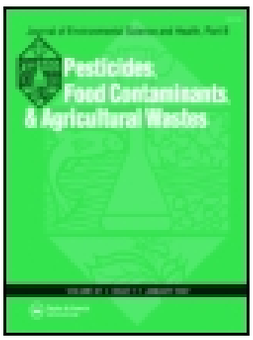

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Biological control of AFB_1-producing *Aspergillus* section *Flavi* strains isolated from brewer's grains, alternative feed intended for swine production in Argentina

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ABSTRACT

The aim of the present study was to investigate the inhibitory activity of lactic acid bacteria (LAB) isolated from brewer's grains on *Aspergillus* section *Flavi* growth and aflatoxin B_1 production. The *Aspergillus* strains tested were inhibited by all the LAB strains assayed. The isolates *Lactobacillus brevis* B20, *P. pentosaceus* B86, *Lactococcus lactis* subsp. *lactis* B87, *L. brevis* B131, and *Lactobacillus* sp. B144 completely suppressed the fungal growth and reduced aflatoxin B_1 production. In conclusion, LAB isolated from brewer's grains show a high inhibitory activity on fungal growth and aflatoxin biosynthesis by *Aspergillus flavus* and *Aspergillus parasiticus*. Further studies must be conducted to evaluate the success of *in vitro* assays under food environment conditions and to elucidate the antifungal mechanism of these strains.

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KEYWORDS
Aflatoxins; antifungal activity; *Aspergillus flavus*; *Aspergillus parasiticus*; lactic acid bacteria

Introduction

Filamentous fungi are ubiquitous organisms distributed in several food commodities for human or animal consumption. These are responsible for undesirable spoilage and represent one of the main causes of economic losses in the food industry worldwide.[1,2] Aflatoxigenic *Aspergillus* species are important as storage deteriorative molds and mycotoxin producers, and the main species involved are *A. flavus*, *A. parasiticus*, and *A. nomious*.[3] In a previous study, aflatoxin (AF)-producing aspergilli species from brewer's grains were isolated; this by-product of the brewery industry is usually used as an alternative feed for animal production. In Villa General Belgrano, Córdoba, Argentina, farmers use brewer's grains for swine feeding due to their high nutrient content and low cost.[4,5]

Contamination of food by AFs frequently occurs in storage environments.[6] Under adequate temperature and humidity conditions, the aflatoxicogenic *Aspergillus* produce these toxins.[7] The economic impact of AFs in pig production includes loss of weight, rough hair coat, anorexia, ataxia, decreased feed conversion efficiency, trembling, coma, and death.[8–10] Indirectly, contaminated animal by-products represent a significant health risk to humans. Aflatoxin B_1 (AFB_1) has the highest toxicity among the four naturally occurring AFs: B_1, B_2, G_1, and G_2. AFB_1 is a potent hepatotoxic, carcinogenic, mutagenic, and teratogenic mycotoxin that causes immunosuppression in animals.[11]

Several strategies, including chemical, physical, and biological control methods, have been investigated to manage AFs in food. Biological strategies imply the application of living microorganisms or their metabolites to provide protection against pathogens or spoilage organisms in food. In this context, lactic acid bacteria (LAB) have long been used in food production and represent an interesting alternative as a bioconservation method. Moreover, most of LAB species are beneficial microorganisms and belong to Qualified Presumption of Safety (QPS) and generally recognized as safe (GRAS) status.[12] Their preserving effect mainly relates to competition for nutrients and space and production of organic acids, hydrogen peroxide, bacteriocins, and antifungal compounds.[13–17] Several researches suggest that LAB have specific mechanisms for reduction of mycotoxins in feeds, which include inhibition of their biosynthesis or decontamination by adsorption after their production.[17] Indeed, the use of antifungal and anti-mycotoxin LAB strains isolated from brewer's grains as biocontrol agents is a promising strategy since they are adapted to the substrate.

The aim of the present study was to investigate the inhibitory activity of LAB isolated from brewer's grains on *Aspergillus* section *Flavi* growth and AFB_1 production.

Materials and methods

Identification of microorganisms and culture conditions

Lactic acid bacteria strains were isolated from brewer's grains used as pig feedstuff in Villa General Belgrano, Córdoba, Argentina. Fourteen isolates were tested for their antifungal activity against aflatoxigenic *Aspergillus*. They were grown in De Man Rogosa Sharpe (MRS) agar at 37°C under microaerobic conditions for 24 h and were stored at −20°C in MRS broth containing 30% (v/v) glycerol. LAB cultures were identified using the following tests: Gram stain, production of catalase and cytochrome oxidase, production of CO_2 from glucose,

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growth at different temperatures (10°C and 45°C), growth at different pH values (4.4 and 9.6), growth at different NaCl concentrations (6.5% and 18%), and production of acid from different carbon sources (glycerol, erythritol, D-arabinose, L-arabinose, D-xylose, D-adonitol, D-galactose, D-glucose, D-fructose, D-mannose, L-rhamnose, Dulcitol, Inositol, D-mannitol, D-celobiose, D-maltose, D-lactose, D-melibiose, D-saccharose, D-trehalose, D-rafinose, and starch).[18]

Three strains of *Aspergillus* section *Flavi* isolated from brewer's grains were selected according to their high capacity to produce AFB_1. The isolates were identified as *A. flavus* AF21, *A. flavus* AF54, and *A. parasiticus* AP60 according to their macroscopic and microscopic morphological characters,[19] and were maintained at 4°C on malt extract agar (MEA) and at −80°C in 15% glycerol.

The microorganisms were kept in the culture collection at the Department of Microbiology and Immunology, National University of Río Cuarto, Córdoba, Argentina.

In vitro *antifungal activity by LAB*

Lactic acid bacteria were tested against *Aspergillus* section *Flavi* strains by the agar diffusion method. Each LAB suspension was prepared in MRS broth and adjusted to a final concentration of 1.5×10^8 CFU/mL. One milliliter of each bacterial suspension was mixed and homogenized with 20 mL of MRS broth added with 1.2% of agar and poured into sterile Petri plates. Plates were then inoculated in the center with a suspension of fungal spores in semisolid agar from seven-day-old cultures on MEA, and incubated for seven days at 25°C. MRS plates with the fungal pathogen that were not inoculated with LAB served as controls.

Determination of fungal growth

Two radii from each colony, perpendicular to each other, were measured daily and the mean radius value was used to calculate fungal growth. The radii of the colonies were plotted against time, the growth rate (mm/day) was calculated by the linear regression of the linear phase for growth, and the time at which the line intercepts the *x*-axis was used to calculate the lag phase.

Aflatoxin B_1 analysis

From each treatment and control, three agar plugs were removed from the central area of the colony, weighed, and introduced into an Eppendorf tube. Then 0.5 mL of chloroform was added, and the mixture was centrifuged at 8154 *g* for 10 min. The chloroform phase was transferred into a clean vial tube and evaporated to dryness under nitrogen flow. Synthesis of AFs was quantitatively determined by high performance liquid chromatography (HPLC), following the method described in a previous study.[4]

Statistical analyses

Results obtained in a triplicate assay were analyzed by using analysis of variance (ANOVA). The Tukey's test was applied to compare means and determine the influence of LAB on lag phase, growth rate, and AFB_1 levels of *A. flavus* and *A. parasiticus*. The analyses were conducted using the INFOSTAT software version 2011 (Córdoba, Argentina).[20]

Results and discussion

Inhibition of fungal growth

The 14 LAB strains used in the *in vitro* antifungal activity assays were identified as *Lactobacillus brevis* B20, *L. plantarum* B29, *L. paracasei* B38, *L. plantarum* B54, *L. plantarum* B57, *Pediococcus pentosaceus* B65, *L. brevis* B72, *P. acidilactici B82, P.* acidilactici B83, P. pentosaceus B86, *Lactococcus lactis subsp.* lactis B87, *L. brevis* B131, *L. brevis* B133, and *Lactobacillus sp.* B144. Isolates B20, B86, B87, B131, and B144 completely inhibited the growth of all of the *Aspergillus* strains tested. On the other hand, *P. pentosaceus* B65 and *L. brevis* B133 inhibited the mycelial growth of *A. flavus* AF21 and *A. parasiticus* AP60, while LAB B82 and B83 and B29, B38, and B57 completely inhibited the growth of AF21 and AP60, respectively.

Table 1 shows fungal lag phases (h) from control treatment and co-inoculation with different LAB. *L. paracasei* B38 and *L. brevis* B72 significantly increased this parameter for *A. flavus* AF21, as compared with controls. *L. plantarum* B29, B54, and B57 decreased mold latency time ($P > 0.05$). The LAB strains B29, B38, B65, B72, B82, B83, and B133 extended the lag phase of AF54, and *L. plantarum* B54 and B57 decreased it. Regarding *A. parasiticus* AP60, *L. plantarum* B54, *P. acidilactici* B82, and *P. acidilactici* B83 significantly extended its lag phase time, while *L. brevis* B72 was the only isolate that reduced the time of this parameter ($P > 0.05$).

The length of the fungal lag phase was variable and depended on the tested *Aspergillus* strain. The bacterial isolates B38, B72, B82, B83, and B133 increased this parameter, and this result might be due to the fact that bacterial metabolism is faster than filamentous fungi developed. Thus, LAB consumed nutrients faster and modified the conditions of the medium.

Table 1. Influence of LAB on AFB_1-producing *Aspergillus* lag phase.

Treatment	Fungal lag phase (h)		
	Aspergillus flavus AF21	*Aspergillus flavus* AF54	*Aspergillus parasiticus* AP60
Control	57.60^{e*}	50.90^{d}	9.60^{c}
LAB B20	ND**	ND	ND
LAB B29	44.20^{d}	102.50^{i}	ND
LAB B38	114.24^{f}	95.30^{h}	ND
LAB B54	1.20^{b}	7.68^{c}	33.12^{f}
LAB B57	10.80^{c}	2.16^{b}	ND
LAB B65	ND	125.76^{j}	ND
LAB B72	118.32^{g}	56.40^{e}	4.80^{b}
LAB B82	ND	87.84^{f}	28.32^{e}
LAB B83	ND	89.52^{g}	16.56^{d}
LAB B86	ND	ND	ND
LAB B87	ND	ND	ND
LAB B131	ND	ND	ND
LAB B133	ND	135.36^{k}	ND
LAB B144	ND	ND	ND

*Mean values with a letter in common are not significantly different according to the Tukey's test ($P < 0.05$).

**ND: No fungal growth was detected in the presence of LAB.

Accordingly, *Aspergillus* section *Flavi* strains must have had more time to adapt to this environment. Fernández-Juri et al.[21] reported that *Enterococcus* species isolated from the faeces of healthy dogs extended lag phases of aspergilla strains. LAB which increase lag phase are suitable for future studies since they delay the production of reproductive structures and the synthesis of secondary metabolites such as aflatoxin.

The mean growth rates of each *Aspergillus* strain that was co-inoculated with different LAB strains and the respective controls are shown in Figure 1. The growth rates of *A. flavus* AF21, *A. flavus* AF54, and *A. parasiticus* AP60 significantly decreased when co-inoculated with LAB strains ($P > 0.05$). The percentage reduction in growth rate caused by different LAB ranged from 4.5–63.7%; 27.1–66.4%, and 2.7–38.7% for *A. flavus* AF21, *A. flavus* AF54, and *A. parasiticus* AP60, respectively (Table 2).

The antimicrobial assays *in vitro* revealed that LAB strains isolated from brewer's grains significantly reduced the growth rate of *A. flavus* and *A. parasiticus*. These results are in agreement with those reported by Gerbaldo et al.,[22] who worked with probiotic *Lactobacillus* strains that showed growth rate inhibition of fungal species. In the present study, the LAB strains tested did not cause an increase of aspergilli growth compared with controls. This important result indicated that lactic bacteria from pig feedstuff samples had a good inhibitory effect *in vitro* against aflatoxigenic fungi isolated from the same substrate. The antimicrobial activity of LAB against fungi could be due to several mechanisms that include competition for nutrients, decrease in pH, and production of antifungal compounds.[17,23] Several compounds with a strong antifungal activity have been isolated from bacterial cultures. They have been identified as organic acids, reuterin, hydrogen peroxide, proteinaceous compounds, cyclic dipeptides, hydroxyl fatty acids, benzeneactic acid, 2-propenyl ester, and phenolic compounds.[17,24–31] Preliminary results of the research group have shown that some of the LAB strains assayed produce organic acids in addition to antimicrobial substances of protein nature (data not published).

Table 2. Decreasing percentages of fungal growth rate by lactic acid bacteria.

LAB strains	Reduction on fungal growth rate (%)		
	Aspergillus flavus AF21	*Aspergillus flavus* AF54	*Aspergillus parasiticus* AP60
B20	ND	ND	ND
B29	46.11	50.80	ND
B38	63.10	56.00	ND
B54	45.20	40.70	2.70
B57	44.50	47.70	ND
B65	ND	66.40	ND
B72	63.70	27.15	16.60
B82	ND	45.10	30.00
B83	ND	45.60	38.70
B86	ND	ND	ND
B87	ND	ND	ND
B131	ND	ND	ND
B133	ND	62.20	ND
B144	ND	ND	ND

ND: No fungal growth was detected in the presence of LAB.

Bacterial influence on aflatoxin B_1 production

Figure 2 shows the influence of LAB on AFB_1 production by aflatoxigenic *Aspergillus* strains. In the control treatment, the mean AFB_1 levels produced by *A. flavus* AF21, *A. flavus* AF54, and *A. parasiticus* AP60 on MRS medium were of 2393.86, 4031.7, and 75.7 ng/g, respectively. In the presence of B29, B38, B54, B57, and B72, mycotoxin synthesis by AF21 was significantly lower compared with the control treatment. The highest inhibition percentage (95.9%) was obtained with *L. brevis* B72. The LAB strains B29, B38, B54, B57, B82, B83, and B133 decreased the production of AFB_1 by *A. flavus* AF54 between 74% and 98.8%. The highest decrease in AFB_1 biosynthesis was observed when AF54 was co-inoculated with *L. paracasei* B38 ($P > 0.05$). The mean toxin level declined from 4031.7 ng/g in the control to 49.20 ng/g in the co-inoculation assay. *A. parasiticus* AP60 showed a significant reduction ($P < 0.05$) in AFB_1 production when grown in the presence of *L. plantarum* B54, *L. brevis* B72, and *P. acidilactici* B83. However, when interacting with *P. acidilactici* B82, AP60 significantly increased the production of AFB_1 compared with the control ($P > 0.05$).

Aflatoxins represent a worldwide problem for food storage that has important repercussions on health, agriculture, and economics. Indeed, prevention of toxin accumulation is a major issue for research groups. In the present work, we highlight that most of our LAB isolates significantly reduced AFB_1 biosynthesis by *A. flavus* and *A. parasiticus* compared with controls. Even though the mechanism of AFB_1 removal is still unknown, it has

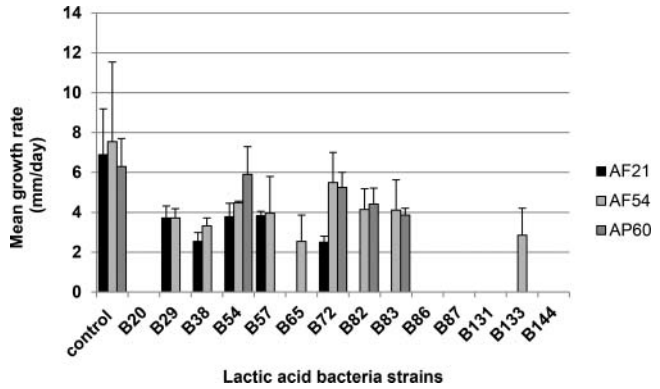

Figure 1. Growth rate (mm/day) of *Aspergillus* section *Flavi* strains co-inoculated with LAB isolated from brewer's grains.

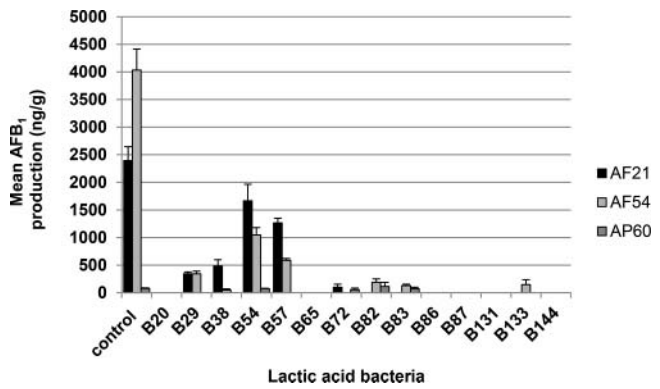

Figure 2. Aflatoxin B_1 (AFB_1) production by *Aspergillus flavus* and *A. parasiticus* isolates in presence of LAB isolates.

been suggested that the observed inhibitory effect on production of AFs by LAB could be attributed to their ability to reduce the growth of aflatoxigenic strains, consequently decreasing the accumulation of toxic metabolites.[32] Mycotoxins could also be removed by adhesion of LAB to cell wall components.[33,34] In addition, Aryantha and Lunggani[35] suggested that in a dynamic ecosystem where microorganisms interact, unfavorable environmental conditions as competition for nutrients and accumulation of toxic metabolites could induce survival mechanisms that involve synthesis of enzymes that use AFB_1 as nutrient. The LAB strains tested showed high reduction percentages of AFB_1 in the medium, with a maximum of 98.8% in the AF54–B38 interaction. In contrast, Zinedine et al.[36] reported that LAB isolated from Moroccan sourdough bread showed low percentages of reduction (1.8–45%). Kim [37] investigated the inhibitory effect of *Lactobacillus* and *Leuconostoc* strains on production of AFs by *A. parasiticus* and showed that reduction of AFB_1 production ranged from 21.6 to 59.7%.

Conclusions

Lactic acid bacteria isolated from brewer's grains show a high inhibitory activity on fungal growth and AF biosynthesis by *A. flavus* and *A. parasiticus*. The ability of lactic bacterial strains to suppress growth of *Aspergillus* section *Flavi* species could provide an alternative approach to overcome the development of contaminating aspergilli or the production of their mycotoxins during food storage. Further studies must be conducted to evaluate the success of *in vitro* assays under food environment conditions and to elucidate the antifungal mechanism of these strains.

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