



The Open Microbiology Journal

Content list available at: www.benthamopen.com/TOMICROJ/

DOI: 10.2174/1874285801610010197



RESEARCH ARTICLE

Antifungal Activity and Aflatoxin Degradation of *Bifidobacterium Bifidum* and *Lactobacillus Fermentum* Against Toxicogenic *Aspergillus Parasiticus*

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Received: November 02, 2015

Revised: May 24, 2016

Accepted: May 26, 2016

Abstract: Food and feedstuff contamination with aflatoxins (AFTs) is a serious health problem for humans and animals, especially in developing countries. The present study evaluated antifungal activities of two lactic acid bacteria (LAB) against growth and aflatoxin production of toxigenic *Aspergillus parasiticus*. The mycelial growth inhibition rate of *A. parasiticus* PTCC 5286 was investigated in the presence of *Bifidobacterium bifidum* PTCC 1644 and *Lactobacillus fermentum* PTCC 1744 by the pour plate method. After seven days incubation in yeast extract sucrose broth at 30°C, the mycelial mass was weighed after drying. The inhibitory activity of LAB metabolites against aflatoxin production by *A. parasiticus* was evaluated using HPLC method. *B. bifidum* and *L. fermentum* significantly reduced aflatoxin production and growth rate of *A. parasiticus* in comparison with the controls ($p \leq 0.05$). LAB reduced total aflatoxins and B₁, B₂, G₁ and G₂ fractions by more than 99%. Moreover, LAB metabolites reduced the level of standard AFB₁, B₂, G₁ and G₂ from 88.8% to 99.8% ($p \leq 0.05$). Based on these findings, *B. bifidum* and *L. fermentum* are recommended as suitable biocontrol agents against the growth and aflatoxin production by aflatoxigenic *Aspergillus* species.

Keywords: *Aspergillus parasiticus*, Aflatoxin, Antifungal activity, *Bifidobacterium bifidum*, *Lactobacillus fermentum*.

1. INTRODUCTION

The presence of various fungal species such as *Aspergillus*, *Penicillium* and *Fusarium* in food commodities can cause serious health problems in humans and animals, which leads to unfavorable economic effects [1]. Some toxigenic *Aspergillus* strains such as *A. flavus* and *A. parasiticus* produce aflatoxin, an important carcinogenic mycotoxin with side effects such as malformations and immune suppression [2, 3]. Physical, chemical and biological methods have been developed for degradation of mycotoxins in food. Since the chemical and biological methods are often regarded as unsafe and expensive, it seems that biodegradation of mycotoxins is the best option.

There are several reports on the interactions between toxigenic fungi and microorganisms that can affect fungal growth and toxin production due to antagonist compounds [2]. Lactic acid bacteria (LAB) are among these microorganisms and *Bifidobacterium* and *Lactobacillus* are two well-known genera of this family [3]. LAB are found in dairy products, meat and cereal products [4]. Several species and subspecies such as *Lactococcus lactis*, *L. cremoris*, *L. diacetylactis*, *L. acidophilus*, *L. plantarum* and *L. curvatus* are able to synthesize antimicrobial peptides or proteins known as bacteriocins [5]. LAB have been used for centuries as starter cultures in the food industry and are able to pro-

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duce different types of bioactive metabolites such as fatty acids, organic acids, aroma compounds, hydrogen peroxide and bacteriocins [6].

In recent years, biopreservation (the use of microorganisms and/or their metabolites to prevent spoilage and to extend the shelf life of foods) has gained a lot of attention because of consumer demand [7].

The aim of the present study was to evaluate the antifungal activities of *B. bifidum* and *L. fermentum* against toxigenic *Aspergillus parasiticus*.

2. MATERIAL AND METHODS

2.1. Microorganisms and Culture Conditions

Standard strains of *B. bifidum* PTCC 1644, *L. fermentum* PTCC 1744, toxigenic *A. parasiticus* PTCC 5286 and nontoxigenic *A. niger* (as negative control) were purchased from the Iranian Research Organization for Science and Technology (IROST). *A. parasiticus* was stored in sterile distilled water at room temperature and then grown on Sabouraud dextrose agar at 30 °C for five days until the start of experiments [8, 9].

LAB were separately incubated in tryptic soy broth (Conda laboratories, Madrid, Spain) at 37 °C for 48 h. Grown bacteria were spread on Man-Rogasa-Sharpe agar and allowed to grow at 38°C for 48 h in an anaerobic jar. Standard aflatoxins (AFT) B1, B2, G1 and G2 were purchased from Sigma-Aldrich Chemical Co.

2.2. Effect of Lactic Acid Bacteria on Growth Rate and AFT Production of *A. Parasiticus*

A loopful of LAB from the Man-Rogasa-Sharpe medium was separately incubated in tryptic soy broth at 37 °C for 18 h. Then, one mL of culture medium (density equivalent to 0.5 MacFarland standard) was added to a 100 mL Erlenmeyer flask containing 25 mL of yeast extract sucrose broth with 5×10^4 spores/mL of *A. parasiticus*. Mycelial mass and AFT production rates were measured according to the method described previously [1, 10, 11].

Fungal mycelial mass was separated by Filter Paper (Whatman No. 1) and weighed after drying in oven at 70°C. The amount of AFTs in the medium was analyzed by HPLC method. In brief, a volume of 10 mL chloroform was added to 50 mL of culture media supernatant, and then agitated vigorously. After 15 minutes, the supernatant was removed and evaporated with N₂ under safety cabinet and reconstituted in 10 mL phosphate buffer saline (pH 7.2), and then passed through a membrane filter (0.45 µm pore size). The filtrate passed through the Immunoaffinity Afla-clean column (LC Tech, Germany) at flow rate of 1 drop/second. The column was washed with 10 mL of deionized water. Finally, AFTs were eluted using methanol through the two following steps: 1 mL methanol was applied to the column and was allowed to pass by gravity after 5 minutes, 1 mL additional methanol was poured into the column after a minute and elute was later collected and 50 µL injected to HPLC. Water/methanol/acetonitrile (60:30:10, v/v/v) were used as the mobile phase with a flow rate of 1.0 mL/min at 25°C [9]. The retention time was 20 min. A five- point calibration curve was drawn for different types of AFTs to compare and determine linear correlations. All tests were carried out in duplicate for each sample.

2.3. Effect of LAB Extracellular Metabolites on Standard AFTs

The culture medium containing LAB was centrifuged at 4000 g for 15 min to precipitate bacterial cells. Supernatant was filtered using 0.22 µm membrane filter and then 0.25 µL of mixture fraction of AFTs (1000 ppb) was added to 1.75 mL of the filtrated supernatant to reach a final concentration of 125 ppb for AFB₁&AFG₁, and 25 ppb for AFB₂&AFG₂. The final solution was incubated at 30°C for 72 h. The amounts of AFTs were analyzed according to the method described previously [9].

2.4. Statistical Analysis

Statistical analysis was performed using paired-samples T-test by SPSS 21 statistical software. P-value of ≤ 0.05 was considered as the statistical significance level.

3. RESULTS

3.1. Antifungal Activity of LAB Against Growth of *A. Parasiticus*

Both *Bifidobacterium* and *Lactobacillus* significantly reduced the growth rate of *A. parasiticus* in comparison with

the positive control ($p < 0.05$). However, there was no significant difference between the antifungal activities of these two LAB (Table 1).

Table 1. Mycelial mass production by *A. parasiticus* in the presence of *L. fermentum* and *B. bifidum*.

Species	Mycelial Mass **	Mycelial Mass Reduction %
<i>A. parasiticus</i> *	1.006 ± 0.06	-
<i>A. parasiticus</i> *+ <i>B. bifidum</i>	0.1853 ± 0.11	81.58
<i>A. parasiticus</i> *+ <i>L. fermentum</i>	0.2313 ± 0.019	77

* Positive control containing 5×10^7 (spore/mL)

** Mycelial mass weight (gram)

3.2. Impact of LAB on Aflatoxin Production by *A. Parasiticus*

Both bacteria significantly reduced AFB₁, AFB₂ and AFG₁ production ($p \leq 0.05$), while this reduction was not significant in the case of AFG₂ (Table 2).

Table 2. Effect of *L. fermentum* and *B. bifidum* on the concentration of AFTs produced by toxigenic *A. parasiticus*.

Species	AFB ₁	AFB ₂	AFG ₁	AFG ₂	Total (ppb)
<i>A. parasiticus</i>	12013.33 ± 2141.4	563.3 ± 144.68	37206.6 ± 14447.2	1460 ± 695.41	51243.3 ± 17421.7
<i>L. fermentum</i>	5.0933 ± 1.193 (99.9)*	0.1167 ± 0.306 (99.9)*	2.783 ± 0.764 (99.9)*	0.323 ± 0.77 (99.9)*	8.3167 ± 1.481 (99.9)*
<i>B. bifidum</i>	4.8167 ± 2.417 (99.9)*	1.110 ± 0.24 (99.8)*	1.1667 ± 0.22 (99.9)*	0.75 ± 0.195 (99.9)*	7.843 ± 2.986 (99.9)*

* Reduction percent

3.3. Effects of LAB Extracellular Metabolites on AFT Standard Solution

The extracellular metabolites filtrated from both LAB significantly reduced the mixture fractions of AFTs from 88.8% to 99.8% (Table 3).

Table 3. Aflatoxin reduction by extracellular metabolites of *L. fermentum* and *B. bifidum* in AFTs mixture solution containing AFB₁ and G₁= 125 ppb, AFB₂ and G₂= 25 ppb.

Species	AFB ₁	AFB ₂	AFG ₁	AFG ₂
<i>L. fermentum</i>	11.53 ± 0.7024 (90.7)*	2.55 ± 0.345 (89.8)*	0.1667 ± 0.0971 (99.8)*	0.1557 ± 0.617 (99.3)*
<i>B. bifidum</i>	12.5 ± 0.60 (90)*	2.786 ± 0.296 (88.8)*	0.66 ± 0.200 (99.4)*	0.7833 ± 0.1305 (96.8)*

* Reduction percentage

4. DISCUSSION

It has been clear that the best effective way to prevention of food with mycotoxins contamination is to avoid of the growth mycotoxigenic fungi [1].

The interaction between mycotoxin-producing fungi and other microorganisms is a suitable biological method to control fungal food poisoning and contamination by blocking the mycotoxins pathway [1, 12]. In the present study, *B. bifidum* and *L. fermentum* caused significant reduction in the mycelial growth of *A. parasiticus*. In addition, these LAB exhibited inhibitory effects on the standard solution of AFTs from 88.8% to 99.8% in comparison with the control. The results of this study are in agreement with the study of Munoz *et al.* in which two LAB of *L. fermentum* and *L. rhamnosus*, and *S. cerevisiae* inhibited the growth of mold and mycotoxin-producing *A. nomius*. However, *L. rhamnosus* showed the highest fungal inhibitory activity compared with the other bacteria tested [10]. The results of the present study are also consistent with the findings of Ghonaimy *et al.* on the growth inhibition of *A. flavus* and *A. parasiticus* and AFT production by *L. acidophilus* ATCC 4495 and ATCC 20552 [13].

Various factors including nutritional competition, secondary metabolites, pH or their combinations have been proposed for the inhibition of fungal growth by the bacteria [14, 15]. El-Nezami *et al.* investigated the reduction of AFT by five *Lactobacillus* species and showed that, the binding of AFT with probiotic strains, such as *L. rhamnosus* GG and *L. rhamnosus* LC-705 was very effective in removing AFB₁ and more than 80% of the toxin trapped [16].

Piotrowska *et al.* reported ochratoxin removal from culture media by *L. plantarum*, *L. brevis* and *L.*

sanfranciscensis. In the mentioned study, ochratoxin reduction appeared with adsorption to bacterial cell wall with hydrophobic phenomenon, electron donor-acceptor and Lewis acid-base properties and no active metabolites were seen in this process [17].

Carbohydrates and/or protein components of LAB cell wall play a major role in the binding of AFT to LAB [18]. Fulgueira *et al.* in 2004 detected a proteinaceous compound from *Streptomyces* sp. with high antagonistic activity on toxigenic fungi [19].

In the present study, LAB extracellular metabolites significantly reduced the standard solution of AFTs. Consistent with these findings, Lindgren *et al.*, Sjogren *et al.* and Mondal *et al.* studies showed that antifungal compounds produced by LAB can significantly reduce AFTs [20 - 22]. Moreover, some *ex vivo* and *in vivo* studies demonstrated that LAB reduce or retard the uptake of AFB₁ in the gastrointestinal tract [23]. Although the biological decontamination and enzymatic degradation of mycotoxins by different microorganisms have been reported [24], the toxicity of enzymatic degradation byproducts and undesired effects of fermentation of non-native microorganisms on food quality remain as problems [25].

CONCLUSION

LAB are naturally associated with many foods and are well recognized for their biopreservative properties. This study shows the ability of *B. bifidum* and *L. fermentum* to reduce aflatoxin levels and prevent growth of mycotoxigenic molds through production of several low-molecular-weight antifungal metabolites, binding to the cell wall or combination of acidity and microbial competition. These antifungal LAB can be used in the food industry instead of chemical preservatives to produce organic foods. Furthermore, the excellent properties of LAB may preserve nutritional value of foods and delay spoilage. The future trends are to include beneficial probiotic microorganisms in a process of dietary detoxification of contaminated foods to constitute an approach for the decrease of the availability of aflatoxins in the human nutrition and animal feed. Due to their economic importance for the food industry and their health-related implications as probiotics safety assessment and risk analysis must be considered.

ETHICAL CONSIDERATIONS

All ethical issues (such as informed consent, plagiarism, misconduct, co-authorship, double submission, *etc.*) were considered carefully.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

This study was derived from MSc thesis supported by the Tehran University of Medical Sciences.

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