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Removal of aflatoxins by viable and heat-killed *Lactobacillus* species isolated from fermented maize

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ABSTRACT
Objectives: Contamination of food and animal feed with aflatoxins produced by *Aspergillus flavus, A. parasiticus* and *A. nomius* is a worldwide problem. Aflatoxins have toxic, carcinogenic and mutagenic potential with adverse effect on human and animal health. This study investigated a novel and cost effective method for reducing aflatoxins content.
Methodology and results: Five strains of *Lactobacillus* species (*L. brevis, L. acidophilus, L. casei, L. delbruekii, L. plantarum*) were isolated from fermented maize (Ogi) and characterized using sugar fermentation and other standard biochemical tests. Viable and heat-killed bacteria were incubated with maize grains artificially contaminated with *A. flavus*. Residual aflatoxins in the maize grains were quantified using enzyme linked immunosorbent assay (ELISA). All viable and heat-killed bacterial strains were able to bind aflatoxins in maize. The binding activities by viable test strains ranged from 32 to 75% and 32 to 95% for live and heat-killed strains, respectively. The ability of the viable and heat-killed strains to bind aflatoxins was most efficient after 72h. The most efficient organism was heat-killed L. *plantarum*, which bound 76ng/g of aflatoxins (95%) out of the original 80ng/g leaving a residual of 4ng/g aflatoxins in the fermented maize.
Conclusion and application of findings: This study demonstrated that aflatoxin can be effectively removed using heat-killed *Lactobacillus* species. This process is also strain specific as *L. plantarum* bound more aflatoxins than other strains tested. The high detoxification rates by *Lactobacillus plantarum* indicate potential for application in food and feed processing industries.

Key words: Aflatoxins, binding activity, detoxification, *Lactobacillus* species, maize-fermentation

INTRODUCTION

Aflatoxin B1(AFB1) is a mycotoxin that frequently contaminates poorly stored products destined for human or animal consumption (Mokoena *et al*., 2006).This toxin, which is one of the most potent naturally occuring mutagens and carcinogens known, is produced by some strains of *Aspergillus flavus, Aspergillus nomius and Aspergillus parasiticus* (Var & Kabak, 2004). It has been estimated by the Food and Agriculture Organization that 25% of the world's crops are affected by mycotoxins (Giessen, 1998). It is one of the most potent naturally occuring mutagens and carcinogens known.

Aflatoxin B1 has been reported in virtually all types of food and feedstuff. Extensive review of human foodstuffs in Nigeria revealed that 50% of yam flour, 30% of garri, 40% of cassava, 20% of beans and melon and 10% of rice were AFB1

positive in a survey carried in Benin-City, Nigeria (Oluwafemi, 2000). Galvano *et a*l. (1998), Battacone *et al.* (2003) and Gunsen & Buyukyoruk (2003) confirmed the presence of AFM1, a derivative of AFB1, in dairy products in Turkey. Aflatoxins came to public prominence after deaths of domesticated animals e.g. the contaminated dog food produced by Diamond Pet Foods in the US in 2005 (FDA, 2005) and people e.g. in 2004 and 2005 in Kenya (Lewis *et al*., 2005).

In Benin City, Nigeria, aflatoxin levels have been reported to be significantly high in male blood and semen (Oluwafemi, 2000). Physiological and toxicological responses of aflatoxins have been demonstrated on rats and day-old chicken (Abdel *et al*., 2002; Oluwafemi& Taiwo, 2004). Dietary exposure of young children to high levels of aflatoxin in West Africa has also been detrimental (Gong *et al*., 2003), with increased risk of liver cancer and impaired growth (Turner *et al*., 2005).

Mycotoxins negatively impact agriculture and associated industries, in different ways, in all parts of the globe (Visconti, 2006). In the US , the estimated annual loss due to fungal and mycotoxin contamination of food and feed is upto 1.6 B $ (Otsuki *et al.,* 2001). A World Bank report estimated that African nations lose about 670 B $ in foreign exchange to trade related effects of aflatoxins (Otsuki *et al*., 2001).

Numerous detoxification methods include use of propionic acid and its salts, sodium bisulfite and sodium hydroxide and nisin (Ghosh *et al*., 1999; Paster *et al*., 1999; Moreno *et al*., 2000). Oatley *et al.* (2000); Pierides *et al*. 2000; Var & Kabak, 2004; Onilude *et al*., 2005; and Abdella *et al*., 2005; Elgerbi *et al.* (2006) suggested that AFB1 can bind to bifidobacteria and lactic acid bacteria. Detoxification of aflatoxin with activated carbon (Galvano *et al.,* 1996), sodium alumino-silcate (Galvano *et al*., 1996), spices (Oluwafemi & Taiwo, 2004) was also achieved. Teniola *et al*. (2005) detoxified AFB1 using *Rhodococcus erthropolis* isolated from contaminated soils.

However, limitations such as products loosing nutritional and organoleptic qualities, undesirable health effects of such treatments and expensive equipment required for degradation techniques has encouraged recent emphasis on biological methods (Dorner *et al*.,1999; Taylor & Draughon, 2001). Limitations of biological methods include long degradation time (≥ 72h), incomplete degradation, non-adaptation to typical food systems, culture pigmentation, and odour production which can reduce their potential for use in the food industry.

Traditional fermentation of maize into *ogi* was found to reduce aflatoxin levels in the product by 50% (Oluwafemi & Ikeowa, 2005). The organisms isolated after 72 hours of fermentation which were predominantly lactic acid bacteria were thought to be responsible for this degradation. Since LAB occurs naturally in many food systems and have been a part of the human diet for centuries, they have been considered as safe organisms to consume. LAB has been documented to specifically inhibit the growth of microorganisms (Gourama & Bullermann, 1997). The mechanisms of removal of aflatoxins by lactic acid bacteria are not clear (Mokoena *et al*., 2006) though some authors suggest that aflatoxins are immobilized on cell.

The current study aimed to study detoxification of aflatoxin and to investigate whether LAB isolated from ogi bind aflatoxins better when viable or heat-killed.

MATERIALS AND METHODS

Collection of samples: Samples of maize were obtained from Abeokuta metropolis, Ogun State, Nigeria. The samples were taken to the laboratory for experimentation.

Microbial analysis: Fifty grammes of maize grains was weighed per bowl into six medium sized bowls and 500 ml of water added to each and labeled according to the hours each of the bowls containing maize grains were left to ferment, i.e. A = 0 hr, B = 24 hrs, C = 48 hrs, D = 72 hrs E = 96 hrs.

The zero hour sample was milled and analyzed immediately, this served as the control. At the end of each fermentation period, maize grains were milled in the laboratory using a warring blender. The

milled maize samples were filtered to separate the Ogi from the bran using white muslin cloth.

Microbial analysis: One gramme of the milled maize was serially diluted with distilled water and 0.1 ml each of the serially diluted sample was inoculated on de Man Rogosa Sharpe agar (MRS agar) and Saboraud Dextrose Agar (SDA) respectively. Plates containing MRS agar were incubated anaerobically at 37 °C for 48 hours while SDA plates were incubated aerobically at 28 °C for 5 days. Observations were recorded and isolates identified using cultural characteristics and morphological characteristics, biochemical test and sugar fermentation tests.

Extraction of aflatoxins: Twenty grammes of Ogi were weighed into a clean conical flask into which 200 ml of chloroform was added to it. The flask was sealed and shaken at 25 (rpm) for 10 minutes. Whatman No 1 filter paper was used to filter the samples and the filtrates were evaporated to dryness in a water-bath.

Enzyme-linked immunosorbent assay: The dilution strips were placed in a microwell strip holder. Equal number of antibody coated microwell strips were plated in a microwell strip holder. Two hundred microlitre of conjugate was dispensed into each dilution well. One hundred microlitre of each standard extract was added using a micropipette. One hundred microlitre of the contents in the dilution well was transferred into a corresponding antibody coated microwell. The samples were incubated at room temperature for fifteen minutes. The contents of the microwell strips were emptied and the microwells were washed three times. One hundred microlitres of stop solution was added into each microwell strip and the color formation noted. The strips were read with a microwell reader using a 450nm filter.

pH determination: The pH of the steep water was determined using a pH meter (Mettler Delta 340). The electrode connected to the pH meter was standardized using buffer solutions (4 and 6.8). Three readings were taken for each sample and the average recorded.

Characterization of Lactobacillus species: Lactic acid bacteria used in this work were isolated from 72h traditional "*Ogi*" fermentation on de Man's Rogosa Sharpe (MRS) agar at 37 °C for 48h to obtain pure culture of the isolates. Pure cultures of the isolates were identified using the conventional scheme of Kandler and Weiss (1986), with complementary fermentation test on API 20 AUX kit (API System, Montalieu Vercieu, France). The lactic acid bacteria were reactivated by culturing on de Man, Rogosa & Sharpe (MRS) agar and broth media at 37 °C for 48h.

Inoculation of artificially contaminated maize with *Lactobacillus species*: Maize grains were autoclaved twice at 121 °C for 15 min and inoculated with toxigenic strain of *A. flavus* for six days (Oluwafemi *et al*., 2008). The activity of toxigenic strain of the *Aspergillus flavus* was stopped by sterilizing the cultured grains for 15 min at 121°C. The grains were washed with water to get rid of spores and mycelia. After washing, grains were dried at 55 °C for 72h and moisture content was lowered to 13%. They were subsequently stored in the cold room at 4 °C prior to detoxification studies.

Inoculation of artificially-contaminated maize with Lactobacillus species: Maize grains containing known amount of aflatoxins were fermented as earlier described and inoculated with viable and heat-killed *Lactobacillus acidophilus, L. brevis, L. casei, L. delbrueki*, and *L. plantarum*. Heat-killed Lactobacillus species were obtained by autoclaving at 121°C for 40 min according to the method described by Haskard *et al.* (2001).

Statistical analysis: The results were analyzed statistically using ANOVA (Analysis of Variance) and Means separated by least significant differences.

RESULTS AND DISCUSSION

Lactic acid bacteria (LAB) are of major importance among the bacteria associated with traditional fermented foods. The largest spectrum and richest variety of lactic fermented food is probably found in Africa (Holzapfel, 2002). The association of LAB with the human environment and their beneficial interactions both in food and in the human intestinal tract, combined with the long tradition of lactic fermented foods in many cultures, have led to the conclusion that these foods may be "generally recognized as safe" (GRAS) (Holzapfel, 2002). Our results (Table 1) show that LAB are associated with the fermentation of maize to produce Ogi. As fermentation progressed, LAB increased in population and there was a progressive reduction of aflatoxins which was significant ($P < 0.05$) after 72 hours. As the concentration of aflatoxin decreases the acidity increases showing that lactic acid bacteria present in the fermented maize is responsible for detoxification. This finding is in agreement with that of Holzapfel (2004). Organic acids, which show antagonistic effects in the un-dissociated form at lower pH values, are particularly effective in inhibiting toxins produced by pathogens.

Fermenting maize for a minimum of three days is an effective form of detoxification method (Table 1) that can reduce aflatoxin significantly. From the results obtained in this work, it is apparent that lactic acid bacteria have the potential to detoxify aflatoxin as earlier showed by Onilude *et al*. (2005) with the suggestion that the end products of fermentation of maize such as lactic acid, diacetyl, and acetaldehyde inhibit spoilage organisms.

Table1: Characterization of lactobacillus species sugar fermentation test.

Fructose	Galactose	Glucose	Gluconate	Lactose	Maltose	Mannitol	Mannose	Raffinose	Rhamnose	Ribose	Salicin	Sorbitol	Sucrose	
+	+	+	+	-	+	-	+	+	-	-	+	-	+	*L. acidophilus*
+	+	+	+	+	-	-	+	+	+	+	+	-	+	*L brevis*
+	+	+	+	+	+	+	+	-	-	+	+	+	+	*L casei*
+	-	+	-	+	-	-	-	-	-	-	-	-	-	*L. delbrueckii*
+	+	+	+	+	+	+	+	+	-	+	+	+	+	*L. plantarum*

+ = Positive, acid and gas produced; - = Negative, no acid no gas produced.

The detoxification of aflatoxin by Lactobacillus species was quantified using ELISA and it was found to be strain dependent (Table 3). According to Onilude *et al*. (2005) the various antimicrobial compounds produced by Lactobacillus species are thought to have contributed to species binding activity of aflatoxins. The aflatoxin binding activities of LAB were demonstrated with reduction in residual aflatoxins from 80 to 4 ng/g and most efficient binding of aflatoxin being observed for the heat- treated *L. plantarum*.

From literature, specific dairy strains of Lactobacillus species remove aflatoxin M_1 from reconstituted milk (Pierides *et al*. 2000; Var & Kabak, 2004; Elgerbi *et al*., 2006). This study also showed that Lactobacillus species isolated from fermented maize used for the production of ogi was able to significantly reduce aflatoxins in maize.

Treatment of Lactobacillus species with heat affected the binding mechanism. Heat treatment significantly enhanced the detoxification process as there was significant difference between heated and non-heat treated Lactobacillus species (Table 3). Autoclaving causes denaturation of bacterial proteins and enzymes leaving behind the peptidoglycan which may be responsible for increasing binding activity observed in this study. Rajendran (1998) reported the binding of 8 different food-borne carcinogenic heterocyclic amines to 25 bacterial isolates and concluded that lyophilized cells and pure cell walls exhibit higher binding activity than bacterial cells which supports our findings. Haskard *et al*. (2001) reported surface binding of aflatoxins to Lactobacillus species. According to Guan *et al*. (2005) peptidoglycans in organisms have the tendency to bind foreign matter coming into the organism so as to give some sort of protection against infections.

Table 2: Detoxification time of aflatoxin by *Lactobacillus* species.

Fermentation period(h)	Aflatoxins(ng/g)	LAB (10^4)	Fungal count(10^4)	pH	pH and titrable acidity
0	160	1	12	6.8	0.124 M
24	160	2	7	5.0	0.134M
48	160	3	4	4.8	0.140M
72	6	5	3	4.6	0.167M
96	6	9	2	4.0	0.169M

Comparison between heat-treated and non heat-treated strains (Table 3) showed statistically significant differences. For heat-treated strains of *Lactobacillus brevis* there was a reduction of aflatoxins levels from 80 ng/g and to 54 ng/g after 24 hours, and to 20 ng/g after 48 hours. However the most prominent detoxification

was seen with *L. plantarum* which had residual aflatoxins levels of 4 ng/g from an initial level of 80 ng/g.

This study showed that aflatoxin levels are reduced by LAB, probably through adsorption to the cell wall, and that that the detoxification is more effective with heat-killed Lactobacillus species Incorporation of *Lactobacillus plantarum* as starter culture can serve to reduce risks associated with aflatoxins and thus increase safety and the nutritional value of foods. The high detoxification rates by *Lactobacillus plantarum* indicate this isolate would be a potential candidate for application in food and feed processing.

Table 3: Detoxification of aflatoxins using heated and non heated strains of *Lactobacillus species*.

Time (h)	*L. acidophilus(ng/g)*		*L. brevis(ng/g)*		*L. casei(ng/g)*		*L. delbruekii(ng/g)*		*L. plantarum(ng/g)*	
	NH	H	NH	H	NH	H	NH	H	NH	H
0	80	80	80	80	80	80	80	80	80	80
24	50	48	54	54	50	48	50	50	80	52
48	50	50	54	20	48	30	40	35	20	4
72	52	50	54	20	48	30	40	35	20	4

N = Non-heat treated; H = Heat-killed

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