifies negative AFs samples. It depends on the illumination of the sample with a UV lamp. The test should be made in a darkened area for best contrast. Fluorescence may be bright or dim, depending on the amount of fluorescing agent present (Irineo Torres, 2011).

2.7. Assessment of the MIC of aflatoxin-producing *Aspergilli*

The antifungal activity of twelve strains of lactic acid bacteria was screened against the two aflatoxin-producing *Aspergilli*. In these tests one milliliter of the final spore suspension containing about 10^4 spores / ml of *A. flavus* and *A. parasiticus* was inoculated individually in 250 ml Erlenmeyer flasks containing 20 ml MRS. Different concentrations of the selected LAB supernatants (10, 15, 20, 25, 50 and 75%) were added to the flasks. The cultures were then incubated for 5 days at 25 °C. In the control experiment, only spore suspension was added to sterile MRS broth. After 5 days of incubation, the fungal mycelium was dried on a pre-weight filter paper in oven at 60°C for 48°h and weighted. The means were calculated from the previous formula.

2.8. Methods for evaluation of antifungal activity

Among many methods available for evaluation of antifungal activity (Parish and Davidson 1993), three methods described below have been used for determining the antifungal activity of the compounds produced by LAB.

2.8.1. Antifungal overlay assay

The antifungal activity of LAB was investigated with an overlay assay (Lind *et al*., 2005; Magnusson and Schnürer, 2001). Among the different fungal species studied, *A. flavus* and *A. parasiticus* were finally selected in the initial screening then further evaluated. LAB bacteria were inoculated in two lines (2-cm) on MRS agar plates and allowed to grow at 30 °C. Ten milliliters of soft (7%) PDA agar containing 1 ml of fungal inoculum were poured and incubated at 30°C. After 48 h, the zone of inhibition was measured. The degree of inhibition was calculated as the area of inhibited

growth in relation to the total area of the Petri dish and the scale was represented as follow: (-) = no visible inhibition, (+) = weak inhibition in the soft agar, (++) = medium inhibition, (+++) = strong inhibition, (++++)= very strong inhibition and (-) = no visible inhibition.

2.8.2. The agar diffusion method

The agar diffusion method has long been used for testing antimicrobial activity. It includes agar well diffusion assay and disc assay. In this test, an antimicrobial compound is applied to an agar plate on a paper disc or in a well. The results of the test are generally qualitative (Parish and Davidson, 1993).

2.8.2.1. The well-diffusion assay

The antifungal activity of cell free supernatants of the selected LAB was used against the aflatoxin-producing *Aspergilli* by the agar well diffusion technique. In this test, an antimicrobial compound is applied to a well in an agar plate containing 20 ml PDA media. The plates were inoculated with the fungal isolate and incubated at 25 °C for 24-48 h and the antimicrobial effect was recorded by measuring the zone of inhibition around the well. The experiment was carried out in duplicates (Parish and Davidson 1993).

2.8.2.2. The disc diffusion assay

The antifungal activity of cell free supernatants of the selected LAB was tested with the filter disk method. In this test, the CFS (100 µl) were applied to a paper disc on an agar plate containing 20 ml of the inoculated PDA media. The plates were incubated at 25 °C for 24-48h and the antimicrobial effect was recorded by measuring the zone of inhibition around the disc. The experiment was carried out in duplicates (Parish and Davidson 1993).

2.8.3. The agar dilution method

The antifungal activity was carried out by agar dilution method (Mitscher *et al.*, 1972). The antifungal activity was evaluated against these fungal isolates (*A. flaves* and *A. parasiticus*) using a concentration of 500 µl of the LAB extract incorporated into the media and poured into the different petriplates and allowed to solidify. Fungal plugs (0.6 mm in diameter) were obtained and placed at the center of Petri dishes containing a Potato Dextrose Agar (PDA) culture media with the LAB extract. The PDA plates containing only fungal plugs were used as control plates. All plates were incubated at 28 ◦C and the radial growth of mycelia was measured daily during 7 days. The control one; presented a fast radial growth of mycelium and reached the edge of the plates. The percentage of inhibition was calculated on the basis of growth in the control plates as:

Percentage of mycelial growth inhibition

$$= \frac{\text{Mycelial growth in control} - \text{Mycelial growth in the treated one}}{\text{Mycelial growth in control}} * 100$$

2.9. Determination of antifungal metabolites produced by LAB strains

2.9.1. Analysis of the organic acids by the High Performance Liquid Chromatography (HPLC)

2.9.1.1. Sample preparation

The LAB cultures were grown in 10 ml MRS broth at 37°C, then the cultures were centrifuged at 10000 rpm, for 15 min. The organic acids were determined in the CFS after 16h, 48h, 20h, 24h and 48h of the bacterial growth. Then 1ml supernatant from each strain was filtered through 0.22 µm membrane filter without dilution to be measured by a HPLC.

2.9.1.2. Analysis by HPLC

Organic acid concentration in the CFCS was measured by a HPLC (Agilent Technologies 1200 series, Calif. US) and the system was equipped with auto sampler, column compartment set at 35˚C, quaternary pump set at flow rate 1 ml /min, and injection volume of sample (15 µl).The organic acid was detected with MWD (Multi Wavelength Detector) at 210 nm. The sample was fractionated by using Ao-1000 column Alltech (300 ×6.5 mm) and Acid identification was performed by comparing the retention times of the samples with that of the standards of organic acid (Sigma Aldrich Co. LLC. US/Canada).

3. RESULT AND DISCUSSION

3.1. Pre-screening the antifungal activity

Based on dry weight measurements of fungal biomass, all tested lactic acid bacterial strains inhibited the growth of four fungal isolates *Aspergillus flavus*, *Aspergillus parasiticus, Aspergillus niger* and *Rhizopus nigricans* to varying extents except *lactobacillus plantarum* strain as shown in (Table 2). The fungal growth on liquid MRS medium inoculated with lactic acid bacteria was expressed as the percentage of the decrease of fungal biomass in comparison to the control.

A high percentage of lactic acid bacteria (LAB) was observed to possess antifungal activity inhabiting all the four spoilage fungi within the incubation period. The percentage inhibition of the fungal growth of *A. parasiticus*

Table (2): Percentage inhibition of fungal growth by using cell-free bacterial supernatants of LAB strains.

Strains of Lactic acid bacteria	% inhibition of fungal growth							
	A. parasiticus		*A. flavus*		*A. niger*		*R. nigricans*	
Control	**0.3154**	**%**	**0.3063**	**%**	**0.352**	**%**	**0.3154**	**%**
***Lactobacillus acidophilus* ATCC 20552**	**0.0548**	**82.6**	**0.102**	**66.7**	**0.113**	**67.9**	**0.1127**	**64.3**
***Lactobacillus acidophilus* NRRL-B-4495**	**0.0866**	**72.54**	**0.0851**	**72.2**	**0.088**	**75.0**	**0.1861**	**41.0**
Lactobacillus bulgaricus	**0.0565**	**82.09**	**0.0507**	**83.5**	**0.055**	**84.4**	**0.056**	**82.2**
***Bifidobacterium lactis* (Bb12)**	**0.0392**	**87.75**	**0.0471**	**84.6**	**0.053**	**85.0**	**0.0383**	**87.9**
Lactobacillus plantarum	**0.3494**	**10.78-**	**0.3555**	**13.8-**	**0.395**	**12.2-**	**0.2009**	**36.3**
***Lactobacillus plantarum* ATCC 14917**	**0.0400**	**87.32**	**0.0805**	**73.7**	**0.084**	**76.2**	**0.0365**	**88.4**
***Streptococcus thermophiles* ATCC-3**	**0.0564**	**82.12**	**0.0403**	**86.8**	**0.049**	**86.0**	**0.0451**	**85.7**

ranged from 72.54 in *Lactobacillus acidophilus* NRRL-B-4495 to 87.75 in *Bifidobacterium lactis* (Bb12), respectively in comparing with the controls grown in MRS. Also, the percentage inhibition of the fungal growth of *A. flavus* and *A. niger* ranged from 66.7 and 67.9 in *Lactobacillus acidophilus* ATCC 20552 to 86.8 and 86.0 in *Streptococcus thermophiles* ATCC-3, respectively. While, the percentage inhibition of fungal growth of *R. nigricans* ranged from 41.0 in *Lactobacillus acidophilus* NRRL-B-4495 to 88.4 in *Lactobacillus plantaru*m ATCC 14917.

The three fungal isolates (*A. flavus, A. parasiticus* and *A. niger*) showed no growth inhibition by cell free culture supernatant from *Lactobacillus plantarum*, while the other strain of *L. plantarum* ATCC 14917 inhibited the fungal growth by 87.3 % in case of *A. parasiticus,* 73.7% in *A. flavus*, 76.2% in *A. niger* and 88.4% in *R. nigricans.*

L. plantarum ATCC 14917 achieved the highest inhibition percentage (88.4%) against *R. nigricans,* also, *Bifidobacterium lactis* (Bb12) achieved the highest inhibition percentage (87.75 %) against *A. parasiticus.* while, *Streptococcus thermophiles* ATCC-3 achieved the highest inhibition percentages (86.8 %) and (86 %) against *A. flavus and R. niger*, respectively.

The results of the dry weight of mycelia indicated the suppressive effect of LAB on the fungal growth due to the production of several metabolites that may act together to inhibit fungal growth in liquid culture.

The antifungal activity of *lactobacilli* may be due to their ability to produce fungistatic bacteriocin-like substance, phenyllactic acid and 4-hydroxyphenyllactic 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; Okkers *et al.*, 1999; Niku-Paavola *et al.,* 1999 and Lavermicocca *et al.*, 2003). Gourama and Bullerman (1995 and 1997) showed that a commercially available silage inoculant with a combination of *Lactobacillus* species had antifungal and antiaflatoxin activity against *A. flavus*. Also, Lavermicocca *et al.*, (2000) reported that phenyllactic acid and 4-hydroxy-phenyllactic acid from *L. plantarum* 21B, were used with their antifungal activity against several species of filamentous fungi. Phenyllactic acid has also been identified from culture supernatants of *L. plantarum* MiLAB 393 (Ström *et al*., 2002). Antifungal metabolites, *e.g.* peptides, phenyllactic acid, proteinaceous compounds and 3-hydroxylated fatty acids have also been isolated from lactic acid bacteria (Schnürer and Magnusson, 2005).

3.2. Detection of aflatoxin- producing fungi

When the fungal isolates were exposed to the UV lamp, it was found that two strains were positive AFs (as they produce blue fluorescence which associated with the aflatoxin production). They include *Aspergillus parasiticus*, produced both B and G aflatoxins while the other identified as *Aspergillus flavus*, produced only B aflatoxins.

3.3. Assessment of the MIC of aflatoxin-producing *Aspergilli*

In this test six different concentrations of the cell-free supernatants of the selected five LAB strains were examined against both aflatoxin-producing *Aspergilli* (*A. flavus* and *A. parasiticus*). The obtained results are summarized in Tables (3&4). The increasing of the supernatant concentration from 10 % to 25 % accompanied a decrease in the dry weights of the tested fungi. But in the case of using supernatant concentrations from 50 % to 75 %, there was no detectable growth for both of the tested fungal isolates.

The highest antifungal activity was obtained

by *B. lactis* (Bb12) which has the MIC at supernatant concentration <10 % against both, *A. flavus* and *A. parasiticus,* respectively. A moderate antifungal activity was detected by both *L. acidophilus* NRRL-B-4495 and *L. plantarum* ATCC 14917, which have the MIC at supernatant concentration at 20 % against *A. flavus* and only *L. acidophilus* NRRL-B-4495 has the MIC at supernatant concentration at 20% against *A. parasiticus*. The lowest antifungal activity was observed by *L. acidophilus* ATCC 20552 and *S. thermophiles* as they have the MIC at supernatant concentration of 50 % against *A. flavus;* also, *L. acidophilus* ATCC 20552 and *L. plantarum* ATCC 14917 have MIC at supernatant concentration of 50% against *A. parasiticu*s. The reduction of fungal sporulation may be referred to the inhibitory activity of lactic acid bacterial strains (Onilude *et al*., 2005).

In this study, *B. lactis* (Bb12), *L. acidophilus* ATCC 20552 and *L. plantarum* ATCC 14917 had a higher antifungal activity against *A. flavus* and *A. parasiticus* than the other tested lactic acid bacteria. *A. flavus* was more sensitive than *A. parasiticus* to the cell-free supernatant and its dilution. The antifungal activity of *lactobacilli* may be due to their ability to produce fungistatic bacteriocin-like substance, phenyllactic acid and 4-hydroxyphenyllactic acid, short-chain fatty acids and low-molecular weight substances, such as benzoic acid, methylhydantoin, mevalonolactone and cyclo (Gly-L-Leu) as reported by (Corsetti *et al*., 1998; Niku *et al*., 1999; Okkers *et al*., 1999; Lavermicocca *et al.,* 2003; Dalié *et al*., 2010). The inhibition effect of *L. plantarum* ATCC 14917 was similar to the inhibition effect that shown by *L. plantarum* YO which was able to inhibit the vegetative and sporulative growth of all aflatoxin producing *Aspergilli* (Ghonaimy *et al*., 2007).

3.4. Methods for evaluation of antifungal activity

The present study was undertaken to demonstrate the mycelial growth inhibition of the two aflatoxin-producing *Aspergilli* (*A. flavus* and *A .parasiticus*) by the presence of both the lactic acid bacterial (LAB) cells and their cell-free culture supernatants (CFNS) using different bioassays. Several methods for detecting activity are available, but since they are not equally sensitive or not based upon the same principle, results will be profoundly influenced by the method. The methodology for testing natural compounds for the determination of antifungal activity is variable and each research group includes different types of tests.

3.4.1. The overlay method

The antifungal activity of twelve LAB strains was evaluated by the overlay method. As shown from the results in (Table 5), there are varying inhibition levels of the growth of each of *A. flavus* and *A. parasiticus* to the antifungal agents produced by lactic acid bacterial strains.

As all LAB strains tested showed antifungal activity. *Streptococcus thermophiles* ATCC-3 was very highly active (++++) against *A. flavus* and has moderate activity (++) against *A. parasiticus. Bifidobacterium lactis* (Bb12) and *Lactobacillus casei* DSM 20011 were highly active (+++) against *A. flavus* and have moderate activity (++) against A. *parasiticus*.

The inhibitory activity of the tested bacteria could be seen because all metabolites; lactic acid, acetic acid, diacetyl, bacteriocin etc., are present and being produced during the assay period and this agreed with (Çon and Gökalp, 2000). Other studies suggested that antifungal activity of LAB is due to a synergistic effect of lactic acid produced by the bacteria and acetic acid from the MRS growth medium (Cabo *et al*., 2002). It might has been due to the fact that the antimicrobial compounds in the mixtures interacted with each other as well as with the tested organisms (Niku-Paavola *et al*., 1999).

3.4.2. The agar diffusion method

3.4.2.1. The well-diffusion assay

The cell-free supernatants (CFS) of the selected LAB strains produced antifungal activities against the two aflatoxin-producing *Aspergilli* as in (Table 6).

The largest zone of inhibition (38.0 mm) was produced by *Bifidobacterium lactis* (Bb12) against *A. flavus* and (35.50 mm) against *A. parasiticus* (Table, 6). While, *Bifidobacterium longum* 15708 produced a moderate inhibition zone (37 mm) against *A. flavus* and (33.0 mm) against *A. parasiticus*. On the other hand, *lactobacillus* and *streptococcus* strains achieved high inhibition zones but less than obtained by *Bifidobacterium* strains.

3.4.2.2. The disc diffusion assay

The assessment of antifungal activity of 12 lactic acid bacterial strains using disc diffusion method is presented in (Table 7).

As shown from the results of (Table, 7), the highest inhibition zone was observed with *Bifidobacterium longum* 15708 against *A. flavus* (11.0 mm) and *A. parasiticus* (7.5 mm). While,

Table (3): Assessment of MIC of lactic acid bacterial supernatants against *Aspergillus flavus*.

Lactic acid bacteria	Supernatant concentration (%)						
	0% (control)	10%	15%	20%	25%	50%	75%
L. acidophilus ATCC 20552	0.31	0.282	0.272	0.242	0.211	ND	ND
L. acidophilus NRRL 4495	0.31	0.233	0.193	ND	ND	ND	ND
L. plantarum ATCC 14917	0.31	0.256	0.212	ND	ND	ND	ND
B. lactis(Bb12)	0.31	ND	ND	ND	ND	ND	ND
S. thermophiles ATCC-3	0.31	0.242	0.210	0.206	0.174	ND	ND

Table (4): Assessment of MIC of lactic acid bacterial supernatants against *Aspergillus parasiticus*.

Lactic acid bacteria	Supernatant concentration (%)						
	0% (control)	10%	15%	20%	25%	50%	75%
L. acidophilus ATCC 20552	0.32	0.282	0.252	0.232	0.218	ND	ND
L. acidophilus NRRL 4495	0.32	0.233	0.160	ND	ND	ND	ND
L. plantarum ATCC 14917	0.32	0.267	0.246	0.240	0.214	ND	ND
B. lactis(Bb12)	0.32	ND	ND	ND	ND	ND	ND
S. thermophiles ATCC-3	0.32	0.246	0.236	0.231	ND	ND	ND

Table (5): The antifungal activity of some lactic acid bacterial strains against the fungal isolates (*A. flavus, A. parasiticus*) using the overlay assay.

Lactic acid bacteria	Fungal isolates	
	Aspergillus flavus	*Aspergillus parasiticus*
Lactobacillus acidophilus NRRL-B-4495	+	+
Lactobacillus acidophilus ATCC 20552	+	+
Lactobacillus acidophilus LA3	+	++
Lactobacillus acidophilus	+	++
Streptococcus thermophiles ATCC-3	++++	++
Streptococcus thermophiles	++	+
Lactobacillus plantarum ATCC 14917	+	++
Lactobacillus rhamnosus	+	+
Bifidobacterium lactis (Bb12)	+++	++
Bifidobacterium longum 15708	+	+
Bifidobacterium bifidium	++	+
Lactobacillus casei DSM 20011	+++	++

(+) = weak inhibition, (++) = medium inhibition, (+++) = strong inhibition, (++++) = very strong inhibition and (-) = no visible inhibition.

Table (6): Assessment of antifungal activity of lactic acid bacterial strains against *A. flavus* and *A. parasiticus* using the well diffusion method.

Strains of lactic acid bacteria	Inhibition zone of fungal strains by (mm)	
	Aspergillus flavus	*Aspergillus parasiticus*
Lactobacillus acidophilus NRRL-B-4495	31.0	32.0
Lactobacillus acidophilus ATCC 20552	32.0	34.0
Lactobacillus acidophilus LA3	35.0	31.0
Lactobacillus acidophilus	34.0	30.0
Streptococcus thermophilus ATCC-3	30.0	32.0
Streptococcus thermophilus	34.0	27.0
Lactobacillus plantarum ATCC 14917	31.0	31.0
Lactobacillus rhamnosus	32.0	22.0
Bifidobacterium lactis (Bb12)	38.0	35.0
Bifidobacterium longum 15708	37.0	33.0
Bifidobacterium bifidum	32.0	32.0
Lactobacillus casei DSM 20011	33.0	20.0

Table (7): Assessment of the antifungal activity of some lactic acid bacterial strains against *A. flavus* and *A. parasiticus* using the disc diffusion method.

Strain of lactic acid bacteria	Inhibition zone of fungal strains by (mm)	
	Aspergillus flavus	*Aspergillus parasiticus*
***Lactobacillus acidophilus* NRRL-B-4495**	**9.0**	**7.0**
***Lactobacillus acidophilus* ATCC 20552**	**7.0**	**6.5**
***Lactobacillus acidophilus* LA3**	**6.5**	**7.0**
Lactobacillus acidophilus	**7.5**	**7.0**
***Streptococcus thermophiles* ATCC-3**	**7.0**	**6.5**
Streptococcus thermophiles	**7.5**	**6.5**
***Lactobacillus plantarum* ATCC 14917**	**7.8**	**6.5**
Lactobacillus rhamnosus	**7.5**	**7.0**
***Bifidobacterium lactis*(Bb12)**	**8.0**	**7.5**
***Bifidobacterium longum* ATCC 15708**	**11.0**	**7.5**
Bifidobacterium bifidum	**8.0**	**7.0**
***Lactobacillus casei* DSM 20011**	**7.6**	**6.5**

the least inhibition zone observed was with *Lactobacillus acidophilus* LA3 against *A. flavus* (6.5mm). In the case of *A. parasiticus, Lactobacillus acidophilus* ATCC 20552**,** *Streptococcus thermophiles* ATCC-3**,** *S. thermophiles***,** *Lactobacillus plantarum* ATCC 14917 and *Lactobacillus casei* DSM 20011 showed the lowest inhibition zones (6.5 mm).

In the diffusion method all LAB inhibited the two aflatoxin-producing *Aspergilli* to varying degrees. It was observed that *B. lactis* (Bb12) exhibited the highest inhibition values in the well-diffusion assay (38.0 mm) against *A. flavus* and (35.0 mm) against *A. parasiticus*. While, *B .longum* ATCC 15708 exhibited the highest inhibition values in the disc-diffusion assay (11.0 mm) against *A. flavus* and (7.5 mm) against *A. parasiticus*, respectively. This may be due to the combined effect of lactic acid and bacteriocin.

Vanne *et al*. (2000) showed that the growth of toxigenic storage fungi was restricted by LAB *in vitro* and attributed this to the combined effect of lactic acid and bacteriocin. Motawee *et al*., (2011) reported that lactic acid bacteria (LAB) and *Bifidobaceria*, due in large part to their Generally Recognized As Safe (GRAS) status and their use as probiotics, are of particular interest for reducing the bioavailability of aflatoxin produced by *Aspergilli*.

Bolognani *et al*., (1997) reported the great efficiency of *Bb. longum* to bind carcinogens produced by aflatoxigenic fungi. A number of studies screened these microorganisms for the ability to bind to aflatoxin and have reported a wide range of genus, species and strain specific binding capacities (Haskard *et al*., 2001; Lee *et.al*., 2003; Hwang *et al*., 2005; Zinedine *et.al*., 2005; Hermandez *et al*., 2009). A previous study showed that the antagonistic activity exhibited by the LAB strains was completely destroyed by treatment with proteolytic enzymes (Adebayo and Aderiye, 2008). As the antifungal effect therefore could be attributed to the production of bacteriocins by the LAB strains.

On the other hands, some strains of LAB have been shown to inhibit both growth of mold and the production of mycotoxins (El- Shafei *et al*., 2010). The antagonistic action was produced by catalase-treated, neutralized, cell–free culture filtrates (CFNS), indicating that the antifungal activity was not due to the action of organic acids or hydrogen-peroxide produced by these LAB strains.

3.4.3. The agar dilution method

As shown from the results of (Table 8), it was observed that the highest inhibition percentage (70.3 %) was obtained by *Bifidobacterium lactis* (Bb12) against *A. flavus. Bifidobacterium bifidium* showed the highest inhibition (84.59 %) against *A. parasiticus.* While the lowest inhibition percentages (21.82 %) and (11.87 %) were obtained by *Lactobacillus acidophilus* LA3 against each of *A. flavus* and *A. parasiticus,* respectively.

The results showed that all *Lactobacillus* strains have antifungal activities in varying degrees against fungal isolates. The reduction in radial mycelial growth of fungi in culture PDA media with the cell-free supernatant of

Table (8): Assessment of the percentage of mycelial growth inhibition by agar dilution assay.

Strains of Lactic acid bacteria	Fungal isolates (%)	
	Aspergillus flavus	*Aspergillus parasiticus*
***Lactobacillus acidophilus* NRRL-B-4495**	**29.05**	**21.82**
***Lactobacillus acidophilus* ATCC 20552**	**45.42**	**37.88**
***Lactobacillus acidophilus* LA3**	**21.82**	**11.87**
Lactobacillus acidophilus	**41.25**	**71.74**
***Streptococcus thermophiles* ATCC-3**	**57.46**	**68.69**
Streptococcus thermophiles	**36.59**	**63.08**
***Lactobacillus plantarum* ATCC 14917**	**47.83**	**54.57**
Lactobacillus rhamnosus	**38.84**	**43.82**
***Bifidobacterium lactis*(Bb12)**	**70.30**	**82.66**
***Bifidobacterium longum* 15708**	**44.62**	**43.33**
Bifidobacterium bifidium	**34.51**	**84.59**
***Lactobacillus casei* DSM 20011**	**57.77**	**43.00**

Bifidobacterium strain (*Bifidobacterium lactis*) was higher than those with *lactobacillus* strains (*L. acidophilus* LA3 and *L. acidophilus* NRRL-B-4495). This may be due to the production of proteinaceous compounds such as bacteriocins or bacteriocin-like compounds beside organic acids, hydrogen peroxide, ethanol, diacetyl, acetaldehyde, carbon dioxide and reuterin against undesirable harmful microorganisms. They are useful in the fields of food preservation or safety, health care, and pharmaceutical applications. The inhibition activity of these substances has been reported to be strain-dependent.

Poltavska and Kovalenko (2012) reported that *Bifidobacterium* sp. 278 and *B. bifidum* 174 strains produced bacteriocins of wide spectrum of activity. Bolognani *et al*. (1997) reported the great efficiency of *Bb. longum* to bind carcinogens that produced by aflatoxigenic fungi. *Bifidobacterium* sp. *Bif.* 4 exerted a biostatic effect on the fungal growth due to the accumulation of lactic and other organic acids which lead to lowering the pH of the medium increasing the titratable acidity in the mixtures. *Bifidobacterium longum* (Bb-46) is a heterofermentative bacterium which produces acetic acid, ethanol, carbonyl compounds and CO2 (Tamime *et al*., 1995, Tratnik, 1998).

3.5. Determination of antifungal metabolites produced by the lactic acid bacterial (LAB) strains

3.5.1. Analysis of the organic acids by the High Performance Liquid Chromatography (HPLC)

The types and contents of organic acids in the supernatant of the twelve lactic acid bacteria were evaluated by reverse-phase High Performance Liquid Chromatography (HPLC). The results revealed that there were ten types of organic acids obtained in the supernatant of the LAB, including oxalic, maleic, acetic, citric, succinic, lactic, formic, propionic, butyric and mallic acids.

From the results in (Table 9), it was observed that the main organic acids produced in the studied strains were lactic and acetic acids. These acids were followed by succinic acid and propionic acids. As the highest organic acids produced by LAB were lactic acid (2.64 mg/ ml) and acetic acid (1.72 mg/ ml) after 48 hours respectively. These acids were followed by propionic acid (1.04 mg/ ml) after 48 hours and citric acid (0.74 mg/ ml).

L. rhamnosus was the highest strain in the production of lactic acid (2.64 mg/ ml) and acetic acid (1.72 mg/ ml) after 48 hours, respectively.

Stiles, (2002) indicated that *L. rhamnosus* VT1 also inhibited mold growth and it has been reported that lactic and acetic acids are the main organic acids involved in antimicrobial activity of *Lactobacillus* strains (Corsetti *et al*., 1998).

Plockova *et al*. (2001) reported that *L. rhamnosus* inhibits the growth of *Aspergillus*, *Penicillium* and *Fusarium.* Cabo *et al*. (2002) revealed that the synergistic effect of the acetic acid and the lactic acid produced were likely the main factor responsible for the antifungal properties of the selected bacteria. These results could explain some discrepancies in reports of the antifungal properties of lactic acid bacteria, since the role of acetic acid has not been considered in previous studies.

Table (9): The main organic acids produced in the supernatants of LAB strains.

Lactic acid bacterial strains	Organic acids (mg/ml)			
	Lactic acid	Acetic acid	Propionic acid	Citric acid
***Lactobacillus acidophilus* NRRL-B-4495**	**1.56**	**0.90**	**0.68**	**0.41**
***Lactobacillus acidophilus* ATCC 20552**	**2.00**	**0.80**	**0.50**	**0.49**
***Lactobacillus acidophilus* LA3**	**1.47**	**0.70**	**0.60**	**0.39**
Lactobacillus acidophilus	**1.21**	**0.92**	**0.65**	**0.74**
***Streptococcus thermopiles* ATCC-3**	**1.60**	**0.57**	**1.04**	**0.48**
Streptococcus thermophiles	**1.90**	**0.78**	**0.56**	**0.60**
***Lactobacillus plantarum* ATCC 14917**	**2.09**	**1.06**	**0.86**	**0.61**
Lactobacillus rhamnosus	**2.64**	**1.72**	**0.67**	**0.68**
Bifidobacterium lactis(Bb12)	**1.77**	**1.07**	**0.47**	**0.39**
Bifidobacterium longum	**1.35**	**0.81**	**0.53**	**0.46**
Bifidobacterium bifidium	**1.99**	**0.81**	**0.47**	**0.62**
***Lactobacillus casei* DSM 20011**	**1.87**	**0.92**	**0.48**	**0.51**

Additionally, Casal *et al*. (1996) showed that some acids, like acetic acid, can be transported across the cell membrane by inducible permeases. While inside the cell, the protonated acid dissociates into anions, which accumulate in the cytoplasm and cause acidification of the cytoplasm (Stratford and Eklund 2003), thereby causing loss of viability and cell destruction (Torino *et al*., 2001 Sathe *et al*., 2007). This fact would explain the differences in activity observed among the acids and the higher antifungal effect obtained at a pH beneath their pKa. The pKa value of the most common acids produced by lactic acid bacteria are below 5.0. The pKa of lactic and acetic acids is 3.8 and 4.7, respectively.

Doyle *et al*. (2002) reported the antifungal activity of acetic acid against *Aspergillus, Penicillium* and *Rhizopus* spp. And some strains of *Saccharomyces.* Lind *et al*. (2005) studied the effect of the three organic acids such as propionic acid, acetic and lactic acid and reported that propionic acid, followed by acetic and lactic acid are the most potent antifungal acids. Lactic acid has shown antifungal activity against the two fungal isolates each of *A. luchuensis* and *A. flavus*.

Conclusions

From the available data on the effect of LAB on mold growth and mycotoxin production, it would appear that LAB strains have the potential as biological control agents in foods to repress aflatoxinogenic mold growth through the production of several antifungal metabolites such as organic acids, bacteriocins, antibiotics and other products like ethanol, hydrogen peroxide, carbon dioxide, diacetyl, acetaldehyde etc. As it could be used as a natural, safe, effective, food-grade biocontrol agent and food industries for management of problems caused by aflatoxin-producing *Aspergilli*.

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النشاط المضاد لبعض سلالات بكتيريا حامض اللاكتيك ضد الفطريات المنتجه للأفلاتوكسين

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ملخص

أجريت هذه الدراسة للتحقق من النشاط المضاد للفطريات لبعض *سلالات بكتيريا حامض* اللاكتيك. تم اختبار سبعة سلالات من بكتيريا حامض اللاكتيك ضد أربعة عزلات فطرية تم عزلها من حبوب الذرة المصابة وتعريفها *Aspergillus flavus* و*Aspergillus parasiticus* و *Aspergillus niger* و*Rhizopus nigricans* عن طريق استخدام طريقة تثبيط الكتلة الحيوية للفطريات بمثابة اختبار قبل الفحص. تم الكشف عن العزلات الفطرية المنتجة للافلاتوكسين باستخدام طريقة الضوء والظلام، حيث أن *Aspergillus parasiticus* تنتج كلا من الأفلاتوكسين B والأفلاتوكسين G، بينما *Aspergillus flavus* ينتج الأفلاتوكسين B فقط. ثم تم فحص مجموع 12 سلالة من سلالات بكتيريا حامض اللاكتيك للنشاط المضاد للفطريات ضد الاثنان من الفطريات المنتجة للأفلاتوكسين ، وسلالات بكتيريا حامض اللاكتيك التى تم اختبارها هى (*Lactobacillus acidophilus* ATCC 20552 و *Lactobacillus acidophilus* NRRL-B-4495 , *Lactobacillus plantarum* ATCC 14917, *Lactobacillus acidophilus* LA3, *Lactobacillus casei* DSM 20011 ، *Lactobacillus rhamnosus* ، *Lactobacillus acidophilus* ، *Bifidobacterium bifidium* ، *Bifidobacterium longum* ATCC 15708،*Streptococcus thermophiles* و *Streptococcus thermophiles* ATCC-3. وقد تم اختبار النشاط المضاد للفطريات بثلاثة طرق. فى طريقة overlay وجد ان أعلى تثبيط لسلالتى الفطر *Aspergillus parasiticus* و *Aspergillus flavus* كان بواسطة الســـــــــلالة *S. Thermophiles ATCC-3*.بينما فى طريقة well diffusion وجد أن أعلى تثبيط لسلالة *Aspergillus flavus* كان بواسطة السلالات *B. lactis* (Bb12) وهى (38.0 mm) و 15708 *B .longum* وهى (37.0 mm) ، بينما كان أعلى تثبيط لفطر*Aspergillus parasiticus* كان بواسطة السلالة (*B. lactis* (Bb12 وهى (35.0 mm). فى حالة استخدام طريقة agar dilution وجد أن أعلى تثبيط لفطر *Aspergillus flavus* كان بواسطة الســــلالات (*B. lactis* (70.3% و (*L. casei* DSM20011 (57.77 % و (57.46 %) *S. thermophiles* ATCC-3 ، بينما اعلى تثبيط لفطر*Aspergillus parasiticus* كان بواسطة السلالات (*B. bifidium* (84.59 % و (*B. lactis* (82.66 % و *S. thermophiles* ATCC-3 (68.69 %) .

قد يكون هذا التثبيط بسبب انتاج المركبات المضادة للفطريات مثل الأحماض العضوية، فوق أكسيد الهيدروجين ،ثنائي الأسيتيل والمواد البروتينية. تم عند تحليل الأحماض العضوية عن طريق جهاز التحليل الكروماتوجرافي السائل ذو الأداء العالى تم ملاحظة أن أهم الأحماض العضوية الأساسية الناتجة هما حمضى اللاكتيك والأستيك.

المجلة العلمية لكلية الزراعة – جامعة القاهرة – المجلد (66) العدد الاول (يناير 2015) :91-104 .