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Full Length Research Paper

Inhibition of aflatoxin-producing aspergilli by lactic acid bacteria isolates from indigenously fermented cereal gruels

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A total of six lactic acid bacteria (LAB) isolates were selected from five indigenously fermented cereal gruels and identified as *Lactobacillus fermentum* OYB, *Lb. fermentum* RS2, *Lb. plantarum* MW, *Lb. plantarum* YO, *Lb. brevis* WS3, and *Lactococcus* spp. RS3. Six aflatoxin- producing aspergilli were also selected from the various food sources. Two of the isolates, identified as *Aspergillus parasiticus* C2 and *A. parasiticus* AF7, produced both aflatoxin B_1 and G while the other four identified as *A. flavus* M1, *A. flavus* B4, *A flavus* B5 and *A. flavus* C6, produced only aflatoxin B_1. Each of the LAB isolates inhibited the growth of at least a toxin-producing *Aspergillus*. The maximum inhibitions were shown by *Lb. plantarum* YO, which was able to inhibit the vegetative and sporulative growth of all the aflatoxin-producing aspergilli. *Lactococcus* spp. RS3 and *Lb. brevis* WS3 were only able to inhibit *A. parasiticus* C7 reasonably and *A. flavus* B5 and C6 mildly.

Key words: Inhibition, Lactic acid bacteria, aflatoxin-producing aspergilli.

INTRODUCTION

The lactic acid bacteria (LAB) are a broad group of gram-positive, catalase-negative, non-sporing rods and cocci, usually non-motile that utilize carbohydrates fermentatively and form lactic acid as the major end product (Aguirre and Collins, 1993). With occasional exception, they are aerotolerant. Several members of the lactic acid bacteria are well known for their ability to act as preservative agents in food products such as sauerkraut, fermented cereal gruels and legumes (Steinkraus et al., 1983). In such food products, LAB has the capacity to perform fermentative activities, which may result in active inhibition of spoilage and pathogenic bacteria. This inhibition is partly due to the production of fermentation end products such as lactic acid, diacetyl, acetaldehyde and acetic acid, which may accumulate to inhibitory levels in certain foods and beverages. In other cases, inhibition may be caused by inadvertent by-products of metabolic activity such as hydrogen peroxide or bacteriocins (Daeschel, 1989).

Aflatoxins are a worldwide important problem in public health, agriculture and economics. They are both acutely and chronically toxic to animals, including man, causing acute liver damage, liver cirrhosis, induction of tumors and teratogenic effects (Stoloff, 1977). The major naturally produced ones are Aflatoxins B_1, B_2, G_1 and G_2. Aflatoxin B_1 has been demonstrated in animals to be the most potent liver carcinogen (Pitt, 2000). Aflatoxins are produced in nature only by *A. flavus, A. parasiticus* and a recently described species *A. nomius* (Klich and Pitt, 1988).

There has recently been a significant commercial interest in using LAB as a natural food preservative to increase food safety and stability. This interest has been driven by the use of artificial preservatives, which has given rise to concerns from consumers, and an increased awareness of the microbiological safety of such foods. Nowadays, consumers favour foods with few chemical

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preservatives (Daeschel, 1993). As a result, there is increased interest in the preservation through LAB because of their safe association with human fermented foods. The common occurrence of LAB in foods and feeds coupled with their long-lived use contribute to their natural acceptance as GRAS (Generally Regarded As Safe) product for human consumption (Aguirre and Collins, 1993).

This present work aimed at studying the *in vitro* inhibition of toxin-producing Aspergilli strains by aqueous suspension of lactic acid bacteria isolates from indigenously fermented cereal gruels which may suggest their use as food preservatives against aflatoxin-producing aspergilli or as aflatoxin detoxificant in tropical foods and feeds.

MATERIALS AND METHODS

Collection of Samples

Samples of maize (*Zea mays*), sorghum (*Sorghum vulgare*) and millet (*Eleusine coracana*), from which the cereal gruels were prepared, were bought from Bodija Market in Ibadan Metropolis, South Western Nigeria. The food samples from which the aflatoxigenic aspergilli were isolated, were collected from different parts of Ibadan while the infected maize cobs were collected from the International Institute of Tropical Agriculture (IITA), Ibadan maize programme. An isolate code-labelled *Aspergillus parasiticus* C7 was obtained from the culture collection of the mycotoxin laboratory of the IITA, Ibadan.

Isolation and identification of lactic acid bacteria

Lactic acid bacteria were freshly isolated from samples of 2-day old fermented cereal gruels of white and yellow maize, white and red sorghum and millet. Sampling and isolations were carried out as described by Halm et al. (1993). Isolates were identified according to Kandler and Weiss (1986) and Garvie (1986) by cell and colony morphology, Gram staining, catalase test, growth at 15°C and 45°C, spore staining, motility test and other biochemical tests like oxidase, indole production, methyl red, Voges-Proskauer, liberation of ammonia from arginine, growth in 4% broth and carbohydrate fermentation pattern.

Fungal isolation and identification

Aspergillus isolates were obtained from maize and other food sources following surface sterilization in 70% alcohol and plating on Potato Dextrose Agar (PDA) medium. Pure culture of each isolate was obtained after incubation at room temperature. Identification was effected by mounting fungal mycelium on lactophenol-cotton blue and observing under x 40 objective lens of the microscope. Colony colour, growth pattern on plates, details of philiades and spores were also used as identification parameters with reference to Raper and Fennel (1965).

Screening for aflatoxin-producing aspergilli

Aspergillus isolates were screened for their aflatoxin production by use of thin layer chromatography (Munimbazi and Bullerman, 1998). The chloroform extracts which were spotted on 20 cm x 20 cm pre-made silica gel plates alongside the standard aflatoxin B_1 spots showed as blue fluorescence which turned to yellow after spraying with 50% (v/v) sulphuric acid (for further confirmation of the aflatoxin B_1) and viewed under the longwave of the ultraviolet light.

Preparation of LAB inoculum

Each of the lactic acid bacteria was suspended first in skimmed milk for upwards of 18 h taking samples every 3 h and determining the state of growth using a Neubauer haemocytometer until they showed steady growth. Thereafter each was transferred to sterile MRS broth in which the steady state was maintained at 10^6 cells/ml.

Inhibition of aflatoxin-producing aspergilli byLAB isolates

The six selected LAB isolates were assayed for inhibition on the six other aflatoxin-producing fungi by the modified overlay method of Magnusson and Schnurer (2000). Here, MRS agar plates on which LAB were inoculated as 2 cm long lines and incubated at 30°C for 48 h in anaerobic jars were overlaid with soft agar (75% by weight agar) preparation of PDA containing known inoculum size (9.5×10^4 spores/ml) of fungal spores determined by counting on a Neubauer haemocytometer. This was carried out in duplicate for each LAB isolate against each of the *Aspergillus* isolate and a control kept for each. The plates were then incubated aerobically at 30°C for five days. They were examined for clear zones of inhibition around the bacterial streaks and the clear zones were scored as -, d, +, ++, noting the dimension of the clear zones.

RESULTS

Several LAB isolates were obtained from the indigenously fermented cereal gruels but six of them were selected for the work. They were identified as *Lb. plantarum* MW, *Lb. plantarum* YO, *Lb. brevis* WS3, Lb. fermentum OYB, Lb. fermentum RS2 and *Lactococcus* spp. RS3. The fungal isolates were coded as M1, C2, B4, B5, C6 and AF7. M1, B4, B5 and C6 were identified as *Aspergillus flavus* and were characteristically greenish yellow on petriplate cultures with colonies reaching 6-8 cm in diameter within 10 days of growth on PDA. All other identification procedures were carried out according to Raper and Fennel (1965). Isolates C2 and AF7 were similarly identified as *Aspergillus parasiticus*.

The LAB isolates were then assayed for possible inhibition of the obtained Aspergilli isolates. The result is as shown in Table 1. All the LAB isolates inhibited at least one aflatoxin-producing fungal isolate to varying extents. Highest inhibition (16 mm) was observed with *Lb. plantarum* YO against *A. parasiticus* AF7. Above 10 mm inhibition zone was observed in *Lb. fermentum* OYB against *A. flavus* C6 (16 mm); *Lactococcus* spp. RS3 against *A. parasiticus* AF7 (14 mm); *Lb. fermentum* RS2 against *A. flavus* M1 (12 mm); and *Lb. plantarum* MW against *A. flavus* M1 (12 mm). The least zone of inhibition (0 mm) was observed with *Lactococcus* spp. RS3 against *A. flavus* B4.

Table 1. Measures of zones of inhibition of selected *Aspergillus** isolates by lactic acid bacteria from indigenous fermented foods.

Lab. isolate	*Lb. fermenutum*	*Lb. brevis*	*brevis Lacobacillus spp*	*Lb. fermentum*	*Lb. plantarun*	*Lb. plantarun*
Fungi isolate	OYB	WS3	RS3	RS2	MW	YO
A. flavus M1	++(7 mm)**	- (0)	+ (3 mm)	++ (12 mm)	++ (12 mm)	++ (15 mm)
A.parasiticus C2	++ (9 mm)	- (0)	d (1 mm)	+ (5 mm)	++ (8 mm)	++(10 mm)
A. flavus B4	d (1 mm)	- (0)	- (0)	++ (8 mm)	d (1 mm)	++ (6 mm)
A. flavus B5	++ (10 mm)	d (1 mm)	+ (5 mm)	++ (9 mm)	++ (14 mm)	++ (12 mm)
A. flavus C6	++ (16 mm)	d (1 mm)	+ (5 mm)	++ (9 mm)	++ (10 mm)	++ (8 mm)
A. parasiticus AF7	+ (5 mm)	++ (8 mm)	++ (14 mm)	++ (8 mm)	d (1 mm)	

*Inoculum size of 9.50 x 10^4 spores/ml.
**Figures in parenthesis represent measures of zone of inhibition (mm).
+: Indicates an inhibition zone $\leq$ 5mm.
++: Indicates an inhibition zone > 5mm
d: Indicates an inhibition zone only over the line of streak.
-: Indicates no inhibition zone.

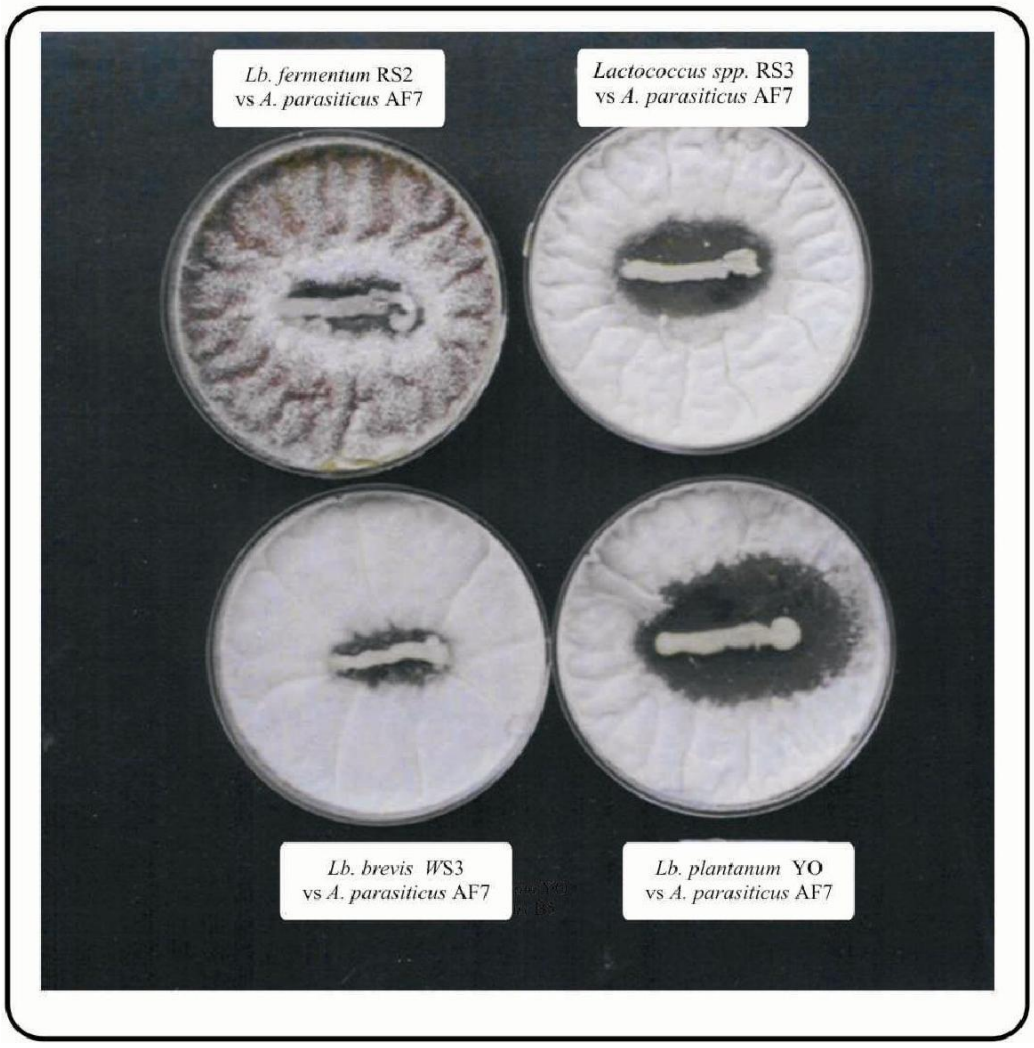

Figure 1. Efffect of isolates *Lb. fermentum* RS2, *Lactococcus* spp RS3, *Lb. brevis* WS3 and *Lb. plantarum* YO on *A. parasiticus* AF7 after 3 days incubation.

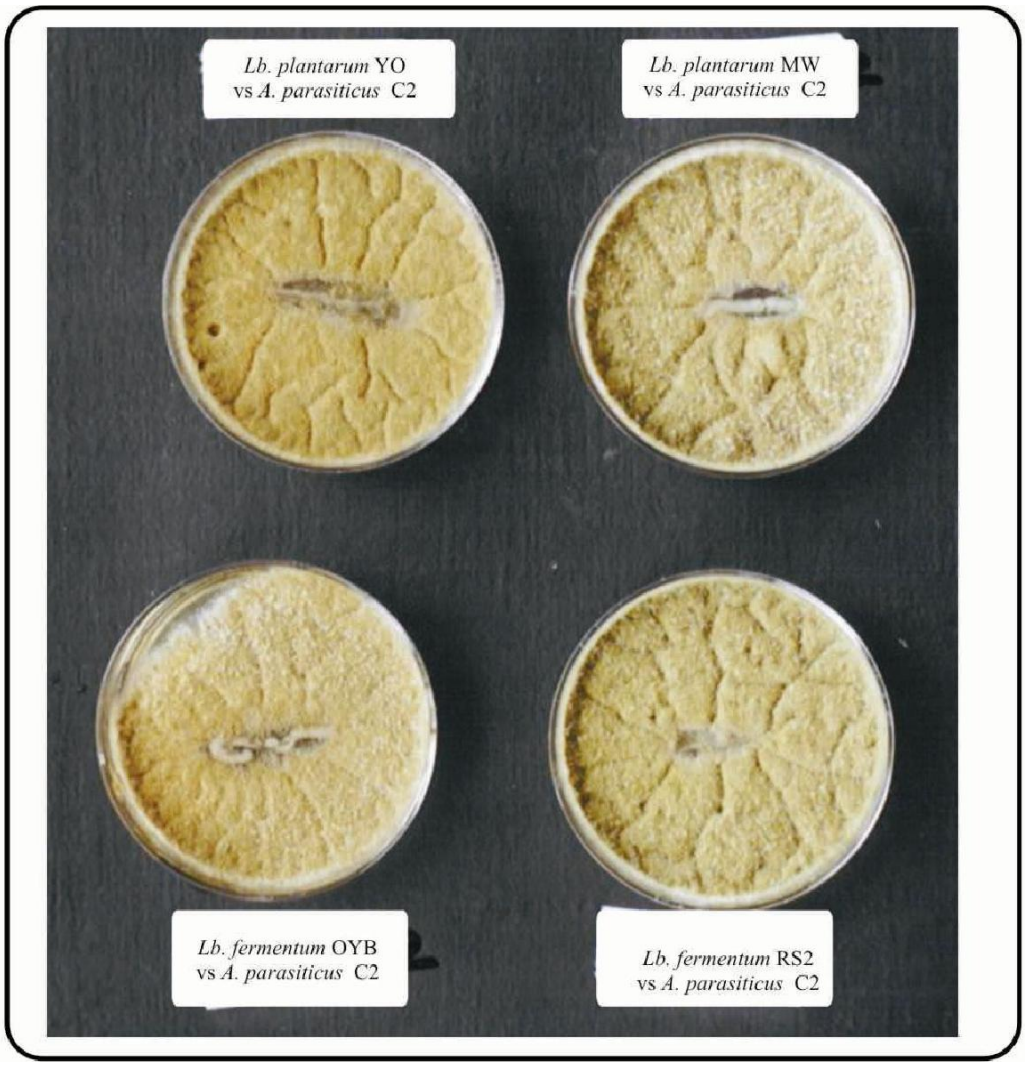

Figure 2. Effect of isolates *Lb. plantarum* MW, *Lb. plantarum* YO, *Lb. fermentum* RS2 and *Lb. fermentum* OYB on *A. parasiticus* C2 after 6 days incubation.

Other results also showed the lactic acid bacteria isolates to have effect on the different *Aspergillus* species prior to the sporulation of the latter. Here, *Lb. fermentum* RS2 was observed to exhibit maximum inhibition on mycelial development for most of the *Aspergillus* species tested while *Lactobacillus* spp. had the lowest.

Figure 1 shows the effect of isolates *Lb. fermentum* RS2, *Lactococcus* spp RS3, *Lb. brevis* WS3 and *Lb. plantarum* YO on *A. parasiticus* AF7 after three days incubation. *Lb. plantarum* YO had the maximum inhibition. It is serially followed by *Lactococcus* spp. RS3 while *Lb. brevis* WS3 and *Lb. fermentum* RS2 had almost equal inhibition. Figure 2 also show the effect that *Lb. plantarum* YO, *Lb. plantarum* MW, *Lb. fermentum* OYB and *Lb. fermentum* RS2 has on sporulating *A.parasiticus* C2. To a good extent, the inhibitory actions of the *Lactobacillus* spp seem to be reduced permitting sporulation to go ahead.

DISCUSSION

Aflatoxins are a worldwide important problem of public health, agriculture and economic concern. The aspergilli producing these aflatoxins grow on a wide range of substrates. The aflatoxins-producing aspergilli used in the current work are those whose occurrence have been reported in various food and feed sources including cereal grains, wheat and its products such as oil seeds and bread (Pitt, 2000). This, therefore, calls for efficient and safe procedures for preservation of foods against invading fungi as well as safe decontamination of aflatoxins-contaminated food and feed sources.

LAB has been reported to be involved in antimicrobial activities but few reports of the antifungal activities have been published. This present work clearly shows the antifungal effects of *Lb. plantarum*, *Lb. fermentum*, *Lb. brevis* and a *Lactococcus* spp. on aflatoxigenic fungal

isolates. Antifungal activities by a *Lb. casei* strain that inhibited both the growth and the aflatoxins production of *A. parasiticus* has been reported (El Gendy and Marth, 1981; Vanne et al., 2000). Indeed, Vanne and co-workers (2000) showed that the growth of toxigenic storage fungi could be restricted by LAB *in vitro*. Karunaratne and co-workers (1990) reported a *Lb. plantarum* that was able to inhibit the growth of *A. flavus* but felt the effect was due to a combination of acidity and microbial competition. This conclusion was further clarified through use of the same inocula strains by Gourama and Bullerman (1995). From the results, it could be safely concluded that the action of the suspensions of lactic acid bacteria used in this work is broad all being active against more than one *Aspergillus* sp. Similar result has been reported by other workers (Lavermicocca et al., 2000).

Further work as to identify as well as characterize the active ingredient involved in the *in vitro* inhibition of the toxigenic *Aspergillus* strains by LAB species needs to be carried out as to prescribe the necessary procedure for its use in the preservation of various food and feed ingredients.

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