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Anti-aflatoxigenic Effect of Some *Lactobacillus* Species on *Aspergillus flavus* by Using Real – Time - q PCR

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THIS STUDY aimed to the potency of two species of *Lactobacillus* (LAB) *i.e. Lactobacillus rhamnosus, L.bulgaricus* and *Streptococcus thermophilus* in inhibition of *A. flavus* growth, production of aflatoxin $B_1$and expression of *aflR* gene of aflatoxin biosynthesis pathway by using real time-q PCR technique. The trail was designed in two variations challenging the fungi with seven treatments of LAB species and S. *thermophilus*, before and at same time (simultaneous) funga linoculation. The results indicated that all treatments exhibited potential antiaflatoxigenic and antifungal effect against *A. flavus.* After 72h of incubation aflatoxin B_1 not detected with some species treatments compared to control which produced aflatoxin B_1 1840±20µg /100 ml medium. Furthermore, the results showed that detection of transcription level of *aflR* gene was not correlated to the actual toxicity of each treatment. The mRNA abundances of *aflR* gene in control was 1± 0.11, while in treatments with (*L. rhamnosus; S. thermophilus; L. rhamnosus & S. thermophilus; L. bulgaricus & L. rhamnosus; L.bulgaricus & S. thermophilus* and *L. rhamnosus & L. bulgaricus & S. thermophilus)* were 3.567± 0.25, 1.564± 0.13, 0.421± 0.05, 0.767±0. 06,0.585± 0.05 and 1.498± 0.12, respectively. These differences in gene expression profiles suggested that there was specificity between gene response and treatment. Finally, the results of real time-q PCR technique for *aflR* gene expression indicated that it was in appropriate method for diagnosis aflatoxin producing and non-producing strains.

Keywords: *Aspergillus flavus, Lactobacillus rhamnosus, Lactobacillus bulgaricus, Streptococcus thermophilus, aflR* gene.

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Aspergillus flavus (*A. flavus)* produces aflatoxins, the most potent naturally occurring toxic and hepatocarcinogenic compounds (Squire, 1981). Aflatoxin contamination in food and feed seriously affects human and animal health (Cary *et al.,* 2011). Outbreaks of aflatoxin poisoning frequently occur and a large percentage of hepatocellular carcinoma cases worldwide are estimated to be attributable to aflatoxin exposure (Wild & Turner, 2002 and Liu & Wu, 2010). *A. flavus* and *A. parasiticus* have complex pathways in biosynthesis of aflatoxins. Enzymes and regulatory proteins for aflatoxin synthesis in these two fungi are encoded by more than 25 clustered genes in a 70-kb region (Yu *et al*., 2004 and Ehrlich *et al*., 2005). A positive regulatory gene, *aflR*, encoding a sequence-specific zinc finger DNA-binding protein, is required for transcriptional activation of most, if not all, of the aflatoxin structural genes (Bhatnagar *et al*., 2006). However, to date, there are few practical methods for preventing aflatoxin contamination, and it is therefore critical to develop effective methods for prevention. A limited number of reports have shown that a good selection of Lactic Acid Bacteria (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.* (1998a) and Gratz *et al.* (2006) demonstrated that the probiotic *Lactobacillus rhamnosus* (*L. rhamnosus*) strain GG is able to bind the potent hepatocarcinogenic aflatoxin B_1 (AFB_1). In addition, Sarimehmetoğlu & Küplülü (2004) and Ayoub *et al.* 2011) reported that both *Lactobacillus bulgaricus* (*L. bulgaricus*) and *Streptococcus thermophilus* (*S. thermophilus*) have binding abilities to aflatoxin M_1 (AFM_1) from food and feed. Therefore, LAB could be used as a biological agent for AFM1 reduction. 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 *L. rhamnosus*, *L. bulgaricus* and *S. thermophilus* on the biosynthesis of aflatoxin and expression of *aflR* gene.

Material and Methods

Isolation and identification of Aspergillus flavus

A. flavus isolates were isolated from different food sources such as peanut, maize flour, corn flour and wheat flour which were randomly collected from Qalubia Governorate, Egypt "winter 2012". Pure isolates of *A. flavus* were identified microscopically and culture as previously described by Raper & Fennell (1965) Davise (1993), Moubasher (1993) and Klich (2002). The ability of aflatoxins production by *A. flavus* strains using liquid media (YES) was investigated according to Singh *et al*. (1991).

Extraction, screening and quantitative estimation of aflatoxins produced by A. flavus

Fifty ml culture filtrate was extracted twice with 100 ml chloroform in 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). AFB1, AFB2, AFGI and AFG2 (Sigma Chemical Co., St Louis, MO, USA) were used as reference standards. Aflatoxins were determined quantitatively according to AOAC (1995). Under U.V. light (365 nm) TLC plates were scanned with the help of densitometer. Densitometer as well as standard peaks determined identified sample peak area comparing with the standard spots. The emission observed at 420-460nm.

Determination of fungal growth

The mycelia were separated from yeast extract sucrose (YES) (2% Yeast extract - 20% Sucrose) broth by filtered through filter paper Whatman No.4 and washed three times with distilled water, dried at 70°C till constant weight, cooled in a desiccator's then weight (Clements, 1968 and Coallier-Ascah & Idziak,1985).

Preparation of inoculum and growth medium

The inoculum of *A. flavus* isolate No.66 was obtained by growing the mold at 30ºC on slants of Sabouraud's glucose agar (Difco Laboratories, Detroit, Mich) until well sporulated (7 days). Spores were harvested by adding 10 ml of sterilized aqueous solution of Tween-80 (Merck, Germany) (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 (Difco Laboratories , Detroit, Mich) plates. Pure culture of lactic acid bacteria *(L. rhamnosus, L. bulgaricus* and *S. thermophilus*) were obtained from Cairo MIRCEN, Ain Shams Univesirty, Egypt. The inoculum of bacteria were obtained by growing isolates in 5 ml of (de Man, Rogosa and Sharpe (MRS), pH 6.5) (Sigma-Aldrich Chemie GmbH) at 37 º C for 24 hr. The concentration of viable cells in the inoculum ($1x10^7$cfu ml^{-1}) (CFU: colony forming unit)was determined by plate counts on Nutrient agar (Difco Laboratories Detroit, Mich) medium) Pulusani *et al.*, (1979).

Experiment design of detoxification effect of lactic acid bacteria (LAB)

Two variation of treatment were conducted as follows: group-1, lactic acid bacteria were grown first for 24hr and then inoculated by *A. flavus* afterwards, group-2, both *A. flavus* and lactic acid bacteria were inoculated at the same time simultaneously. One ml spore suspension of *A. flavus* and 1.0 ml of LAB using for the following treatments: (*L. rhamnosus, L. bulgaricus, S.*

thermophilus, L. rhamnosus & L. bulgaricus, L. rhamnosus & S. thermophilus, L. bulgaricus & S. thermophilus and L. rhamnosus & L. bulgaricus & S. thermophilus) All of treatments were incubated for (3, 5, 7, 10, 14, and 21) days at 30ºC 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 Kites #K0731 protocol,USA) (Chomczynski & Sacchi, 1987 and Boom *et al.*, 1990). First strand cDNA was synthesis according to (Fermentas Kites #K1621 protoco, USA) (Wiame *et al.*, 2000). *aflR* primer designed by primer 3 program and processed by Promega Germany. The glyceraldehydes-3phosphate dehydrogenase (GAPDH) was chosen as a system control for reverse transcription. The primer sets were *aflR* forward 5'-AAAAGTGCGATGCACCAAG-3' *&* *aflR* reverse 5'-AACACTGACCCACCTCTTCC-3' and *GAPDH* forward 5'-CAAGGTCATCCATGACAACTTTG-3' *&* *GAPDH* reverse 5'-GTCCACCACCCTGTTGCTGTAG-3'. *aflR* primer generated 636 bp RT-PCR product while *GAPDH* primer generate 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 Kites, #K0221 protocol, USA (O'Brian *et al.*, 2003). Maxima® SYBR Green/ROX qPCR Master Mix was added in 25µl for each reaction. Positive and negative control reactions should be 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 (Chang, 2003). 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 YES medium. Seven isolates only of *A. flavus* recovered in this study were aflatoxigenic. These are No.13 from peanut 1 produced aflatoxin B_1 1800 µg/100 ml medium. Another two isolates of *A. flavus* were isolated from peanut 2, isolate No.32 produced AFB_1 800 µg/100 ml medium and isolate No. 39 1000 µg/100 ml

medium. Also, *A. flavus* isolate No.60 from corn flour AFB_2 600 µg/100 ml medium, *A. flavus* isolate No.62 and from wheat flour1produced AFB_1 600 µg/100 ml medium. In addition, two isolate of *A. flavus* were isolated from wheat flour1 produced aflatoxins, one of them was isolate No. 65 produced AFB_2 800 µg/100 ml medium. The other was isolate No.66 which gave the highest value of AFB_1 1900 µg/100 ml medium. All other isolates of *A. flavus* cannot produce aflatoxin. This result is in agreement with those reported by many others who reported that not all *A. flavus* strains 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 liver cancer 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).

The influence of lactic acid bacteria on the growth and the aflatoxin B_1 production by A. flavus No. 66

The interaction between all LAB species with different time of inoculation gave nearly the same growth pattern on *A. flavus.* All treatments can inhibit the growth of *A. flavus* over 21 days compared with control (1.50 and 1.51gm/100 ml) as can be seen on Tables 1and 2, respectively. The growth interaction between *A. flavus* with two different variation of incubation gave nearly the same respond on aflatoxin B_1 production by *A. flavus*.

Overall results as can be seen on Tables 1 and 2 indicated that all lactic acid bacteria species gave a reduction on aflatoxin B_1 production by *A. flavus* compared with control. The highest antiaflatoxigenic activity was achieved by treatment with *L. rhamnosus, S. thermophilus,* and (*L. rhamnosus & S. thermophilus*) (Table 1) and (*L. bulgaricus & L. rhamnosus*), (*L. bulgaricus & S. thermophilus*) and (*L. bulgaricus & L. rhamnosus & S. thermophilus*) (Table 2) with total inhibition to aflatoxin B_1 production after 3 days of incubation, while control produced up to 1860 and 1820 µg/100 ml, respectively. It was obviously observed that the antiaflatoxigenic activity reduced with prolongation of incubation periods in all inoculation treatments.

TABLE 1. Effect of LAB on aflatoxin production and growth of *A. flavus* (The fungus inoculated after LAB by 24 hr).

Treatment	Incubation In (days)	Final pH	Mycelial dry weight (g/100ml)	Aflatoxin (µg/100ml)
Control *(A. flavus)*	3	5.0	1.35	1860
	5	4.8	1.38	1900
	7	5.1	1.42	1960
	10	5.1	1.45	1600
	14	6.0	1.47	1200
	21	6.5	1.50	1000
L. rhamnosus +(A.flavus)	3	5.3	1.30	ND*
	5	4.9	1.32	ND*
	7	5.1	1.33	200
	10	5.3	0.86	600
	14	6.0	0.81	600
	21	6.0	0.71	850
L. bulgaricus +(A. flavus)	3	4.5	1.31	460
	5	4.9	1.32	600
	7	5.2	1.40	1060
	10	5.2	0.83	1080
	14	5.8	0.76	1090
	21	6.0	0.69	800
S. thermophilus +(A. flavus)	3	5.2	1.23	ND*
	5	5.1	1.30	200
	7	5.5	1.28	300
	10	5.7	1.01	1200
	14	6.2	0.92	1200
	21	6.8	0.68	850
L. bulgaricus & L. rhamnosus+ (A. flavus)	3	4.8	1.23	200
	5	4.9	1.30	400
	7	5.1	1.31	1000
	10	5.4	0.91	740
	14	6.1	0.83	840
	21	6.1	0.71	560
L.bulgaricus ,S.thermophilus+ (A.flavus)	3	4.8	1.24	200
	5	4.8	1.34	240
	7	5.4	1.31	740
	10	5.8	0.89	1060
	14	5.9	0.69	960
	21	6.3	0.70	560
L.rhamnosus ,S.thermophilus + (A.flavus)	3	4.6	1.10	ND*
	5	4.5	1.21	200
	7	4.7	1.23	400
	10	5.2	0.79	1000
	14	6.1	0.71	1040
	21	6.2	0.70	520
L.bulgaricus&, L.rhamnosus& S.thermophilus) + (A.flavus)	3	4.8	1.11	240
	5	4.8	1.20	360
	7	5.1	1.15	600
	10	5.1	1.03	1050
	14	6.1	0.77	1100
	21	5.0	0.70	360

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

TABLE 2. effect of lab on aflatoxin production and growth of *a. flavus* (simultaneously inoculation).

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	1.43	1980
	10	6.0	1.46	1700
	14	5.8	1.48	1560
	21	6.0	1.51	1200
L.rhamnosus +(A.flavus)	3	5.0	1.21	600
	5	5.0	1.23	100
	7	4.8	1.20	ND*
	10	5.8	0.91	460
	14	5.0	0.83	800
	21	6.0	0.70	860
L.bulgaricus +(A.flavus)	3	4.9	1.30	600
	5	5.0	1.33	500
	7	4.9	1.20	100
	10	5.9	0.83	680
	14	5.5	0.75	780
	21	6.0	0.68	1040
S.thermophilus +(A.flavus)	3	4.8	1.30	600
	5	5.0	1.22	150
	7	4.6	1.21	ND*
	10	6.0	0.98	800
	14	5.6	0.83	840
	21	6.1	0.70	690
L.bulgaricus &L.rhamnosus+ (A.flavus)	3	4.9	1.20	ND*
	5	5.0	1.13	1500
	7	4.7	1.11	1160
	10	5.8	1.05	1020
	14	6.0	0.81	1020
	21	6.3	0.72	1040
L.bulgaricus ,S.thermophilus+ (A.flavus)	3	4.8	1.20	ND*
	5	5.0	1.23	440
	7	5.1	1.24	500
	10	5.7	1.10	820
	14	5.7	0.79	1080
	21	6.3	0.68	1080
L.rhamnosus ,S.thermophilus + (A.flavus)	3	6.0	1.12	400
	5	5.0	1.21	740
	7	5.1	1.22	960
	10	5.9	0.90	600
	14	5.7	0.80	1040
	21	6.5	0.70	1040
L.bulgaricus&, L.rhamnosus& S.thermophilus +(A.flavus)	3	4.7	1.02	ND*
	5	5.0	1.04	500
	7	5.0	1.10	420
	10	6.0	1.09	800
	14	5.6	0.81	900
	21	6.0	0.71	1100

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

On conclusion of the above results, through the first 3 days of growth there is a competition between *A. flavus* and species of lactic acid bacteria used, in addition these species of LAB secrets metabolites affects and retards the growth of *A. flavus*. The effect causing reduced AFL in the presence of LAB is due to the binding by bacteriocins from metabolites of LAB (binding means active uptake) and this is a type of bioprocesses to get rid of aflatoxins , as clarified by Oluwafemi *et al*. (2010) reported that combined cultures gave the same results as individual cultures for lactic acid bacteria detoxification. Who conclude that each bacterial strain is releasing proteins that have an antagonistic effect on the other strain. Bacteriocins have strong toxin-binding ability. It is worthy to mentioned that Sezer *et al*. (2013) reported that the mixture of liquid culture, pellet, and bacteriocin of *L. lactis* had the strongest effect (59%) in group mix-sub, followed by liquid culture alone, pellet alone, and bacteriocin.

Our result in agreement with EL-Gendy & Marth (1981) who investigated the interaction between *A. parasiticus* and *Lactobacillus casei*. They suggested that such reduction was due to some nutritional change in the medium after the growth of *L. casei* and *A. flavus* also affected the metabolism and shape of lactic acid bacteria cells, which became elongated. Aryantha & Arina (2007) found that *L. delbrueckii, L. fermentum* and *L. plantarum* are potential to inhibit fungal growth and reduce aflatoxin-B produced by *A. flavus.*

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). In addition, Bueno *et al.* 2006) tested the ability of lactic acid bacteria and *Saccharomyces cerevisiae* to remove aflatoxin from liquid medium. The results revealed that AFB_1 binding to microorganisms was a rapid process. Haskard *et al.* (2001) stated that a high concentration of lactic acid in the medium induced the formation of attachment sites in lactic acid bacteria cell wall. They noticed also continued incubation of the culture resulted in an apparent inactivation of the inhibitor.

The influence of LAB on aflR gene expression responsible for regulating aflatoxin biosynthesis:

After 72h of cultivation, the aflatoxin concentration of the control in the two variation treatments was 1860 & 1820 (µg/100ml), respectively. Whereas in the following treatments (*L. rhamnosus; S. thermophilus; L. rhamnosus & S. thermophilus; L. bulgaricus & L. rhamnosus; L. bulgaricus & S. thermophilus; L. rhamnosus & L. bulgaricus & S. thermophilus)* grow in companion to *A. flavus*, aflatoxin B_1 were not detected. As shown in Fig. 1 moderate (Fig. 2 level of GAPDH transcription Compared to Control> Moreover, as shown in Fig. 2 unexpected high level of *aflR* transcription compared to control.The results showed that detection of transcription level of *aflR* gene was not correlated to the actual toxicity of each treatment. The mRNA abundances of *aflR* gene in control was 1± 0.11, while in experiments were 3.567± 0.25, 1.564± 0.13, 0.421± 0.05, 0.767±0. 06, 0.585± 0.05 and 1.498± 0.12, respectively. Finally, these differences in gene expression profiles by different treatments further suggest that there was specificity between gene response and treatment. 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* was 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) shown that the *A. nidulans aflR* was required for the transcription of the pathway genes leading to sterigmatocystin production.

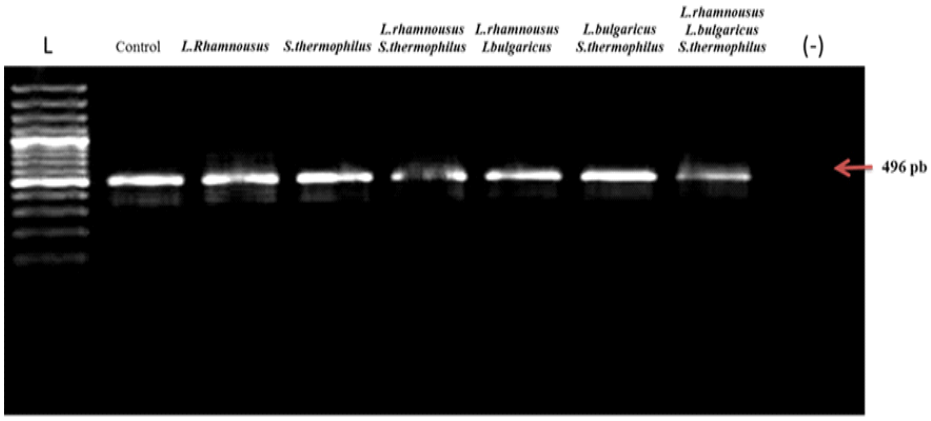

Fig.1: Agarose gel electrophoresis of *GAPDH* gene transcript during incubation of LAB with *A. flavus* as indused by real time – q PCR.

Fig. 2. Agarose gel electrophoresis of *aflR* gene transcript during incubation of LAB with *A. flavus* as indused by real time – q PCR.

L = 100:3000 bp
Control= Mycelium of *A. flavus* harvested after 3days of incubation at 30^{o}C
(-) = Negative control (contains all components for real time-qPCR except the sample).

The present results indicated that *aflR* gene expression was found in all treatments. A transcription 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 Lui & Chu (1998) suggest that a high temperature suppresses aflatoxin formation by down regulating AflR primer, 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* gene by real-time RT-PCR was also detected in *A. sojae* strains, which have been proven non aflatoxigenic 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* gene was expressed slightly or AflR primer 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). 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). However, Watson *et al.* (1999) and Matsushima *et al.* (2001) revealed that the lack of a functional AflRs was insufficient to explain the complete repression of *aflRs* and other aflatoxin related genes in *A. sojae*.

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 belongs 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).

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 AFB_1 inhibition by the fungus, but also for down regulating other genes studied. 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.,* 1999 and Price *et al.,* 2006).

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.

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 & Thomas, 1998; Flaherty and 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, thus the regulation of aflatoxin biosynthesis was more complicated than previously considered and did not solely involve the transcription activation of the pathway.

Conclusion

The three species of LAB used in these study exhibited potential biological activity against aflatoxin biosynthesis caused by *A. flavus*. The results of *aflR* gene obtained from real time q PCR indicated that 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. 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. The results of *aflR* gene obtained from real time q PCR needed further genetic studies.

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تاثيربعض انواع *Lactobacillus* على جين *aflR* في *Aspergillus flavus* باستخدام طريقة Real –Time – q PCR

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هذه الدراسة تهدف لقياس جهد نوعين من *اللاكتوباسللس* هما. *L , bulgaricus L, rhamnosus* وكذلك *Streptococcus thermophilus* في تثبيط نمو فطر *A flavus*.وانتاج الافلاتوكسين B1 وكذلك مسارالتخليق الحيوي لجين *aflR* باستخدام طريقة Real – Time – q PCR .

, كما .*A flavus*اظهرت النتائج ان كل المعاملات لها تاثير مضاد لنمو فطر. بعد 72 ساعة تحضين مقارنة B1 اظهرت النتائج اختفاء تواجد الافلاتوكسين *aflR* 1840 . بينت النتائج ايضا ان نسخ جين µg/100 mlبالكنترول الذي ينتج في mRNA في *aflR* لم يرتبط باكتمال السمية في كل معاملة. تراكم جين *L.rhamnosus ;* الكنترول كان 1±0.11 , بينما في المعاملات مع *S.thermophilus ; L.rhamnosus & S.thermophilus ; L.bulgaricus & L.rhamnosus ; L.bulgaricus & S.thermophilus and L.rhamnosus & L.bulgaricus & S.thermophilus* كانت ±3.567 0.25and , 0.13 ±1.564 , 0.05±0.421 , 0.0±0.767 . 06 , 0.05 ±0.585 بالتتالي . هذه الاختلافات في ملف التعبير الجيني تدل على ان هناك 0.12 ±1.498 Real- Time – تخصصية بين استجابة الجين والمعاملة . في النهاية نتائج طريقة بينت ان هناك طريقة لتشخيص العزلات المفرزه والغير مفرزه للسموم.q PCR