In Vitro Efficacy of Selected LAB Strains against Aflatoxigenic Fungi

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Abstract

Aflatoxins are secondary metabolites produced by various species of Aspergillus and are responsible for toxic effects on farm animals. The limitation of the mycotoxin contamination of food and feed could be achieved by specific methods that inhibit the fungal growth. The aim of our work was the evaluation the effects of different lactic acid bacteria strains (as potential biocontrol agents) on *Aspergillus flavus* growth and aflatoxin biosynthesis. Two toxigenic isolates of *A. flavus* were selected as high aflatoxin producers, based on *in vitro* observations (fluorescence on coconut medium, after exposure to UV light, and large opaque ring surrounding the fungal colonies cultivated on PDA medium with β-cyclodextrine and sodium deoxycholate). As inhibitory microorganisms were used strains of lactic acid bacteria. Significant morphological effects of lactic acid bacteria on *A. flavus* mycelium were observed microscopically. For separation of aflatoxins from samples, was used thin-layer chromatography (TLC). *Lactobacilus plantarum* 58, 35, 45 had inhibitory effects both on fungal growth and aflatoxin level.

Keywords: *Aspergillus flavus*, aflatotoxins, antagonistic, lactic acid bacteria, TLC

1. Introduction

Feed spoilage moulds cause great economic losses worldwide. It is estimated that between 5 and 10% of the world's feed production is wasted due to fungal deterioration [1]. These fungi cause losses in dry matter or quality and some species can produce health-damaging mycotoxins. These toxins comprise a group of chemically diverse compounds originating from secondary metabolism by moulds (filamentous fungi) and are mainly produced by: *Aspergillus*, *Penicillium*, *Fusarium*, *Alternaria* and *Claviceps* genus [2]. The mycotoxins produced by fungi can be carcinogenic, hepatotoxic, teratogenic or immuno-suppressing [3,4]. The mycotoxigenic potential depends on species and strains of fungus, composition of matrix and environmental factors (temperature and moisture). These substances are quite common in grains, and have a high incidence of aflatoxins. Aflatoxins are mainly secondary metabolites produced by strains of *Aspergillus parasiticus* and *A. flavus*. The most commonly found substances in grains are aflatoxins B1, B2, G1 and G2 [5,6]. Aflatoxin B1 (AFB1) is extremely mutagenic, toxic and a potent carcinogen to both humans and livestock and chronic exposure to low levels of AFB1 is a concern [7,8]. Mycotoxins could be eliminated after their production or by growth inhibition of the fungus-producing strain. Current strategies to destroy mycotoxins in food include heating, treatment with ammonia, screening and radiation, but they are too expensive, impractical for commercial application or destroy vital nutrients of the grain [9]. Chemical preservatives and natural antimicrobial compounds. Therefore, reduction of mould growth during the production and storage of food and feed is of great importance. *Aspergillus* species produces

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mycotoxins in grains and consequently represents a direct threat to the crops. The primary method of control is the use of chemical fungicides. However, nowadays most of them are not authorized due to the toxicological risks (Directive 91/414/CEE of the EU). After consumption of contaminated food, the microorganism also threatens human and animal health. The interaction between mycotoxin producing fungi and other microorganisms is a common phenomenon in nature that can affect fungal growth and/or production of mycotoxins. For this reason, research is now focused, to some degree, on inhibition of fungal growth. For many years it has been clear that the most effective means to prevent contamination of food. At the same time, the general public demands are to reduce the use of chemical preservatives and additives in food and feed. Among the different potential decontaminating microorganisms, yeast and lactic acid bacteria (LAB) represent unique groups, which are widely used in food fermentation and preservation. Many LAB strains have shown probiotic activity. Lactic acid bacteria (LAB) are generally recognized as safe microorganisms that modify the food enhancing quality, and additionally they possess nutritional and therapeutic benefits. The majority of the numerous reports on antimicrobial activity by LAB have focused on antibacterial effects, whereas there are only few reports on antifungal effects.
The aim of our work was to evaluate the effects of different lactic acid bacteria strains (as potential biocontrol agents) on *Aspergillus flavus* growth and aflatoxin biosynthesis.

2. Materials and methods

Fungal isolates

The *Aspergillus flavus* strains T11 and GE2 were isolated from seeds of wheat or triticale and maintained on PDA (Potato dextrose agar) [13].

LAB strains

10 strains of LAB from the Faculty of Biotechnology collections of the USAMV Bucharest, were used as in experiments.

Detection of aflatoxigenic *Aspergillus* strains

For the detection of mycotoxigenic abilities of fungal strains three different culture media were used for: Potato Dextrose Agar (PDA) supplemented with 0.3% methyl-β-Cyclodextrin and 0.6% Sodium Deoxycholate (PDACD) [10; 11], YES medium supplemented with 0.3% β-Cyclodextrin and 0.6% Sodium Deoxycholate (CCD) [10] and Coconut agar medium [9].
The presence of a beige opaque ring surrounding the fungal colonies cultivated on PDACD and CCD is associated to aflatoxin production [12]. On coconut medium the production of mycotoxins was revealed by examination on UV light (365nm): blue florescence is associated with aflatoxin AFB1 and AFB2 production, and blue-green fluorescence with AFG1 and AFG2 production after the isolates were evaluated by TLC. The plate was developed with toluene/ethylic acetate/ formic acid (5:4:1, v/v/v) [15] Based on previous results two fungal strains of *Aspergillus flavus* (T11 and GE2) were selected to be used in this experiment. These strains were chosen due to the fact that they produced the biggest quantity of aflatoxin [13].

Detection of antifungal action of LAB strains

The antifungal activity of LAB strains against *Aspergillus* was examined using the double layer technique. Plates with MRS medium were spotted with fresh LAB suspensions (5 µl) and after 48 h of incubation at 36^{o}C on the plates were poured 5 ml of 0.6% PDA containing fungal spores. Plates were incubated at 27^{o}C for 3 days, and up to 14 days at room temperature. Antifungal activity of the LAB strains was appreciated based on the fungal growth inhibition around the bacterial spot.
For evaluation of LAB effects on aflatoxin production/degradation, co-cultivation of bacteria and fungi was performed. Equal volumes of LAB suspension (in MRS) and fungal suspension (in PD broth) were mixed and incubated for 48 h at 37^{o}C and then aflatoxins were extracted with chloroform [14] and subjected to TLC analysis.

TLC analysis of aflatoxins

Aflatoxins extracted with chloroform from liquid medium (1/2, v/v) from each experimental variant (10 µl) were placed on TLC Silica gel 60 Merck

[15]. The developing solvent toluene/ethyl acetate/formic acid (5:4:1, v/v/v) was used as a mobile phase, and B1, B2, G1 and G2 aflatoxins as standards. The visualization of spots corresponding to aflatoxins was performed under UV light (365 nm). The semi-quantitative determination was done by comparing the fluorescence ratios and the frequency (Rfs). The Rf can provide corroborative evidence as to the identity of a compound. If the identity of a compound is suspected but not yet proven, an authentic sample of the compound, or standard, is spotted and run on a TLC plate side by side (or on top of each other) with the compound in question. If two substances have the same Rf value, they are likely (but not necessarily) the same compound. If they have different Rf values, they are definitely different compounds [16].

3. Results and discussion

Mycotoxins are very hazardous to animals and humans. There is a need to study the structures of mycotoxin and toxicity of detoxifying agents. Special attention is given in recent years to potential biological control (bacteria, yeasts) able to inhibit fungal growth, aflatoxin biosynthesis or degradation. In this respect, based on previous results related to the screening of lactic acid bacteria with antifungal properties [17], 10 strains of LAB were selected and used for ability to inhibit *A.flavus* GE2 and T11 strains. The best results registered after 5 days of incubation were obtained with 3 LAB strains, designated as 35, 43 and 58, isolated from fermented wheat bran and from sauerkraut (fig 1).

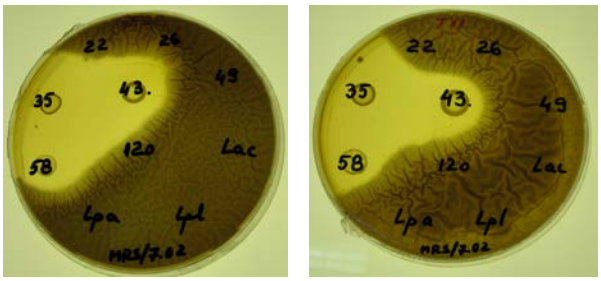

Figure 1. Inhibitory action of LAB strains against *A. flavus* GE2 (left) and T11 (right)

It should be noted that the inhibitory action of these LAB strains was stable even after 14 days of maintaining at room temperature, suggesting a potential fungicide effect of bacteria.

Important damages of the fungal hyphae (fragmentation, loss of the cellular content, twisting of hyphae or apical swelling) due to interactions with the strains LAB 58 and LAB 35 were observed for both of the *Aspergillus flavus* strains used in experiments (fig.2).

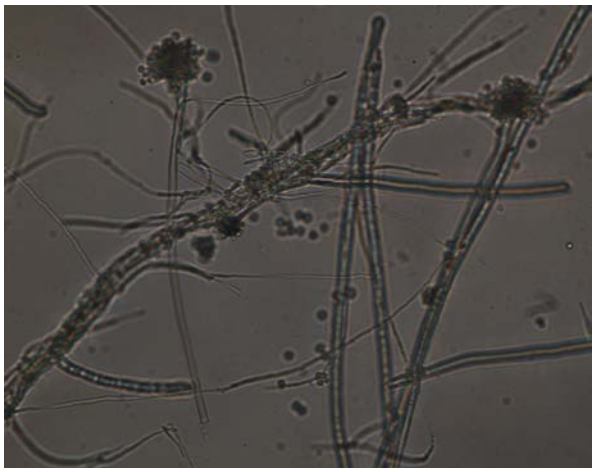

Figure 2. Molphological modification of T11 fungal hyphae induced by LAB 35

It was also shown that the LAB strains inhibited the sporulation of target fungi (fig.3).

Figure 3. *A. flavus* (GE2) strain inhibited by LAB strains (after 5 days of incubation at 27^{o}C).

In order to evaluate the impact of LAB on mycotoxin level, TLC analysis was performed after co-cultivation of bacteria and fungi. The results obtained (based on Rf calculation) and comparison with the data from literature [15] confirm the presence of aflatoxins in fungal samples, but not in the mixed ones (*Aspergillus flavus* with LAB strains) (fig.4)

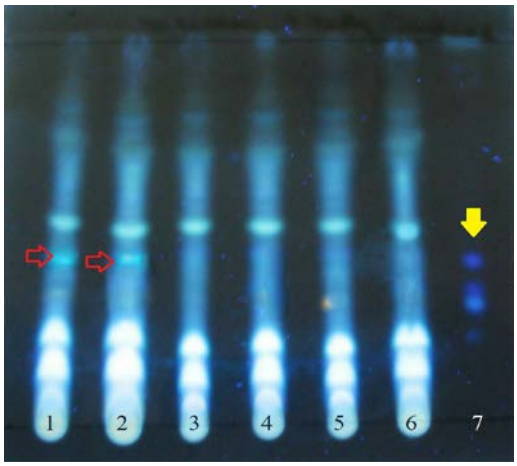

Figure 4. TLC analysis of aflatoxins produced by *A.flavus* strains in various experimental conditions. 1 - GE2; 2 -T11; 3- LAB 58; 4 - LAB 35; 5 - GE2+ mixture of LAB; 6 - T11+ mixture of LAB; 7 - aflatoxin standard (from top to bottom AFB1; AFB2; AFG1; AFG2). The red arrow indicates the position of AFB1.

According to our results the lactic acid bacteria used in the experiments had not only inhibitory action against the fungal growth but also against the level of aflatoxins in producing strains, at least of AFB1 aflatoxin. At this moment we are not able to say if this action is due to the inhibition of aflatoxin biosynthesis or to degradation of the mycotoxins. It is possible that both mechanisms could be involved, but further experiments are necessary.

4. Conclusions

In these experiments it was proved that three LAB strains, designated as 35, 43 and 58 were able to inhibit the development of two aflatoxigenic strains of *Aspergillus flavus* (GE2 and T11).

Important damages at cellular level (fragmentation, loss of the cellular content, twisting of hyphae or apical swelling) due to interactions with the strains LAB58 and LAB35 were observed.

Inhibition of aflatoxin biosynthesis and/or degradation of AFB1 was detected for the strains LAB 35 and LAB 58, as revealed by TLC analysis.

The selected LAB strains could represent good candidates as biocontrol agents for grains/animal feed protection and prevention of contamination with aflatoxigenic fungi.

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