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Suppression of Aflatoxin Production by Essential Oil and *Lactobacillus rhamnosus* and Molecular Detection of Aflatoxin Biosynthesis *aflR* Gene Expression in *Aspergillus flavus*

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ASPERGILLUS flavus produces many secondary metabolites including aflatoxin B_1, the most toxic and most potent carcinogenic natural compound that causes contamination on a variety of food and feed commodities. The aim of this study was to investigate the effect of *Lactobacillus rhamnosus*, the essential oils anise, caraway, fennel and potassium sorbate on growth rate, and aflatoxin B_1 accumulation, and also to measure expression of *aflR a regulatory* gene of aflatoxin biosynthesis pathway by using real time-q PCR technique. Our results indicated that all treatments exhibited potential antiaflatoxigenic and antifungal effect against *A.flavus.* Furthermore, *L. rhamnosus* after 7days of incubation, anise oil at concentration 100µl/100ml medium, and potassium sorbate at concentration 4.0µg /100ml medium at pH 6.0 showed a lowered transcription level of *aflR* gene as compared to control. The results indicated that *L. rhamnosus* may be useful as a natural biocontrol and strengthens the utilization of anise oil as an ideal antimicrobial against *A.flavus.* Visually, the results of real time-q PCR technique for *aflR* gene expression indicated that it was not a useful method for diagnosis of non-aflatoxin producing strains or vise viscera.

Keywords: *Aspergillus flavus, Lactobacillus rhamnosus,* Anise oil, *aflR* gene

Aspergillus flavus produces many secondary metabolites including aflatoxins, the most toxic and most potent carcinogenic natural compound that causes aflatoxicoses and induces cancer in mammals. In addition, it is a weak and opportunistic pathogen of many crops (corn, cotton, peanuts, and tree nuts) and contaminates them with aflatoxins. This ubiquitous mold not only reduces yield of agricultural crops but decreases the quality of the harvested grains. The economic losses and the health hazards of the mycotoxins produced by spoilage fungi are of great concern to the food industry (Gray & Bemiller, 2003 and Yu *et al*., 2005). Moreover, the diseases caused by aflatoxin consumption are loosely called aflatoxicoses. Acute aflatoxicoses results in death, chronic aflatoxicoses results in cancer, immune suppression, and other slow pathological conditions (Hsieh, 1988 , Calvo *et al.*, 2002 and Yu *et al*., 2005). Aflatoxin biosynthesis has

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been proposed to involve at least 23 enzymatic reaction and 25 genes or open reading frames (ORFs) representing a well defined aflatoxin pathway gene cluster (Bhatnagar *et al.,* 2003; Dutton, 1988 and Yabe & Nakajima, 2004). Among them, the *aflR* gene is known to encode a major transcriptional regulator of aflatoxin biosynthesis genes (Cary *et al.,* 2000; Woloshuk *et al.,* 1994 and Yu *et al.* (1996). AFLR binds to the consensus sequence 5'-TCGN_5CGR-3' Ehrlich *et al.,* (1999) found in the promoters of most, if not all, of the aflatoxin biosynthesis genes (Yu *et al.,* 2004 and Bhatnagar *et al.,* 2006). In recent years consumers have become more concerned about the processed food they buy and eat. Demands for natural, high quality preservative − free products that at the same time are safe and stable poses a challenge for the food industry, (Brul & Coote, 1999).Considerable pressure from consumers to reduce or eliminate chemically synthesized additives in their food has led to a renewal of scientific interest in natural substances (Nychas, 1995; Tuley De Silva, 1996 and Druvefors *et al.,* 2005). Essential oils (EOs) have been proven to be inhibitory against a wide range of food spoiling microorganism dependent upon their concentration, testing method, and active constituents (Paster *et al.,* 1995; Lis−Balch *et al.,* 1999 and Smith−palmer *et al.,* 2001). Biopreservation, the control of one organism by another, has received much attention (Magnusson *et al.,* 2003). Lactic Acid Bacteria (LAB) have a GRAS status (generally recognized as safe) status and it has been estimated that 25% of the European diet and 60% of the diet in many developing countries consist of fermented foods (Stiles, 1996). LAB are well known as starter cultures in the manufacture of dairy products (Carr *et al.,* 2002), so LAB have several potential applications. Using LAB to control mould growth could be an interesting alternative to physical and chemical methods because these bacteria have been reported to have strong antimicrobial properties. However, the antifungal activity of lactic strains remains to be elucidated. A limited number of reports have shown that a good selection of LAB could allow the control of mould growth and improve the shelf life of many fermented products and, therefore, reduce health risks due to exposure to mycotoxins (Gourama & Bullerman, 1995b and Dal Bello *et al.,* 2007). El-Nezami *et al.* (1998) and Gratz *et al,* (2006) demonstrated that the probiotic *Lactobacillus rhamnosus* strain GG is able to bind the potent hepatocarcinogenic B_1 (AFB_1). Also, Bueno *et al.* (2006) indicated that *L. casei* CRL 431 and *L. rhamnosus* CRL 1224 may be useful as potential biocontrol agent against *A. flavus*. The aim of this study was to investigate the effect of *Lactobacillus rhamnosus*, some essential oils and potassium sorbate on the biosynthesis of aflatoxin and expression of *aflR* gene.

Material and Methods

Isolation and identification of Aspergillus flavus

Aspergillus flavus isolates were obtained from different food sources such as peanut, maize flour, corn flour and wheat flour which were randomly collected from Qalubia Governorate Egypt "winter 2011". Pure isolates of *A. flavus* were

identified microscopically and cultured as previously described by Raper & Fennell (1965), Davise (1993), Moubasher (1993), Samson (2000) and Klich (2002).

Determination of fungal growth

The mycelia were separated from yeast extract sucrose (YES) broth by filterering through filter paper (Whatman No.4) and washed three times with distilled water, dried at 70°C till constant weight, cooled in a desiccator and then weighed.

Production of AFs by isolated A. flavus strains

The production of AFs by *A. flavus* strains on liquid media (YES) was investigated according to Singh *et al.* (1991).

Extraction, screening and detection of aflatoxins produced by A.flavus

50 ml culture filtrate was extracted twice with 100 ml chloroform in a separating funnel. The chloroform extracts (lower layer) were then filtered through anhydrous sodium sulphate, evaporated to dryness by rotary evaporator under vacuum at 40°C and stored at 0 °C for later chromatographic analysis.

Aflatoxins were determined using pre-coated TLC plates (Merck aluminium backed silica gel D 60 without fluorescent indicator) developed with chloroform: acetone: isopropanol: water (88:12:5:1 v/v). The intensity of the aflatoxin spots was measured with a fluorodensitometer (TLD-100 Vitatron) at an excitation wavelength of 365 nm and emission wavelength of 443 nm and by HPLC method. Aflatoxin extraction and quantification were carried out using standard procedures (AOAC, 1995; 2003). AFB_1, AFB_2, AFG_I and AFG_2 (Sigma Chemical Co., St Louis, MO, USA) were used as reference standards. Aflatoxins B_1, B_2 were detected as blue fluorescence and G_1, G_2 as yellow green fluorescence.

Quantitative estimation of aflatoxins

Aflatoxins were determined quantitatively according to AOAC (1995). Under U.V. light (365 nm), TLC plates were scanned using a densitometer to identify and measure sample peak areas by comparing with the standard spots. Emission was observed at 420-460 nm.

Preparation of inoculum and growth medium

The inoculum of *A. flavus* isolate No.66 was obtained by growing the mold at 30 ºC on slants of Sabouraud's glucose agar until well sporulated (7 days). Spores were harvested by adding 10 ml of sterilized aqueous solution of Tween-80 (0.05% v/v) to cultures and gently dislodging spores from conidiophores with an inoculation loop. The spore suspension was filtered through 4 layers of sterile cheesecloth to remove mycelial debris. The total spore count was of the suspension ($\approx 10^6$-10^7 spore ml^{-1}) was determined using a spread plate technique on Potato Dextrose Agar (PDA) plates. *Lactobacillus rhamnosus* was obtained from Cairo MIRCEN, Ain Shams Univesirty, Egypt. The inoculum of *L. rhamnosus* was obtained by growing isolates in 5 ml of (MRS, pH 6.5) at 37 º C

for 24 h. The concentration of viable cells in the inoculum ($1x10^7$cfu ml^{-1}) was determined by plate counts on Nutrient agar (NA) medium.

Detoxification effect of potassium sorbate

Potassium sorbate salts obtained from (El-Nasr Pharmaceutical Chemical Co., Egypt) as chemical preservative was added in 0.06, 0.12, 0.25, 0.5, 1.0, 2.0, 4.0, and 6.0 µg /100ml to YES medium at different pHs 4.0, 6.0, 8.0, and 10.0. After inoculating with 1.0 ml of *A. flavus* isolate No.66 (10^7 spores $m1^{-1}$) the flasks were incubated at 30°C ±1°C for 7 days. Mycelium dry weight, purification and detection of aflatoxin B_1 were then done as mentioned previously. Fungistatic and fungicide effects were determined according to the quantity of aflatoxin B_1 after incubation period by TLC and HPLC method (AOAC, 2003).

Detoxification effect of essential oils (EOs)

The essential oils (anise oil, caraway oil, and fennel oil) were purchased from Sekem Co. The crude oils were prepared by dissolving separately its requisite amount in 0.5 ml 5% tween-20 at concentrations (5, 10, 15, 20, 25 ,30 ,35 , 40 ,45 ,50 ,100 ,150 and 200 oil) µl/100 (YES) medium and then mixed with 100 ml of YES. Flasks were inoculated aseptically with 1.0 ml spore suspension (≈ 10^7 spores ml^{-1}) prepared in 0.1% tween-80 (Rosengaus *et al.*, 2000) and incubated at 30°C ±1°C for 7 days with triplicates. Mycelium dry weight, purification and detection of aflatoxin B1 were then done as mentioned previously. The nature of toxicity (fungistatic/fungicidal) of the essential oil was determined following Kumar *et al.* (2007). Fungal discs inhibited in growth in oil treated sets were re-inoculated on fresh medium after washing with distilled water and revival of their growth was observed to determine the fungicidal or fungistatic effect.

Detoxification effect of Lactobacillus rhamnosus

One ml spore suspension of *A. flavus* isolate No.66 (10^7 spores $m1^{-1}$) 7 days old culture at 30°C ±1°C and 1.0 ml *Lactobacillus rhamnosus* obtained from ($1x10^7$CFU ml^{-1}) (CFU: colony forming unit) grown on MRS broth for 24h at 37 ±1°C were added to 100 ml YES medium in 250 ml Erlenmeyer flasks. Both micro-organisms were added simultaneously and were incubated for (3, 5, 7, 10, 14, and 21) days at 30 °C ±1°C with triplicates. At the end of incubation period, final pH, mycelium dry weight, purification and detection of aflatoxin B_1 were then done as mentioned previously.

Total RNA extraction and RT-PCR

Total RNA was extracted from *A. flavus* isolate No.66 grown in YES medium. Mycelia were ground in liquid N_2 with sterile mortar and pestle. Total RNA was purified from the homogenized fungal mycelia using Fermentas Kit #K0731 protocol (Chomczynski & Sacchi, 1987) First strand cDNA was synthesized according to Fermentas Kits #K1621 protocol (Wiame *et al.*, 2000). *aflR* primer designed by Primer3 program and processed by Promega Germany. The glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was chosen as a system control for reverse transcription. The primer sets were *aflR* 5'-AAAAGTGCGATGCACCAAG-3' *aflR* 5' AACACTGACCCA CCTCTTCC-3' and *GAPDH5*'-CAAGGTCATCCA

TGACAACTTTG-3' and reverse. GAPDH5'- GTCCACCACCC TGTTGCTGTAG-3'. Amplication of the *aflr* gene generated a 636 bp RT-PCR product while GAPDH gene generated a 496 bp RT-PCR product. Quantitative detection of aflR expression was carried out by using real-time PCR (Stratagene Modul MX 3000P) according to Fermentas Kit, #K0221 protocol Maxima® SYBR Green/ROX qPCR Master Mix was added in 25µl for each reaction. Positive and negative control reactions were used to verify the results of the first strand cDNA synthesis steps. No template negative control (NTC) was important to assess for reagent contamination. The NTC reaction contained every reagent for the reverse transcription reaction except for RNA template. Data acquisition was performed during the annealing/extension step. Each PCR reaction was replicated three times and the experiment was repeated twice.

Statistical analysis

All experiments were carried out in triplicate and results represent mean ± standard error.

Result and Discussion

Isolation and screening of A.flavus isolates produced aflatoxin

In this study, twenty- seven isolates of *A.flavus* from 7 food samples were isolated and screened for their ability to produce aflatoxins on yeast extract sucrose medium. Seven isolates only of *A.flavus* recovered in this study were aflatoxigenic. These are No.13 from peanut 1 that produced 1800 µg/100ml medium aflatoxin B_1. Another two isolates of *A.flavus* were isolated from peanut 2. Isolate No.32 produced 800 µg/100ml medium AFB_1 and isolate No. 39, 1000 µg/100ml medium. Also, *A.flavus* isolate No.60 from corn flour produced AFB_2 (600 µg/100ml medium), *A. flavus* isolate No.62 and from wheat flour1produced AFB_1 600 µg/100ml medium. In addition, two isolate of *A. flavus* isolated from wheat flour1 produced aflatoxins, one of them was isolate No. 65 that produced 800 µg/100ml mediumAFB_2. The other was isolate No.66 which gave the highest value of AFB_1, 1900 µg/100ml medium. All other isolates of *A. flavus* did not produce aflatoxin. This result is in agreement with those reported by many others who reported that not all *A. flavus* are capable of producing aflatoxins (Koehler *et al.*, 1975 and Varma &Verma, 1987). Among all aflatoxins, Aflatoxin B_1 is the most abundant aflatoxin and is considered to be the most toxic. Colonization of food with aflatoxigenic *A. flavus* is of importance because of its potential to produce aflatoxins which are potent toxic, carcinogenic, mutagenic, immunosuppressive agents (Calvo *et al.*, 2002 and Krishnamurthy & Shashikala, 2006). Aflatoxin not only gives rise to cases of poisoning but is also associated with cancer (liver), kwashiorkor, and growth retardation among children (Gong *et al.*, 2004 and Tsugane, 2004). The correlation between aflatoxins and hepatocellular carcinoma was studied by Polychronaki *et al.* (2008).

Effect of potassium sorbate

In this study, our results in Table 1 indicated that pH 4 the optimum pH value for maximum antiaflatoxigenic effect of potassium sorbate against *A. flavus* isolate No.66 then followed in descending order by pH 6 >8>10 in all concentrations used. Potassium sorbate is more effective on aflatoxin production

more the pH, however pH is more effective on growth of *A. flavus*. Also, at pH 4 potassium sorbate shows fungicide effects, as well as inhibiting the production of AFB_1 of the tested isolate completely at concentrations 4.0, and 6.0 µg /100ml medium. Our results are similar to those reported by Marín *et al.* (2002a) who found that potassium sorbate was effective to inhibit some isolates from bakery products at pH 4.5. However, other studies showed that the addition of the same weak-organic acid salts in a sponge cake analogue of pH 6 appeared to be effective only at low water activity levels (Guynot *et al.*, 2002 and Marín *et al.*, 2002b). Guynot *et al.* (2004) indicate that potassium sorbate is a suitable preserving agent to inhibit growth of xerophilic fungi in bakery products of pH near 4.5 regardless water activity levels. For products of slightly higher, pH 5.5, the addition of this preservative must be combined with low water activity levels. The current accepted theory of weak acid preservative action suggests an inhibition via internal pH depression by directly inhibiting glycolysis enzymes (Lück, 1981 and Krebs *et al.*, 1983). Moreover, (López-Malo *et al.*, 2005) noticed that increasing antimicrobial concentration in the case of sodium benzoate and potassium sorbate had a dramatic effect of *A.flavus* germination time.

TABLE 1. Effect of potassium sorbate on aflatoxin on production and growth of *A. flavus* isolate No. 66.

Concentration (µg/100ml) medium	Final pH				Average of mycelial dry weight (mg/100ml)				Amount of aflatoxin (µg/100ml)			
	4	6	8	10	4	6	8	10	4	6	8	10
Control	5.0	5.5	7.3	9.0	1.33	1.63	1.54	1.45	1600	1980	1080	780
0.06	No growth fungicide effect	5.5	5.5	5.8	No growth fungicide effect	1.62	1.51	1.42	No growth fungicide effect	1700	1030	650
0.12		4.5	5.0	6.0		1.60	1.50	1.41		1460	1000	600
0.25		4.8	5.0	6.0		1.55	1.48	1.38		1340	960	525
0.5		5.0	5.0	6.0		1.50	1.43	1.35		1300	560	485
1.0		5.8	5.6	6.3		0.39	0.91	0.93		840	360	360
2.0		6.0	6.6	6.7		0.22	0.41	0.61		440	200	100
4.0		7.0	6.8	7.7		0.03	0.08	0.06		ND	160	ND
6.0		7.0	7.0	9.0		0.01	0.03	0.02		ND	100	ND

ND= Not Detected

Effect of essential oils

Use of chemicals as preservative has led to a number of environmental and health problems due to their residual toxicity, carcinogenicity, hormonal imbalance and spermatotoxicity (Pandey, 2003 and Kumar *et al.*, 2007). Plant essential oils have been used for hundreds of years for controlling fungus in food preservation (Bullerman *et al.*, 1977 and Kumar *et al.*, 2008). The results in Table 2 indicate that the essential oils tested had inhibitory effect on growth and aflatoxin accumulation by *A .flavus*. The data indicate that anise oil gave the best results with complete inhibition effect on both AFB_1 and growth of the experimental organism with fungicide at lower concentration and caraway and fennel which showed lower fungitoxicity. Anise oil is thus considered the best oil used in this work. Both mycelial biomass and aflatoxin B_1 production were recorded to decrease on increasing the concentration of the oils. It was found that reduction in mycelial growth causes reduction in aflatoxin. In the present study,

the concentration of 150µl/100ml medium of anise, caraway or fennel inhibited AFB_1 production by *A. flavus*. Similar results were reported by Kumar *et al.* (2008) who revealed that the same component of the oil is responsible for both activities. Moreover, these results are in agreement with the results obtained by Soliman & Badeaa (2002) Bluma *et al. (*2008) and Bluma & Etcheverry (2008) who demonstrated that the inhibitory effect of anise oil on *A. flavus*, *A. parasiticus* and aflatoxin production was higher than the remaining members of the *Umbellifereae* family like caraway and fennel. Also, many studies have revealed that some EOs of plants could potentially provide protection against aflatoxins especially AFB_1 (Webster *et al.*, 1996; Rasoli & Owlia, 2005; Agar *et al.*, 2005; Alpsoy *et al.*, 2009 and Alpsoy, 2010). In addition, essential oils such as anise and boldus could be safely used as a preservative material on some food because they stopped fungal growth and aflatoxin accumulation B_1 (AFB_1) (Soliman & Badeaa, 2002 and Bluma *et al.*, 2008). The EOs composition of plants varies significantly in different genera and species. A variation in fungitoxicity of tested plant EOs against toxigenic strain of *A. flavus* may be due to considerable variation in EO constituents (Pina-Vaz *et al.*, 2004 and Cavaleiro *et al.*, 2006). The effect of anise as fungicide is much greater than that produced by the other members of the same family; the difference in anethole concentration may explain the antifungal effect observed. Also, many investigators have demonstrated the fungistatic and fungicidal effects of anise, caraway and fennel essential oils against *A. flavus* and *A. parasiticus* (Farag, 1989 and Hasan, 1994 and . In addition, the physical nature of essential oils, that is, low molecular weight combined with pronounced lipophilic tendencies allow them to penetrate cell membrane more quickly than other substances (Pawar & Thaker, 2007).

TABLE 2. Effect of essential oils on aflatoxin production and growth of *A. flavus* isolate No.66.

Concentration µl/100ml medium	Final pH			Average of mycelial dry weight mg/100ml			Amount of aflatoxin µg/100ml		
	Anise	Carawy	fennel	Anise	Carawy	fennel	Anise	Carawy	Fennel
Control	5.1	5.0	5.5	1.65	1.62	1.66	2000	1960	1960
5	5.0	4.7	5.0	1.45	1.36	1.66	1700	1860	1660
10	5.0	4.8	5.0	1.44	1.10	1.63	1540	1820	1600
15	5.0	4.9	5.0	1.42	0.96	1.60	1500	1820	1560
20	5.0	5.8	6.0	1.40	0.82	0.45	1400	1800	1300
25	5.0	5.9	6.0	1.25	0.63	0.30	1360	1740	1000
30	5.0	5.8	6.0	1.23	0.42	0.22	1300	1700	800
35	5.0	6.0	5.0	1.30	0.38	0.18	800	1460	560
40	5.0	6.0	5.0	1.23	0.36	0.09	560	1300	400
50	5.0	5.0	5.0	1.18	0.12	0.03	160	1140	360
100	5.2	5.0	5.0	1.00	0.09	0.01	ND	ND	200
150	No. growth	No. growth	No. growth	No. growth	No. growth	No. growth	ND	ND	ND
200	No. growth	No. growth	No. growth	No. growth	No. growth	No. growth	ND	ND	ND

ND= Not Detected

Effect of Lactobacillus rahmnosus

Since LAB occur naturally in many food systems and they have been a part of the human diet for centuries, they can be regarded as safe organisms to consume. They have a great potential for extended use in biopreservation of both food and feed products. It would appear that lactic acid bacteria have the potential to be used as biological control agents in foods to prevent mould growth (Haskard *et al.,* 2001). Data presented in Table 3 indicated that *Lactobacillus rhamnosus* inhibited both aflatoxin production by 100% after 7 days and the growth of *A. flavus* isolate No.66 by 60.76% after 14 days of incubation compared to the control. It was apparent that the anti-aflatoxigenic activity of *L. rhamnosus* decreased with prolongation of incubation periods. Our result in agreement with Bueno *et al.* (2006) mycelia dry weight of *A. flavus* was reduced to 73 and 85% by using *L. casei* CRL 431 and *L. rhamnosus* CRL 1224, respectively. Previous study also reported *that L. rhamnosus* was able to remove up to 80% of aflatoxin-B from liquid media (El-Nezami *et al*., 1998). Moreover, the probiotic strain *Lactobacillus rhamnosus* strain GG (ATCC 53013) efficiently binds several mycotoxins, including aflatoxin B_1 (AFB_1) and aflatoxin M_1 (AFM_1), its hydroxylated metabolite, *in vitro* (El-Nezami *et al.,*1998; El-Nezami *et al.,* 2000; Pierides *et al.,* 2000 and El-Nezami *et al.,* 2002). Lactobacillus strains could remove more AFB_1 than *Pediococcus* and *Leuconostoc* strains. Five strains of *L. rhamnosus*, one strain of *L. lactis* and one strain of *L. casei* reduced AFB_1 by more than 20%. *L. rhamnosus* strain Lb50 reduced AFB_1 by 45% (Zinedine *et al.,* 2005). Bagherzadeh Kasmani *et al.* (2012) reported that *L.rhamnosus*TMU094 and *L. fermentum* were the most efficient species in AFB1 binding by 75.06 and 72.15%, respectively.

TABLE 3. Effect of *L. rhamnosus* on aflatoxin production and growth of *A. flavus* isolate No. 66 (Both *A.flavus* and LAB were inoculated simultaneously) on (YES).

Treatment	Incubation in (days)	Final pH	Average of mycelial dry weight (g/100ml)	Amount of aflatoxin (µg/100ml)
Control *(A.flavus)*	3	5.0	1.30	1820
	5	5.0	1.40	1920
	7	5.3	1.57	1980
	10	6.0	1.61	1700
	14	5.8	1.69	1560
	21	6.0	1.73	1200
Lb.rhamnosus +(A.flavus)	3	5.0	1.21	600
	5	5.0	1.23	100
	7	4.8	1.20	ND
	10	5.1	1.01	460
	14	5.7	1.01	800
	21	5.8	0.79	860

The table expresses the final mycelial dry weight after subtraction the bacterial dry weight of cultures parallely cultivated under the same conditions. ND= Not Detected.

Effect of Lactobacillus rahmnosus, anise oil, and potassium sorbate on aflR gene expression

Data shown in Fig. 1 and 2 (all treatments 7 days age) show a high transcription level of *aflR* gene compared to control. The highest transcription level observed with potassium sorbate 4.0µg /100ml medium at pH 6.0. In spite of *aflR* gene expressed no aflatoxin was detected in (YES) culture filtrates with these treatments. The basic assumption that determined the design of the experiment in this study was that *aflR* was the transcriptional regulator of the aflatoxin biosynthesis genes. If this was true, altered expression of *aflR* transcription should lead to altered transcription of the pathway genes. Further, if aflatoxin biosynthesis was regulated only by *aflR*, altered transcription of *aflR* should lead to altered timing and accumulation of aflatoxin and production of aflatoxin under nonconductive conditions. This hypothesis was based on previous research showing that *aflR* is required for the transcription of the pathway genes *nor-1* and *ver-1* in *A. flavus* (Payne *et al.,* 1993) and that an additional copy of *aflR* in *A. parasiticus* leads to increased aflatoxin production and elevated transcript accumulation of *nor-1*, *ver-1*, and *pksA* (Chang *et al.,* 1995). Additionally, Yu *et al.* (1996) showed that *A. nidulans aflR* was required for the transcription of the pathway genes leading to sterigmatocystin production. The present results indicate that *aflR* gene expression was found in all treatments. However, the treatments recorded (not detected) for AFB_1 in (YES) culture filtrate. A transformant of *A. parasiticus* containing an extra copy of the *aflR* gene did not overcome the inhibitory effect of a high temperature on aflatoxin formation (Chang *et al.,* 1995). Thus, although Liu & Chu, (1998) suggest that a high temperature suppresses aflatoxin formation by down regulating *aflR*, it is likely that, in addition to *aflR*, another factor(s) may also play a critical role in the temperature-induced regulation of aflatoxin biosynthesis. Expression of *aflR* by real-time RT-PCR or RT-PCR was also detected in *A. sojae* strains, which have been proven nonaflatoxigenic and *A. flavus* strains, which did not produce aflatoxin. It was thought that the reason for the lack of expression of *avnA*, *vbs*, *verB*, and *omtA* genes was a lower transcription level of the regulatory gene, *aflR*. However, it was possible that translation was not performed even if *aflR* mRNA was expressed slightly or that *aflR* was degraded (Chang, 2004; Scherm *et al.,* 2005 and Tominaga *et al.,* 2006). *A. flavus* 194A strain was described as a "false positive" on the basis of the lack of any correlation between the gene(s) expression profile, as assessed by the multiplex RT-PCR and the aflatoxin production phenotype (Degola *et al.,* 2007). Surprisingly enough, 16 AF biosynthesis genes analysed were readily transcribed in the 194A strain. However, non producers (Afla−) strains, in which most of the aflatoxin biosynthetic pathway is intact, have already been described (Ehrlich & Cotty, 2004) and many events (mutations, post-transcriptional or post-translational events) that allowed gene transcription but not aflatoxin production might be envisaged. In addition, other genes not belonging to the aflatoxin gene cluster might directly or indirectly control toxin production, as reported for *VeA* and *LexA* in *A. nidulans* and *A. parasiticus* (Cary *et al.,* 2006). O'Brian *et al.* (2007) reported that all the aflatoxin biosynthetic genes were much more highly expressed at 28 °C relative to 37°C. To our surprise expression of the pathway regulatory genes *aflR* and *aflS*, as well as *aflR* antisense, did not differ between the two temperatures. These data indicate that the failure of *A. flavus* to produce aflatoxin at 37°C is not due to lack of

transcription of *aflR* or *aflS*. One explanation is that *aflR* is nonfunctional at high temperatures. However, Watson *et al.* (1999) and Matsushima *et al.* (2001) revealed that the lack of a functional *aflR* was insufficient to explain the complete repression of *aflRs* and other aflatoxin related genes in *A. sojae.* The lack of aflatoxin production by *A. sojae* can be attributed, at least in part, to the premature termination defect in *aflRs,* which deletes the C- terminal transcription of aflatoxin activation domain that was critical for expression of aflatoxin biosynthetic genes (Takahashi *et al.,* 2002). Adjacent to the *aflR* gene in the aflatoxin gene cluster, *aflS*, a divergently transcribed gene, has been shown to be responsible of enhanced transcription of other genes in the cluster through its interaction with *aflR*, though its exact function was still unclear (Chang, 2003 and Yu & Keller, 2005). Clustered biosynthetic genes for fungal secondary metabolism were not only regulated by specific transcription factors, as a global epigenetic control mechanism might be conducted by genes, beyond the biosynthetic cluster, which were able to regulate multiple physiological processes and the response to environmental and nutritional factors such as temperature, pH, light, carbon and nitrogen sources (Georgianna & Payne, 2009). Accinelli *et al.* (2008) analyzed expression of five AF genes (*aflD, aflG, aflP, aflR, and aflS*) by RT-PCR. They did not find a correlation between gene expression profiles of aflatoxigenic *A. flavus* isolates and AFB_1 concentrations in the soil. Moreover, Jamali *et al.* (2013) purpose that a significant reduction in the expression of *aflR* gene in curcumin-exposed *A.parasiticus* is responsible in part not only for AFB1 inhibition by the fungus, but also for down regulating other genes studied. In contrast, Kong *et al.* (2010) reported that the mRNA abundances of *aflR* and *aflS* genes in control were 1.11±0.24 and 0.18±0.05, respectively, while in experiment group were 0.28±0.03 and 0.024±0.005, respectively indicating that *Bacillus megaterium* could suppress the expression of these two genes. The concentration of aflatoxins and the mRNA abundances of these two genes in the control experiment corresponded: the lower the expression of *aflR* gene and *aflS* gene, the lower the aflatoxin concentration detected. In addition, Sweeney *et al.* (2000) demonstrated that aflatoxin production monitored by thin layer chromatography was correlated with transcription of *aflR* and *aflQ* in *A. parasiticus* strain 439. Prieto *et al.* (1996) and Woloshuk *et al.* (1995) showed that *aflR* was sufficient to initiate gene transcription of early, mid, and late genes in the pathway, and that AflS enhances the transcription of early and mid aflatoxin pathway genes. Another possible explanation was that regulation of AF cluster gene expression was complex, and factors other than transcript levels of *aflR* and *aflS* were important in its regulation. *afls* transcript was thought to be dependent on *aflR* (Du *et al.,* 2007; Ehrlich *et al.,* 1999b and Price *et al.,* 2006).The expression of the majority aflatoxin biosynthetic genes including *aflR* and *aflS* of all strains varied with regarded to the aflatoxin-producing ability and the growth conditions (Scherm *et al.,* 2005). In addition, the possibility exists that some of the genes involved in aflatoxin and ST biosynthesis were located somewhere outside the gene clusters. The genetic control of aflatoxin biosynthesis in relation to primary metabolism and environmental stimuli was apparently beyond this defined gene cluster (Calvo *et al.,* 2002; Feng & Leonard, 1998; Flaherty & Payne, 1997 and Yu *et al.,* 2002). Flaherty & Payne (1997) concluded that transcriptional activation of the pathway was not the only requirement for the initiation of aflatoxin biosynthesis. The mechanism of this regulation was not known, but it was provided two lines of evidence that it was mediated through fungal metabolism. Under normal conditions, transcription of the pathway required specific

conducive conditions, for example, the presence of simple sugars. If the regulation of pathway transcription was bypassed by the constitutive transcription of *aflR*, the induction profile for aflatoxin was the same as in wild-type strains. This argues that additional medium-dependent factors were required for initiation of aflatoxin biosynthesis. The second line of evidence was the addition of a single compound affects the biosynthesis of aflatoxin, and we now known that it was not at the level of *aflR* transcription. Several studies were suggested that the physiology of the fungus played an important role in the regulation of aflatoxin biosynthesis (Buchanan *et al.*, 1987; Buchanan & Stahl, 1984 and Shih & Marth, 1974). Recent evidence for the complex regulation by media was supported by the observation in *A. niger* that intracellular cyclic AMP levels were associated with the initial sucrose levels in the media (Gradisnik-Grapulin & Legisa, 1997). Thus the regulation of aflatoxin biosynthesis was more complicated than previously considered and did not solely involve the transcription activation of the pathway. Identification of all of the genes and global regulators involved in and related to aflatoxin biosynthesis in the fungal system is a daunting challenge. *A. flavus* genomics and microarray technologies (Yu *et al.*, 2002 and O'Brian *et al.*, 2003) will provide a new avenue for deciphering such mechanisms and unraveling these regulatory elements governing aflatoxin biosynthesis.

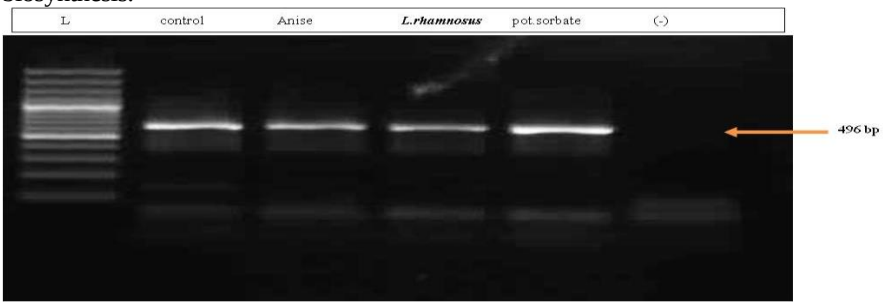

Fig.1. Agarose gel electrophoresis of *GAPDH* gene transcript during incubation of *L. rahmnosus*, anise oil, and potassium sorbate with *A. flavus* as indused by real time – q PCR.

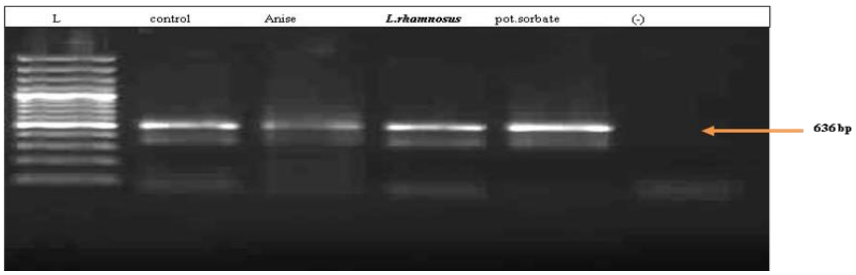

Fig.2. Agarose gel electrophoresis of *aflR* gene transcript during incubation of *L. rahmnosus*, anise oil, and potassium sorbate with *A. flavus* as indused by real time – q PCR.

Control= mycelium of *A. flavus* harvested after 3days of incubation at 30°C.
(-) = Negative control (contains all components for real time-q PCR except the sample).
L = 100:3000 bp standards

There is no relation between the growth of *A. flavus* and production of aflatoxins in spite of the presence of *aflR* gene in some strains of *Aspergillus* which not secretes aflatoxins *i. e., A. sojae* used in Japanese foods. These results agreed with the results of Jorgensen (2007) who reported that mold strain belong to the species *A. oryzae*, *A. sojae* are highly valued as Koji molds in the traditional preparation of fermented foods and as protein production hosts in modern industrial processes. As close relatives of aflatoxin-producing wild molds, koji molds possess an aflatoxin gene homolog cluster. Some strains identified as *A. oryzae* and *A. sojae* have been implicated in aflatoxin production. Identification of a strain as *A. oryzae* or *A. sojae* is no guarantee of its inability to produce aflatoxins or other toxic metabolites. Toxigenic potential must be determined specifically for individual strains. The species taxa, *A. oryzae* and *A. sojae*, are currently conserved by societal issues.

Clustered biosynthetic genes for fungal secondary metabolism were not only regulated by specific transcription factors, as a global epigenetic control mechanism might be conducted by genes, beyond the biosynthetic cluster, which were able to regulate multiple physiological processes and the response to environmental and nutritional factors such as temperature, pH, light, carbon and nitrogen sources (Georgianna & Payne, 2009).

Conclusion

The high anti-fungitoxic and antifungal effect exhibited by EO in the present study strengthens its utilization as an ideal antimicrobial for food preservation compared to the traditional chemical preservatives. *L.rhamnosus* has great potential biocontrol activity against the decay and aflatoxin biosynthesis caused by *A.flavus*. This potential may extend to use as a natural biopreservatives of both food and feed products. Our results obtained from real time-q PCR technique for *aflR* gene expression indicated that it was inappropriate method for diagnosis non-aflatoxin producing strains or vise viscera. Further genetic studies are needed to evaluate the role of each genes internal and external AF biosynthesis cluster genes during different phases of *A. flavus* development and aflatoxin biosynthesis and different detoxification treatments.

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تثبيط انتاج الافلاتوكسين بواسطة الزيوت الطبيعية و *L.rhamnosus* والاكتشاف الجزيئي للتخليق الحيوي للافلاتوكسين لجين *aflR* في فطر *A. flavus*

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ينتج فطر *Aspergillus flavus* العديد من نواتج ايضية ثانوية تشمل الافلاتوكسين B1 الاكثر سمية واكفر المركبات المسببة للسرطان والملوثة للاغذية والاعلاف .

الهدف من هذه الدراسة هو تحري تاثير *Lactobacillus rhamnosus* , وتاثير بعض الزيوت الطبيعية للينسون والكراوية والشمر وكذلك سوربات البوتاسيوم علي معدل النمو , وتراكم الافلاتوكسين B1 وكذلك الجين *aflR* المسئول عن مسار التخليق الحيوي للافلاتوكسين B1 باستخدام طريقة real time – q PCR.

دلت النتائج علي ان المعاملات اظهرت تاثير مضاد لنمو الفطر *A. flavus* . *L. rhamnosus* بعد 7 ايام تحضين مع *A. flavus* , وزيت الينسون بتركيز 100 µl لكل 100 مللي ميديا , وسوربات البوتاسيوم بتركيز 4 µg لكل100 مللي ميديا عند 6 pH , اظهرت مستوي نسخ قليل لجين *aflR* مقارنة بالكنترول. بينت النتائج ان *L. rhamnosus* قد تنفع في المكافحة الحيوية الطبيعية – وكذلك استخدام الينسون كمضاد ميكروبي مثالي ضد *A. flavus* .