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ANTIFUNGAL ACTION OF LACTIC ACID BACTERIA ISOLATED FROM PLANT MATERIALS AGAINST MYCOTOXIGENIC FUNGI

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Abstract

The contamination of food and feed by mycotoxigenic fungi that belong to genera Aspergillus, Alternaria, Fusarium and Penicillium poses significant risks for animal and human health. The mycotoxins (aflatoxins, trichothecenes, ochratoxins, fumonisin etc) produced by such spoilage fungi can be carcinogenic, hepatotoxic, teratogenic or immunosuppressing (Logrieco et al., 2003; Atanda et al., 2013). The limitation of the mycotoxin contamination of food and feed could be achieved by specific methods that inhibit the fungal growth. Several approaches are described by now to avoid the accumulation of mycotoxins, directed towards: prevention of contamination; decontamination of mycotoxin-containing food and feed; and inhibition or absorption of mycotoxin content of consumed food into the digestive tract (Juodeikiene et al., 2012). The use of biological control microorganisms (bacteria, yeasts or fungi) to inhibit the growth of mycotoxin producing fungi and/or degrade the mycotoxins is an important strategy for prevention/decontamination of products (Tsitsigiannis et al., 2012).
The aim of this work was the screening of new lactic acid bacteria (LAB) strains isolated from plant materials or some Romanian traditional foods for antifungal action againstplant pathogenic fungi and mycotoxin producing Aspergillus strains. 123 strains of LAB were examined for anti-Aspergillus action and 24 out of them (more than 21%) exhibit strong inhibitory activity against the growth of A.flavus, A.ochraceus and A.niger strains after 3 days of incubation. For most of the LAB strains the inhibitory action was maintained even after 14 days of incubation. The principal antifungal compound is represented by lactic acid, quantified by HPLC. Important damages of the fungal hyphae were observed at microscopic level (fragmentation, loss of the cellular content, twisting of hyphae or apical swelling). 17 new isolate of Aspergillus spp. were obtained and their ability to produce mycotoxin was evaluated by qualitative and quantitative methods. Four of them were used for interactions with LAB: strong and stable inhibition was obtained with the LAB strains designated as 35, 58 and 26. The results suggest that selected LAB could be used for fungal growth inhibition and possible for mycotoxin content reduction.

Key words: *antifungal LAB, mycotoxin producing Aspergillus*

INTRODUCTION

Biological means for yield protection and food preservation are becoming increasingly interesting for both food industry and consumers. Spoilage fungi not only that determine quantitative looses but could induce qualitative depreciations such as mycotoxin contamination. The contamination of food and feed by mycotoxigenic fungi that belong to *Aspergillus, Alternaria, Fusarium* and *Penicillium* poses significant risks for animal

and human health. The mycotoxins (aflatoxins, trichothecenes, ochratoxins, fumonisin, etc) produced by such spoilage fungi can be carcinogenic, hepatotoxic, teratogenic or immuno-suppressing (Logrieco et al., 2003; Atanda et al., 2013). Aflatoxins (B1, B2, G1 and G2) are a group of secondary metabolites predominantly produced by *Aspergillus flavus* and *Aspergillus parasiticus*, considered as the most potent hepato-carcinogens affecting animal species, including humans (Mishra and Das, 2003). The limitation of the mycotoxin contamination of food and feed could be achieved by specific methods that inhibit the fungal growth. To avoid fungal contamination and mycotoxins accumulation in foods and feeds, several approaches are described by now and directed towards: prevention of food and feedstuff contamination, decontamination of mycotoxins-containing products, and inhibiting the absorption of mycotoxin content into the digestive tract when consuming contaminated aliments (Griessler et al., 2010; Juodeikiene et al., 2012). The use of biological control microorganisms to inhibit the growth of mycotoxin producing fungi and/or degrade their mycotoxins is an important strategy to prevent infections or aliment decontamination (Tsitsigiannis et al., 2012).

Several microorganisms, especially bacteria, have been examined for their potential to inhibit fungal growth and production of aflatoxins, the best results being obtained, under laboratory conditions, with spcies of *Bacillus*, *Lactobacillus*, *Pseudomonas*, *Ralstonia, Burkholderia, Enterococcus or Rhodococcus* (Alberts et al., 2006; Reddy et al., 2010; Topcu et al., 2010). It was shown that microorganisms (both living or dead cells) could bind aflatoxins to their cell walls components or could produce extra- or intracellular enzymes involved in degradation/modification of aflatoxins. The results obtained by various authors proved that the methods of biodegradation can be o promising choice to reduce or eliminate the contaminations of aflatoxins in food and feed (Wu et al., 2009).

The inhibition of spoilage fungi using beneficial microorganisms such as lactic acid bacteria (LAB) has been the subject of various investigations due to the practical importance of LAB as active ingredients in food and feed preservation. LAB not only that are widely used in food and feed processing but they have probiotic activity and, in some cases, they can produce antimicrobial metabolites with antifungal effects, inhibiting also mycotoxigenic fungi (Nyoman Pugeg Aryantha and Lunggani, 2007). Several studies reported LAB strains that could bind aflatoxin (Peltonen et al., 2001; Kankaanpää et al., 2000, Gratz et al., 2004; Shah and Wu, 1999, El-Nezami et al., 2002), inhibit aflatoxin accumulation (Coallier-Ascah and Idziak, 1985; Onilude et al., 2005), or even remove AFB1 (Shah and Wu, 1999; El-Nezami et al., 2002). Fuchs et al. (2008) reported LAB strains that could remove ochratoxin A mycotoxin from solutions.

The aim of this work was the screening of new lactic acid bacteria (LAB) strains isolated from plant materials or some Romanian traditional foods for antifungal action against plant pathogenic fungi and mycotoxins producing *Aspergillus* strains.

MATERIALS AND METHODS

Microbial strains

One hundred and twenty three lactic acid bacteria were used in this study. The LAB were isolated from plant materials or some Romanian traditional foods such as borş (fermented wheat bran), sauerkraut or pickles. Three collection strains of LAB were used as reference strains: *Lactobacillus plantarum* IC12353, *Lb.paracasei* CCM 1837 and *Lb.acidophilus* IC11692. Routinely, LAB strains were grown on MRS broth at 36°C.

Fungal isolation and identification

Two groups of fungi were used in experiments: plant pathogenic fungi and aspergilli. The plant pathogens, like *Botrytis cinerea, Alternaria alternata, Penicillium spp., Monilinia fructigena, Fusarium spp.* were isolated from fruits, seeds or vegetables (Cornea et al., 2013). *Aspergillus spp.* strains were isolated from cereal seeds (wheat, Triticale, barley, oats) on Potato Dextrose Agar (PDA), and their identification was based on macroscopically and microscopically features. 17 strains of aspergilli were isolated and identified as *A.flavus/A.parasiticus* (13 strains), and *A.niger* (4 strains). Other eight *Aspergillus spp* strains,

previously isolated and identified as *A.niger* (An4 and An5), *A.ochraceus* (2 strains), *A.oryzae* MI156, *Aspergillus spp.* (Asp1 and Asp 4.3)(Cornea et al., 2011) and *A.flavus* (Judet et al., 2006) were also used in our experiments.

Screening for mycotoxin-producing aspergilli

The mycotoxin producing capability of the fungal strains was evidenced on coconut agar medium (Davis et al., 1987; Heenan et al., 1998; Lin and Dianese, 1976), under UV light, after 7 days of incubation in the dark, at 26°C, and by cultivation of fungi on PDA supplemented with 0.3% methyl-β-cyclodextrin (MβCD) and 0.6% sodium deoxycholate (a beige opaque ring surrounding the fungal colonies is associated to aflatoxin production)(Jaimez Ordaz et al., 2003; Yazdani et al., 2010).

Mycotoxin production and quantification

For mycotoxin production, fungi were grown in APA broth (NH_4 H_2PO_4 10g, K_2HPO_4 1g, $MgSO_4 \cdot 7H_2O$ 0.5g, KCl 0.5g, $FeSO_4 \cdot 7H_2O$ 0.01g, sucrose 30g, $HgCl_2$ 5×10^{-4} M, corn steep liqueur 0.5g, distilled water 1L) (Hara *et al*., 1974) for 4 days at 26°C. Quantitative analysis of aflatoxin production was performed with RIDA® QUICK Aflatoxin RQS kit from R-Biofarm AG, according to kit protocol.

Antagonistic interactions

The antifungal activity of LAB strains against *Aspergillus* was first examined using the double layer technique. MRS plates were first spotted with fresh LAB suspensions (5 µl) and after 48 h of incubation at 36°C there were covered with 5ml of 0.6% PDA containing fungal spores. Plates were than incubated at 27°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 all around the bacterial spot. Microbial interactions were microscopically examined.

In a second method, the fungi were streaked diagonal in plates containing MRS or PDA. The LAB were subsequently placed at the both sides o the fungi. The incubation was performed at 37°C or 30°C for at least 5 days, and inhibitory areas were examined.

HPLC analysis of LAB antifungal compound

Using HPLC method two antifungal compounds were determined, lactic and acetic acids. Therefore, LAB broth cultures were centrifuged, and clear supernatant was diluted 10 times. Samples were filtered through 0.22µm PVDF filter discs before HPLC analysis. Chromatographic separation and quantification were done using Alliance HPLC System (Waters). Data acquisition and management were completed with the Empower 2 software.

RESULTS AND DISCUSSIONS

1. Screening of antifungal action of new LAB isolates

123 isolates of LAB strains were selected from various plant materials. Among them, about 45% presented inhibitory action against at least one plant pathogenic fungi (fig.1).

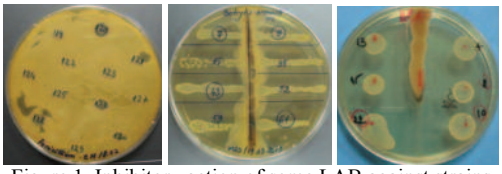
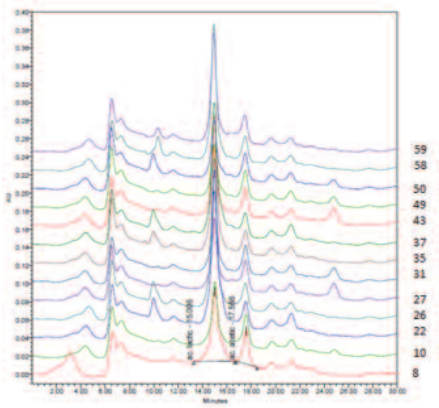


Figure 1. Inhibitory action of some LAB against strains of *Penicillium spp., Botrytis cinerea* and *Alternaria alternata* (Alt)

The best results were obtained with 12 LAB strains isolated from fermented wheat bran and from sauerkraut: they presented a broad spectrum of activity against selected fungi. The production of organic acids is clearly involved in antifungal action, at least for the most active LAB: strains 22, 26, 35 and 58 (fig.2).

These results suggest that inhibitory activity of the selected LAB is due mainly to the production of fermentation end products such as lactic acid, acetic acid (as it was proved by HPLC analysis) or diacetyl, acetaldehyde and hydrogen peroxide. Although, some of the selected LAB strains used in these experiments produce bacteriocins, no antifungal activity was associated with this (data not shown).



Sample	Lactic Acid (µg/ml)	Acetic Acid (µg/ml)
8	18,325	6,228
10	12,002	5,723
22	**32,781**	**3,831**
26	**28,754**	**4,105**
27	12,095	3,991
31	15,303	4,814
35	26,82	3,513
37	25,662	2,764
40	17,479	4,821
43	11,687	4,459
49	9,278	5,042
50	10,118	4,342
58	**25,689**	**3,805**
59	18,969	3,648

Figure 2. HPLC quantification of lactic and acetic acids produced by selected LAB strains

2. Inhibition of mycotoxigenic fungi by selected LAB

Aflatoxin and ochratoxin contamination of food and feed cause negative effects on human and animal health and the prevention of mycotoxins production/accumulation could be achieved by LAB, as presented various authors (Reddy et al., 2010). For these reason, the ability of selected LAB to inhibit the growth of several *Aspergillus flavus*, *A.oryzae* and *A.ochraceus* strains was examined. Results shown that, among the 123 LAB isolates tested, at least 24 were able to inhibit almost all the aspergilli strains tested (table 1).

Table 1. Growth inhibition of different aspergilli by LAB

LAB	*Afl*	Asp 4.3	Asp1	MI156	Ochra5	*Ochra* Am
7	+	±	+	+	+	+
8	+	+	+	+	+	+
9	+	±	+	+	+	+
10	+	+	+	+	+	+
13	+	+	+	+	+	+
15	+	±	+	+	+	+
22	+	+	+	+	+	+
26	+	±	+	+	+	+
27	±	+	+	+	+	+
31	±	+	+	+	+	+
35	+	+	+	+	+	+
37	+	±	+	+	+	+
40	±	+	±	+	+	+
43	+	+	+	+	+	+
49	+	+	+	+	+	+
50	±	±	+	+	+	+
53	+	±	+	+	+	+
58	+	+	+	+	+	+
61	+	+	+	+	+	+
94	–	–	–	+	–	+
115	+	±	+	+	+/±	+
120	+/±	+	+	+	+	+
126	+	+/±	+/±	+	±	+
135	+/±	+	+	+	+	+
Lpl	+	+	+	+	+	+

Where: + = inhibition of the fungal growth; ± = slight inhibition of the fungal growth; – = no inhibition

Moreover, important damages of the fungal hyphae (fragmentation, loss of the cellular content, twisting of hyphae or apical swelling) due to interactions with the strains LAB58 and LAB35 were observed for *A.oryzae* and *A.ochraceus* (fig.3).

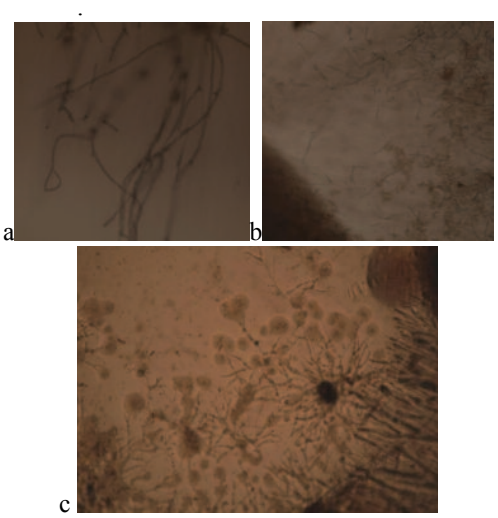


Figure 3. Microscopically aspects of LAB action against *A.oryzae* mycelium (a, b) and *A.ochraceus* mycelium (c)

Similar effects were also observed against *A.niger* strains, growth inhibition (fig.4, left) and mycelium alterations were induced by LAB (Fig.4, right).

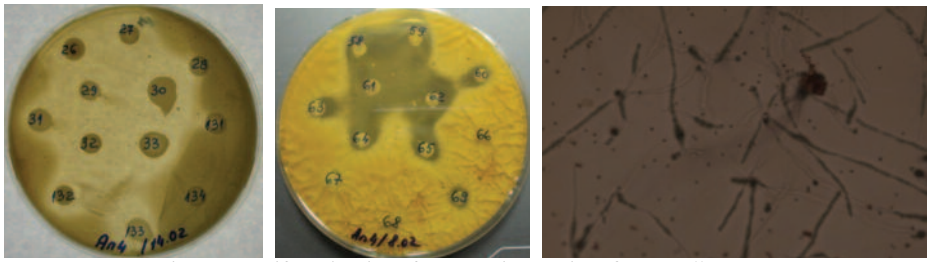


Figure 4. Antifungal action of LAB against strains of *Aspergillus niger*

The ability of natural *Aspergillus spp.* to synthesize mycotoxins is not a general feature, but higly mycotoxigenic strains could be isolate from infected materials and products. The most rapid and simple screening methods of aflatoxin producing fungi involve cultivation on selective media and/or examination of culture characteristics. In this respects, 17 natural isolates of *Aspergillus spp.* were selected from cereal seeds and preliminary identified as *A.flavus/A.parasiticus* (13 strains), and *A.niger* (4 strains). Their ability to produce mycotoxins was examined on selective media. On coconut medium, blue fluorescence (associated with aflatoxin production) on reverse side of some fungal colonies, as well as green-blue fluorescence (associated with ochratoxin production) was observed in UV light (Fig. 5).

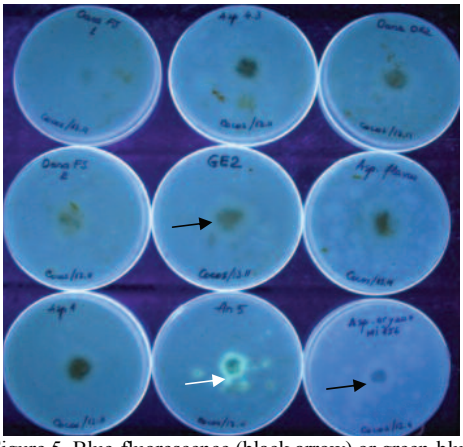


Figure 5. Blue-fluorescence (black arrow) or green-blue fluorescence (white arrow) on reverse side of some fungal colonies grown on coconut medium

Better results were obtained when fungi were cultivated on PDA supplemented with MβCD and sodium deoxycholate, were the presence of an opaque (white) halo around the colonies suggest aflatoxin production. Significant differences were noticed between the tested fungal strains, when this medium was used (Fig. 6).

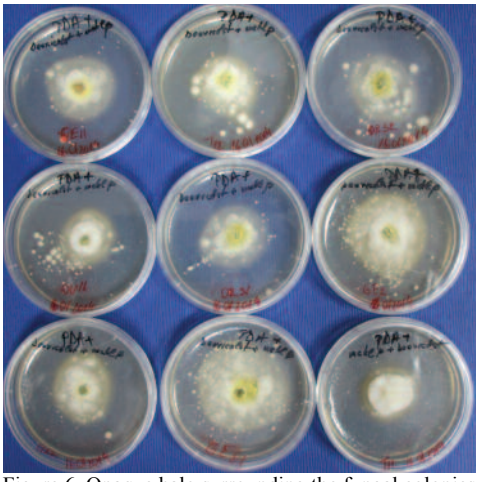


Figure 6. Opaque halo surrounding the fungal colonies associated with aflatoxin production.

The high mycotoxin producing strains selected in these experiments were GE21, T11, GE2 and G32. Significant amounts of aflatoxin were detected using RIDA® QUICK Aflatoxin RQS kit, when these strains were grown in APA broth for 4 days. The aflatoxin level was between 6.1-6.5 ppb, comparing with 5.2. ppb detected for *A.flavus* used as reference.

The effect of LAB against the four aflatoxigenic strains was examined (fig.7) and at least three LAB strains (35, 58 and 26) confirm their high and stable inhibitory action (the areas of inhibition were maintained even after 14 days of incubation). These results suggest that the antifungal action could be due not only to organic acids, but to other possible mechanisms.

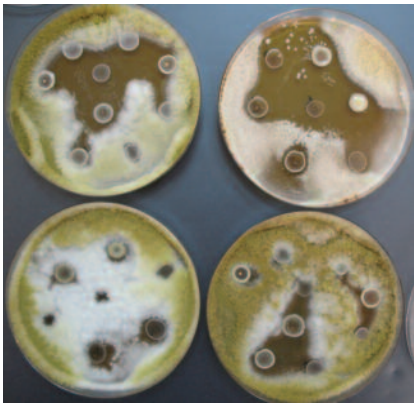


Figure 7. Inhibitory action of some LAB against selected aflatoxigenic strains

CONCLUSIONS

Among the 123 LAB isolates, 45% presented inhibitory action against at least one plant pathogenic fungi and 21% of the strains were active against *Aspergillus spp*.

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.

The production of organic acids is clearly involved in antifungal action, at least for the most active LAB strains: 22, 26, 35 and 58.

17 natural isolates of *Aspergillus spp.* were selected from cereal seeds and preliminary identified as *A.flavus/A.parasiticus* (13 strains), and *A.niger* (4 strains).

Their ability to produce mycotoxins was determined on coconut medium and on PDA medium supplemented with 0.3% MβCD and 0.6% sodium deoxycholate, and quantified using RIDA® QUICK Aflatoxin RQS kit.

Four isolates, designated as GE21, T11, GE2 and G32 were selected for their higher level of aflatoxin and used for interactions with LAB.

At least three LAB presented a broad spectrum of activity against almost all fungi used in experiments, including the natural aflatoxigenic isolates (GE21, T11, GE2 and G32), the inhibitory action being strong and stable even after 14 days of cultivation

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

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