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The inhibitory effect of lactic acid bacteria on aflatoxin production and expression of aflR gene in *Aspergillus parasiticus*

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Abstract

Recent studies indicated the inhibitory effects of *lactic acid bacteria* (*LAB*) strains isolated from different origins on both fungal growth and aflatoxin production. This study aimed to investigate the inhibitory effect of *Lactobacillus plantarum* and *L. delbrueckii* subsp. *Lactis* on *Aspergillus parasiticus* ATCC15517 growth. Quantification of aflatoxin was performed using high-performance liquid chromatography technique. Quantitative changes in the expression of the aflR gene were analyzed by measuring the cognate aflR mRNA level with quantitative real-time reverse-transcription polymerase chain reaction. Our results demonstrated the inhibitory effect of *LAB* on *A. parasiticus* growth at 2×10^3 cfu/ml of *L. plantarum* and *L. delbrueckii* subsp. *Lactis*. Aflatoxin G2 production was significantly inhibited to lower than level of detection. The level of aflR gene expression was considerably decreased after the exposure of fungal cells with used *LAB*s. Assessment of the used *LAB*s on *A. parasiticus* revealed antifungal properties and effective ability to decrease aflatoxin production.

Practical applications

Fungi spoilage is widespread among different types of food that may result in huge amounts of waste and economical lost. In addition, side effects raised by using chemical additives as preservative, are a serious source of health concern. Therefore, natural agents such as lactic acid bacteria which are also commonly used as probiotics in recent years, could play a good role as inhibitors of fungal growth as well as mycotoxin production both in food and feed. Different media like dairy foods and so on, can be formulated with *Lactobacillus plantarum* and *L. delbrueckii* subsp. *Lactis*, to prevent *Aspergillus parasiticus* growth and mycotoxin excretion. *LAB* can be added to processed food during inoculation step and even for protection, they can be capsulated with edible materials to improve their efficiency.

1 | INTRODUCTION

Fungi are potent organisms able to grow on all types of foods, including cereals, fruits, and meats. This type of fungi affects about 25% of the world product annually and cause considerable economic problems due to loss in various types of products (Azab et al., 2005). Furthermore, the consequent hazardous effects of mycotoxins and intoxication of moldy foods and feeds in human and animal have been known for centuries (Chu, 1991). Aflatoxin is the most toxic mycotoxin for mammals (Mohseni, Noorbakhsh, Moazeni, Nasrollahi Omran, & Rezaie, 2014) and is a food safety concern for crops worldwide (Thippeswamy, Mohana, Abhishek, & Manjunath, 2014).

Aflatoxin B1 is one of the most common and dangerous mycotoxin that is hepatotoxic, teratogenic, mutagenic, which may lead to several diseases such as toxic hepatitis, edema, hemorrhage, immunosuppression, and hepatic carcinoma (Mohseni et al., 2014). Aflatoxins are found in different types of food such as corn, peanut, cotton seeds, and other grains around the world, and it is estimated to about 4.5 billion people in developing countries suffer from uncontrolled exposure to aflatoxin worldwide (Thippeswamy et al., 2014).

Aflatoxin B1 (AFB1) is the most potent hepatotoxic and carcino-genic compound of the four naturally occurring aflatoxins namely (AFB1, AFB2, AFG1, and AFG2) (Martins, Kluczkovski, de Souza, Savi, & Scussel, 2014; Mohseni et al., 2014) and the International Agency for Research on Cancer classifies it as a group I carcinogen (Alpsoy, 2010).

Aflatoxins are secondary metabolites produced by certain strains of *Aspergillus parasiticus*, *A. flavus*, and *A. nominus* under certain conditions (Alpsoy, 2010).

These fungi can also produce aflatoxin in postharvest conditions: storage, transportation, and food processing which could be found in cereal grains, oil seeds, fermented beverages made from grains, milk, cheese, nut crops, meat, fruit juice, and numerous other agricultural products (Alpsoy, 2010; Khlangwiset & Wu, 2010).

Maize and groundnuts are the main toxic sources for human due to the high rate consumption of these foods in the worldwide (Strosnider et al., 2006).

Some techniques have been developed for preservation of crops against mycotoxins. Drying, freeze- drying, cold storage, modified atmosphere storage and heat treatments are all means of physical methods of food preservation (Schnürer & Magnusson, 2005). Several chemical fungicides also used as preservatives; however, these synthetic compounds have been shown to be dangerous for human health (Tripathi & Dubey, 2004). Moreover, consumers increasingly require high quality products and foods that are free from chemical preservatives with extended shelf life. Use of natural biopreserva-tives or microorganisms to prevent spoilage and to extend the shelf life of foods has attracted the interest of producers resulted from consumers' demands (Gerez, Torres, de Valdez, & Rollán, 2013).

Many microorganisms including bacteria, yeasts, molds, actinomy-cetes, and algae are capable to remove or reduce aflatoxins in foods and feeds (Azab et al., 2005). Among natural biological antagonists, lactic acid bacteria (*LAB*) have various potential applications (Dalié, Deschamps, & Richard-Forget, 2010). *LAB* have been used traditionally as natural biopreservatives of food and feed preventing the growth of spoilage microorganisms (Gerez et al., 2013; Ross, Morgan, & Hill, 2002).

LAB have a generally recognized as safe status where researches have shown that they may improve human and animal health as probi-otics (Bianchini, 2014). *LAB* are widely used for the production of fer-mented foods such as dairy, meat products, and vegetables (Carr, Chill, & Maida, 2002).

LAB produce some antagonistic compounds which are able to con-trol pathogenic bacteria and undesirable spoilage microflora, such as lactic acid, organic acids, fatty acids, hydrogen peroxide, and bacterio-cins (Gerez et al., 2013). These bacteria are generally divided into four genera: *Lactococcus*, *Lactobacillus*, *Leuconostoc*, and *Pediococcus* (Dalié et al., 2010).

According to Magnusson (2003) three mechanisms may explain the antimicrobial efficiency of *LAB*: the yield of organic acid, competi-tion for nutrients and production of antagonistic compounds.

A few number of study have reported that a good selection of *LAB* could allow the control of mold growth and extend the shelf life of many fermented products and, therefore, reduce health risks due to exposure to mycotoxins (Dalié et al., 2010).

The aim of this study was evaluation of the effect of *L. plantarum* and *L. delbrueckii* subsp. *Lactis* on *A. parasiticus* growth as well as aflatoxin production in these fungi.

2 | MATERIALS AND METHODS

2.1 | Fungal strain

In this study, *A. parasiticus* American Type Culture Collection (ATCC) 15517 having ability to produce AFB1, AFB2, AFG1, and AFG2 was selected as a test organism for determining the antifungal and anti-aflatoxigenic efficacies. The strain was cultured on Sabouraud Dextrose Agar culture medium (Merck, Darmstadt, Germany) and was incubated at 25°C for 72 hr.

2.2 | *LAB* strains and culture conditions

Lactobacillus plantarum 1058 and *L. delbrueckii* subsp. *Lactis* 1057 were obtained from Iranian Research Organization for Science and Technol-ogy. According to their specific needs of temperature and aeration level, they were cultured on deMan, Rogosa, and Sharpe agar plates and incubated at 37°C for 48 hr. *LABs* were approved of biochemical tests condition.

2.3 | Preparation of fungal inoculums

The fungal strain was sub-cultured on potato dextrose agar (PDA) (Merck) and incubated at 25°C for 7 days to allow sporulation. Spore inoculum was prepared by immersing in 2 ml of sterile saline solution, and the culture surface was smoothly scraped with the tip of a transfer pipette to harvest the spores. The spore concentration was determined using a hemocytometer showing a concentration of 5×10^4 spore/ml of *A. parasiticus*.

2.4 | Determination of minimal inhibitory concentration

Antifungal susceptibility test was carried out by the broth microdilution method described in the Clinical and Laboratory Standards Institute (CLSI) document M27-A3 with some correction (CLSI 2008). Bacterial suspensions were prepared in saline buffer, according to the standard CLSI protocol, the final concentration was equal to 2×10^3–10^3 cfu/ml in the mentioned medium. All tests were performed in duplicate. Negative and positive control (only fungus without *LAB*) were also used along with each test. The final concentration of fungal spores was calcu-lated using the hemocytometery procedure so that each test well con-tained 5×10^4 spore/ml. Plates were incubated at 35°C and assessed visually after 48 hr of incubation. Minimal inhibitory concentrations (MICs) were determined based on the low concentration in which the growth of the isolates was prevented significantly based on antifungal ability of *LAB*. Endpoint was determined as the lowest concentrations

that prevented any detectable growth, based on the antifungal capacity of *LAB* (100% inhibition).

2.5 | Detection of aflatoxin

The aflatoxin analytical quantification in normal as well as *LAB*-treated PDB culture mediums of *A. parasiticus* were determined by high-performance liquid chromatography (HPLC) technique.

Several PDB plates containing 2×10^3 cfu/ml of *L. plantarum* and *L. delbrueckii* subsp. *Lactis* were inoculated with 5×10^4 fungal spores.

The plates, containing positive (PDB + 5×10^4 cfu/ml fungal spores) and negative (only PDB) controls, were then incubated at 30°C for 7 days without agitating. Then, obtained fungal biomass was harvested and its weight was measured. Afterward, 20 g of each fungal biomass with 2.5 g of sodium chloride were added into 100 ml of HPLC grade methanol 80% and blended for 3 min at 18,000 rpm. Cell-free filtrates were obtained using Whatman filter paper No. 4 and 200 μL of product injected to analysis by HPLC method. HPLC machine was scanning fluorescence detector (Waters e2659, Milford, Massachusetts, USA) and toxin was detected in λem = 435 and λex = 365.

2.6 | RNA extraction and quantitative real-time reverse-transcription polymerase chain reaction assay

RNA expression is one way of measuring gene activity, therefore to determine the probable changes in the aflR gene expression, the level of cognate aflR mRNA in *A. parasiticus* was measured by quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR).

Total cytoplasmic RNA molecules were isolated from normal as well as *LAB*-treated fungal cells that were incubated at 35°C for 48 hr and generated large quantities of mycelia.

In summary, the part of the cultured mycelium was washed twice with ice-cold sterile phosphate buffered saline (pH 7.2) followed by centrifugation at 1,600 g for 10 min. Then it was grounded to fine powder in a porcelain mortar in liquid nitrogen.

Then, the samples were transferred to a tube and 1 ml ice cold RNX was added by a standard method that reported by CinnaGen Co (RNX-Plus, solution for total RNA isolation, CinnaGen Co., Tehran, Iran).

The samples were vortexed 5–10 s and incubated at room temperature for 5 min. Then 200 μL of Chloroform was added. The tubes were shaken 15 s (do not vortex) and incubated on ice or 4°C for 5 min. Afterward they were centrifuged at 12,000 rpm at 4°C for 15 min. The aqueous phase was transferred to new RNase-free 1.5 ml tube (do not disturb the midphase) and added equal volume of Isopropanol with gentle mix and incubated on ice for 15 min. The mixture was centrifuged at 12,000 rpm at 4°C for 15 min and discarded the supernatant and 1 ml of 75% ethanol, shortly vortexed to dislodge the pellet and then centrifuged at 4°C for 8 min at 7,500 rpm. The supernatant was discarded and the pellet was left to be dried at room temperature for few minutes (complete drying was prevented since it will decrease the solubility of the pellet). Pellet was dissolved in 50 μL of DEPC treated water. To help dissolving, place the tube in 55–60°C water bath for 10 min.

The quantity and quality of the isolated total RNA were measured spectrophotometrically (bio photometer, Eppendorf, Hamburg, Germany) and equal concentrations of RNA (1 μg in 20 μL) were subjected to cDNA synthesis using the primescript RT reagent kit (TaKaRa, Kyoto, Japan).

The primers of aflR were designed following the published sequence of the aflR gene in *A. parasiticus* (NCBI, accessionnr: AF441438).

The β-actin gene (ACT1) was applied in present study as an endogenous reference gene. The sequences of forward and reverse primer pairs, which were designed as the amplification of a 200-basepair sequence of aflR and a 110-basepair sequence of ACT1 genes were used.

3 | RESULTS AND DISCUSSION

3.1 | Determination of MIC

In this study, broth microdilution method was applied to identify the antifungal activity of *LAB* (CLSI document M38-A2). A considerable antifungal effect was observed in which MIC value of $> 2 \times 10^3$ cfu/ml.

3.2 | Detection of aflatoxin

Aflatoxin production was analyzed by HPLC. In the presence of 2×10^3 cfu/ml of the *LAB*, production of AFG2 was decreased to lower level than LOD (0.1 ppb) of HPLC. Moreover, the production of AFG1 was decreased at the explained concentration and was inhibited again to lower level than LOD (0.1 ppb) of HPLC with higher concentrations of *LAB*. In addition, no significant changes were observed in AFB2. Nevertheless, the production of AFB1, which is as the most toxic aflatoxin, was not inhibited completely and observed to have decreased in a dose-dependent manner. Table 1 shows the inhibitory effects of the *LAB*s on the rate of aflatoxin production.

3.3 | Effect of *LAB*s on aflR gene expression

ACT1 and aflR mRNA levels were assayed after 48 hr of incubation with *LAB*s by real time PCR. ACT1 and aflR primers had similar

TABLE 1 Effect of the *LAB*s on the aflatoxin production (ppb)

Sample number	AFB1	AFB2	AFG1	AEG2	Total
1	ND	ND	ND	ND	ND
2	99,000	2,200	49,100	1000	151,300
3	50,600	2,200	25,600	ND	78,400
4	64,700	2,700	33,500	ND	100,900

1, Negative control (PDA medium only); 2, positive control (PDA containing fungal spores); 3, *Aspergillus parasiticus* exposed to *Lactobacillus plantarum*; 4, *Aspergillus parasiticus* exposed to *Lactobacillus delbrueckii* subsp. Lactis.
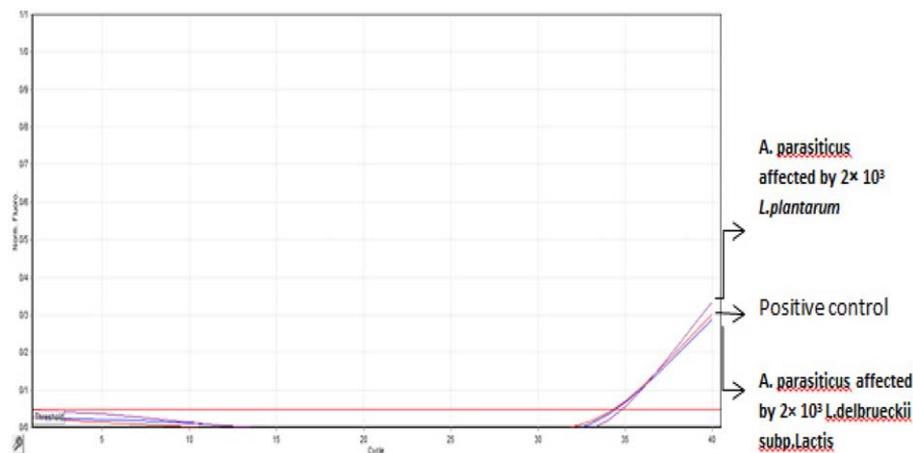AFB = aflatoxin B; AFG = aflatoxin G; ND = not detectable.

FIGURE 1 aflR gene expression analysis in *A. parasiticus* after incubation with *L. plantarum* and *L. delbrueckii* subsp. *Lactis*

performance in a titration experiment using *A. parasiticus* cDNA in serial dilutions. The REST software (2008 V2.0.7) calculated the expression of aflR1 as 0.064 after normalization of aflR gene expression to the housekeeping gene ACT1 (1 for relative expression). Figure 1 shows the effect of *LABs* on aflR gene expression. A considerable decrease in aflR gene expression was revealed after using 2×10^3 cfu/ml of *LABs* ($p < .05$). Table 2 shows the results from the data analysis using REST software.

Further studies on the natural biological antagonists that can lead to reduction in aflatoxin production have been extensively considered during recent years. Among natural factors, *LAB* could have inhibitory effects on mold growth and improve the shelf life of many fermented products and therefore, reduce health risks due to exposure to mycotoxins (Gourama & Bullerman, 1995).

Studies demonstrated that *LAB* have beneficial health effects in human and have a long history of use in food (Dalié et al., 2010). The study of antifungal effect of *LAB* is still novel. Several publications on the antibacterial and antifungal activity of *LAB* have illustrated their inhibitory effects recently (Asurmendi, Pascual, Dalcero, & Barberis, 2014; Gerez et al., 2013; Gonçalves, Rosim, de Oliveira, & Corassin, 2015; Sahebghalam, Mohamadi Sani, & Mehraban, 2013) however, they have rarely identified active compounds or probable reasons for the inhibitory activity.

According to our findings, *LAB* may be considered as an interesting alternative for bio-conservation with antifungal and antitoxin activity against *A. parasiticus*. Overall, obtained results revealed significant reduction in the aflatoxin B1 production and mycelial growth in the presence of the mentioned *LAB*. In addition, gene expression was inhibited when compared with the control. Although the production of aflatoxin was not completely inhibited in the concentration of 2×10^3 cfu/ml, a significant reduction in growth rate of *A. parasiticus* was observed in the interaction with *LAB*.

The antifungal strains have been isolated from various environments such as grass silage (Magnusson & Schnürer, 2001; Magnusson, 2003), sourdough (Corsetti, Gobetti, Rossi, & Damiani, 1998; Hassan & Bullerman, 2008) and vegetable products (Sathe, Nawani, Dhakephalkar, & Kapadnis, 2007). *A. parasiticus* growth was inhibited by *Pistacia atlantica* subsp. *Kurdica* plant at a concentration of 125 mg/ml. furthermore, a significant decrease was observed in aflatoxin excretion with above concentration of *P. atlantica* subsp. kurdica, especially AFL-B1 was completely prohibited. Real-time PCR showed also a considerable decrease in aflR gene expression (Khodavaisy et al., 2016).

Rafaat M. Elsanhoty assessed the potential of fourteen strains of *LAB* to inhibit the outgrowth of aflatoxin-producing *aspergilli*. Between the tested *LAB*, 12 of them had inhibitory effect on the *aspergilli* and *L. plantarum* and *L. acidophilus* ATCC 20552 indicated maximum activity against fungus (Elsanhoty, 2008).

Moreover, Gomah, Ragab, and Bullerman (2009) reported inhibitory effects of the five *Lactobacillus* strains (Three strains of *L. plantarum*, *L. paracasei* subsp. *paracasei*, and *L. rhamnosus* VT1) on production of aflatoxin B1 by *A. parasiticus* (Gomah et al., 2009).

In 2014, Asurmendi et al., evaluated potential antifungal activity of *LAB* on *A. flavus* in brewer's grains and reported that all bacteria tested inhibited two *A. flavus* strains. Moreover, it was observed that the

TABLE 2 Results for relative expression of the aflR gene using the $\Delta\Delta$Ct method (REST, 2008 V2.0.7)

LAB	Gene	Type	Reaction Efficiency	Expression	SE	95% C.I.	P(H1)	Result
Lactobacillus plantarum	β-actin	REF	1.0	1.000				
	aflR	TRG	1.0	0.342	0.342–0.342	0.342–0.342	0.000	DOWN[a]
Lactobacillus delbrueckii subsp. Lactis	β-actin	REF	1.0	1.000				
	aflR	TRG	1.0	0.053	0.053–0.053	0.053–0.053	0.000	DOWN[a]

[a]Indicates down-regulation for aflR gene after treating with *LABs*.
CI = confidence interval; REF = reference gene; SE = standard error; TRG = target gene.

macroscopic appearance of the fungal colonies changed compared with the fungal control (Asurmendi et al., 2014). According to Sathe et al. (2007) *L. plantarum* significantly postponed the growth of *A. flavus* and *Fusarium graminearum* in cucumber.

The potentiality of *LAB* strains isolated from different origins to inhibit mold growth evaluated by Gerez et al. (2013). From all of 91 *LAB* strains examined, 10 were selected due to their high inhibitory effect (>80%). This study was the first report on antifungal peptides produced by a *L. fermentum* strain (Gerez et al., 2013). In this study, in the presence of 2×10^3 CFU/ml of both *LAB*, the relative expression of aflR gene significantly reduced when compared to control.

During the primary stages of fungal cell growth, genes involve in aflatoxin biosynthesis. Aflatoxin is inhibited predominantly in this stage, when the maximum activity of genes within cells occurs (Rasooli & Abyaneh, 2004). Different reasons have led to investigation for new alternatives to reduce the risks related to the presence of fungal spoilage in foods and feed such as consumers' demands regarding quality and food safety.

These results compared to other studies, demonstrate that *L. plantarum* and *L. delbrueckii* subsp. *Lactis* have great ability for inhibition on growth as well as aflatoxin production in *A. parasiticus* and aflR gene expression in low concentration. As a conclusion, *L. plantarum* and *L. delbrueckii* subsp. *Lactis* may be used in food industry combined with processing materials with their beneficial effects on health, to reduce fungal growth and aflatoxin production.

4 | CONCLUSION

L. plantarum and *L. delbrueckii* subsp. *Lactis* inhibit the growth of *A. parasiticus* and reduce aflatoxin production. Additionally, analysis of aflatoxin genes pathway by real time PCR revealed that both *LAB* showed inhibitory effect on expression of aflR genes. *L. plantarum* and *L. delbrueckii* subsp. *Lactis* due to inhibitory effects on fungal contamination may be suitable candidates in controlling the *Aspergillus parasiticus* in food and feed. *LAB* may be added to processed food during inoculation step such as in dairy industry and even for protection, they can be capsulated with suitable materials to extend their survival.

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