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Original Article

Investigation the Effects of *Lactobacillus acidophilus* and *Lactobacillus casei* on aflR Gene expression in *Aspergillus parasiticus* by Real Time-PCR

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Abstract

Background: The effect of probiotic bacteria (*Lactobacillus acidophilus* and *L. casei*) as safe organisms was examined on fungal growth and aflatoxin gene regulation in *Aspergillus parasiticus*.

Methods: The fungus was cultured in presence of two different concentrations of *L. acidophilus* and *L. casei* in MRS broth medium. Mycelia dry weight is indicated as criteria to evaluate fungal growth. Besides, investigation of aflR gene expression by Real Time PCR was performed for analysis of gene regulatory effects in aflatoxin biosynthetic pathway.

Results: Both *Lactobacillus* strongly inhibited fungal growth in the concentrations of 1. 5×10^2, 1. 5×10^3 $\frac{CFU}{ml}$. Expression analysis of aflatoxin genes pathway by real time PCR showed inhibitory effect of *L. acidophilus* and *L. casei* on expression of aflR gene. The gene expression revealed to be reduced at the approximate rates of 99. 7% and 98% respectively by *L. acidopholus* and *L. casei* in concentrations of 1. 5×10^2 $\frac{CFU}{ml}$ and more.

Conclusion: *L. acidophilus* and *L. casei* may be used successfully as suitable candidates in controlling of *A. parasiticus* growth on food and feed as well as reducing of aflatoxin contamination.

Keywords: Aflatoxin, *Aspergillus parasiticus*, *Lactobacilus acidophilus*, *Lactobacilus casei*, aflR gene, Real Time-PCR

Introduction

Aflatoxins are a group of mycotoxins which are toxic, carcinogenic and mutagenic. These secondary metabolites produced by *Aspergillus flavus*, *A. parasiticus*, *A. nomius*, *A. pseudotamarii*, *A. bombycis*, and *A. ochraceoroseus* (1). These organisms are able to grow on grains, nuts, spices, dried fruits, maize (2), and many other foods and are capable to produce aflatoxin. At least 14 different types of aflatoxin are produced in nature including AFB1, AFB2, AFG1, AFG2. Aflatoxin B1 is known as the most potent hepatotoxic and hepatocarcinogenic chemical (3).

During 30 yr of research to identify aflatoxin biosynthesis pathways, more than 20 enzymes has identified and most genes associated with aflatoxin production are clustered within a 75kb region of the genome of the toxin-producing fungi (4-6) . The aflR gene has been identified in *A. flavus*, *A. parasiticus*, *A. sojae* and *A. oryzae* , involved in the regulation of aflatoxin biosynthesis (7-11). The *aflR* gene encoding AFLR protein that contains a

GAL4-type zinc-finger motif, required for transcriptional activation of most structural genes for toxin biosynthetic pathways, such as ver-1 and nor-1(12).

Different bacteria have been studied for their ability to degrade or reduce Aflatoxin. Among these bacteria, probiotic lactic acid bacteria mentioned as useful bacteria in health effects, have been identified as safe organisms that have potential to reduce of aflatoxin (13, 14).

To our knowledge, very little studies have been documented about effects of probiotic lactic acid bacteria on expression of aflR gene. In the present study, antifungal activities of *L. acidophilus* and *L. casei* as well as their ability to decrease aflR expression were studied in *A. parasiticus*.

Materials and Methods

This study was performed in Molecular Biology Lab School of Public Health, Tehran University of Medical Science in 2014.

Fungal cell preparation

Aspergillus parasiticus (ATCC 1551117) was cultured on Sabouraud Dextrose Agar (Merck, Germany) and incubated for 3 days at 30°C. Fungal suspension with concentration of $1.5\times10^{8}\ \frac{CFU}{ml}$ was then produced based on standard protocol (McFarland Turbidity No. 0. 5).

Bacterial cell preparation

The bacterial strains of *Lactobacillus acidophilus* and *Lactobacillus casei*, were cultured on MRS broth medium at 30°C for 48 h. The concentrations of $1.5\times10^{3}\ \frac{CFU}{ml}$ as well as $1.5\times10^{4}\ \frac{CFU}{ml}$ were prepared in saline buffer for both mentioned strains.

A. parasiticus cultivated in the presence of L. acidophilus and L. casei

To study the effect of *L. acidophilus* and *L. casei* on aflR gene expression in *A. parasiticus*, 100 μl of fungal suspension was cultured with 100 µl of bacterial suspension in 10 ml of MRS broth medium. The control culture was obtained without *Lactobacillus* strain. These cultures were then incubated at 30 °C for 3 days.

Fungal growth analysis

After separating the mycelia from the culture medium, the mycelial weight was measured and the fungal growth inhibition was calculated by the following formula:

Inhibition of growth (%) = Dc- Ds / Dc × 100

In which Dc represent the dry weight of colony in control sample, and Ds represent the dry weight of colony in treated sample.

RNA extraction

A. parasiticus, was cultured with bacterial suspension as described above. After incubation at 30 °C for 3 days, the mycelia mass was harvested and frozen in liquid nitrogen. Total cytoplasmic RNA was then extracted according to Guanidine Isothiocyanate method as described previously (15).

cDNA synthesis

cDNA molecules were prepared by using random hexamer primers and reverse transcriptase enzyme, according to the defined protocol (Revert Aid, Fermentase, Germany).

Real Time-PCR

Real-Time PCR was carried out using the SYBR Green Master Mix (Applied Biosystem, USA) and Corbett thermal cycler (Thermocycler Rotor-Gene 6000 Corbett Research, Australia). B-actin gene was used as an internal control (House keeping gene). PCR conditions were as follows: 94 °C for 5 min, 45 cycles of 95 °C for 50s, 58 °C for 20s and 72 °C for 30s. Final holding was done in 72 °C for 1min. The result was analyzed by rotor-gene software.

Results

Effects of L. acidophilus and L. casei on A. parasiticus growth

Two strains of probiotic bacteria; *L. acidophilus* (DVS) and *L. casei* (DVS), were used. The inhibitory effects of these two strains on the growth of *A. parasiticus* were summarized in Table 1.

Table 1: Inhibitory effects of *L. acidophilus* and *L. casei* on the growth of *A. parasiticus*

Bacterial concentration(CFU/ml)	***A. parasiticus* effected *by L. acidophilus***		***A. parasiticus* effected by *L. casei***	
	Dry weigh (gr)	**Inhibitory percentage**	**Dry weigh (gr)**	**Inhibitory percentage**
0	0. 261	0.0	0. 261	0.0
1. 5X10¹	0. 219	16. 09	0. 234	10. 34
1. 5X10²	0. 084	67. 81	0. 102	60. 92
1. 5X10³	0. 037	85. 82	0. 078	70. 11

Both used strains had significant inhibitory effects on *A. parasiticus* growth, however, *L. acidophilus* revealed more efficiency to inhibit the growth of this fungus.

The expression of aflR gene

Real time-PCR results indicated significant decrease in aflR gene expression in *A. parasiticus* after incubation with *L. acidophilus* as well as with *L. casei*. The highest inhibition was observed in 1. $5\times10^3 \frac{CFU}{ml}$ concentration of *L. casei*, however, the minimum inhibitory effect was related to 1. 5×10^2 concentration of *L. casei*. Both concentrations of *L. acidophilus* showed similar inhibitory effect on aflR expression in *A. parasiticus* (Fig. 1). β-actin as a House keeping gene was used in order to compare gene expression. B-actin showed staibility in *A. parasiticus* in the presence of *L. acidophilus* and *L. casei* (Fig. 2).

PFAFFL method was used to determine the range of reduction in aflR expression. In this way the formula $2^{-\Delta\Delta CT}$ was used to calculate gene expression (Table 2).

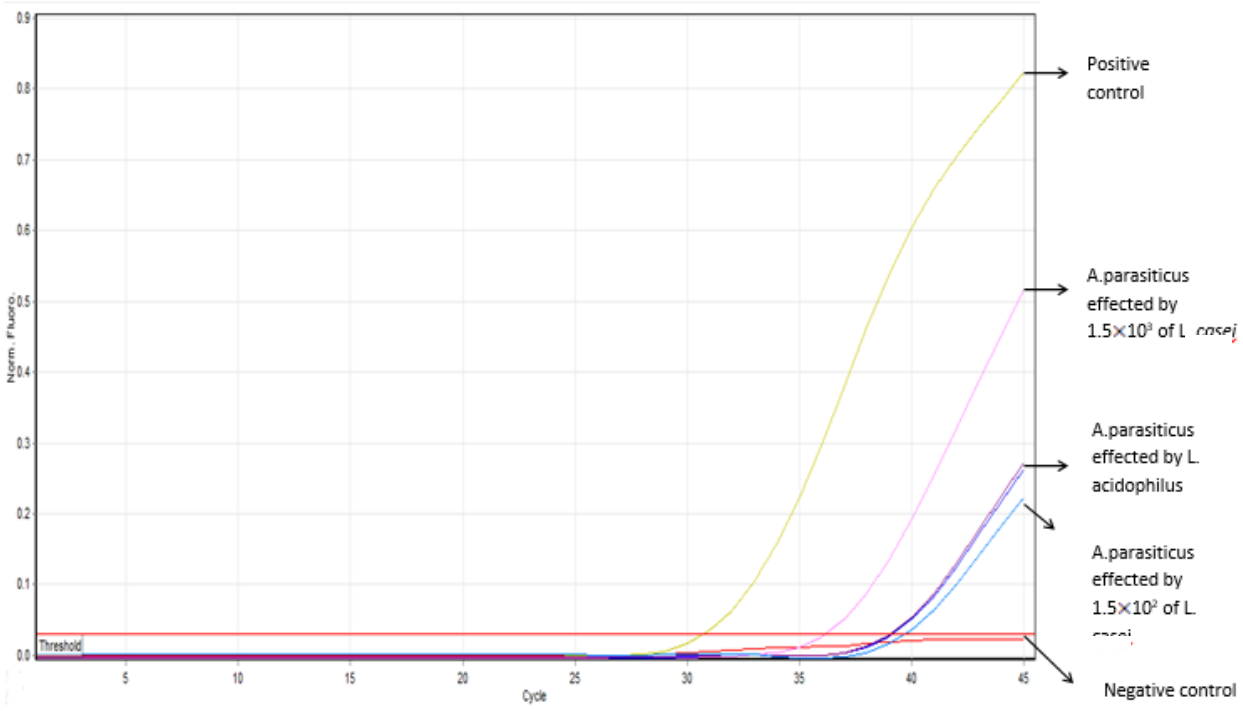

Fig. 1: aflR gene expression analysis in *A. parasiticus* after incubation with *L. acidophilus* and *L. casei*

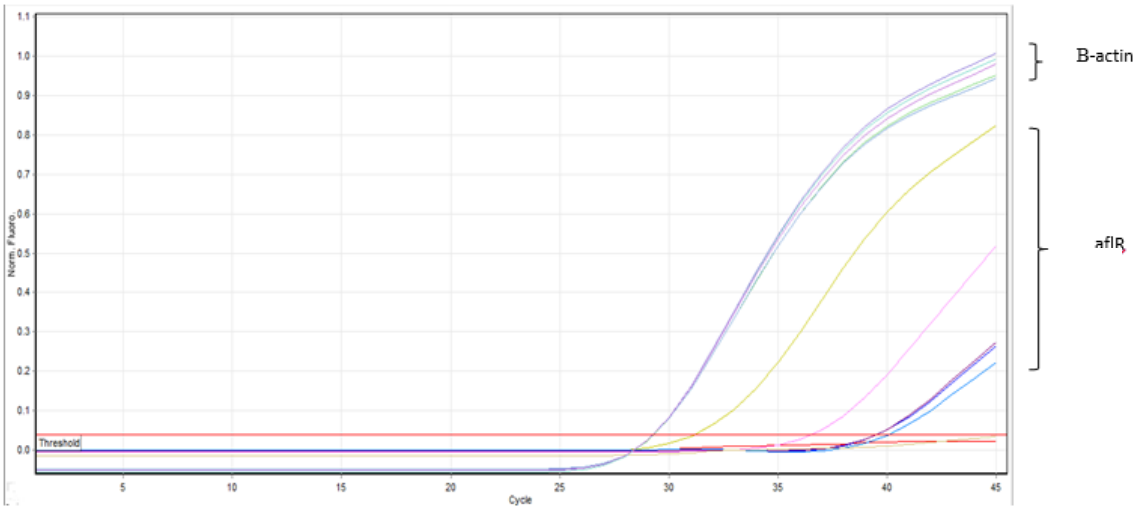

Fig. 2: aflR expression compaired with β-actin in *A. parasiticus*

Table 2: aflR expression in *A. parasiticus* after incubation with *L. acidophilus* and *L. casei*

Sample	aflR	β -actin	ΔC_T (C_{TaflR}-$C_{T\beta\text{-actin}}$)	$\Delta\Delta C_T$ ($\Delta C_{TSAMPLE}$-$\Delta C_{TCONTROL}$)	$2^{-\Delta\Delta CT}$
1. 5X10^2 *A. parasiticus* effected by L. *acidophilus* 1. 5X10^3	39. 5	29	10. 5	8. 5	0. 003
	39. 5	29	10. 5	8. 5	0. 003
A. Parasiticus 1. 5X10^2 effected by *L. casei* 1. 5X10^3	36. 5	29	7. 5	5. 5	0. 02
	40	29	11	9	0. 002
A. parasiticus	31	29	2	0	1

Discussion

Contamination of food crops by different types of toxic fungi has become a serious concern recently. Several researches have been developed for evaluation the inhibitory effects of probiotic bacteria on the growth and on the toxin production process in toxin producing fungi (3, 13, 14, 16). The results mostly indicated efficiency of these bacteria in inhibition of toxin production. In the present study, antifungal activities as well as gene-regulatory effects of *L. acidophilus* and *L. casei* were established against an aflatoxin-producing *A. parasiticus*. Our results revealed both inhibitory in growth as well as aflR gene down-regulation in this fungus due to combine growth with the mentioned bacteria. Compare to these findings, the results of Chang & Kim indicated an inhibition in aflatoxin B1 biosynthesis and mycelial growth of *A. flavus* after incubation with the cell-free supernatant fluids of *L. casei* (16). Besides, obtained results of Oluwafemi et al. showed significant detoxification effects of *L. acidophilus*, *L. brevis*, *L. casei, L. delbruekii* and *L. plantarum* on aflatoxin B in *A. flavus* which revealed the inhibitory effects of mentioned probi-

otic bacteria in toxin production (17). In addition, the role of genes involved in aflatoxin production pathways have been investigated recently and revealed a major role for aflR gene as a regulator in aflatoxin biosynthesis.

Dioctatin A, a metabolite of *Streptomyces*, significantly inhibited aflatoxin production in *A. parasiticus* and it can inhibit the transcription of pksA, ver-1 and omtA and also suppress aflR gene in *A. parasiticus* (18).

Jahanshiri et al. have tested the effect of curcumin on fungal growth and aflatoxin production in *A. parasiticus*. They investigated ver-1, nor-1, pksA, omtA and aflR expression by Real Time-PCR and revealed that fungal growth as well as aflatoxin production inhibited by curcumin. Besides, analysis of the gene expression in aflatoxin pathway showed inhibitory effect of curcumin in aflR gene expression (12).

In this study, effect of *L. acidophilus* and *L. casei* as probitic bacteria was studied on aflR expression in *A. parasiticus*, the obtained results demonstrated high ability of *L. acidophilus* and *L. casei* in inhibition of aflR gene expression in *A. parasiticus*. In addition, both tested *Lactobacillus* revealed remarkable decrease (approximately 67-85%) in *A. parasiticus* growth in two concentration of 1. $5X10^2$ and 1. $5X10^3 \frac{CFU}{ml}$. These results compare to other studies, demonstrate that *L. acidophilus* and *L. casei* have great ability to inhibit mycelia growth and aflR gene expression in low concentration. As a conclusion, *L. acidophilus* and *L. casei* may be used in food industries as safe microorganisms with beneficial effects on health, in order to reduce or inhibit saprophytic-fungal growth and aflatoxin biosynthesis.

Conclusion

Expression analysis of aflatoxin genes pathway by real time PCR showed inhibitory effect of *L. acidophilus* and *L. casei* on expression of aflR gene. Lactic acid bacteria inhibit the growth of *A. parasiticus* and reduce aflatoxin production, therefore may be used successfully as suitable candidates in controlling of *Aspergillus parasiticus* contamination on food and feed.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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