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POTENTIAL OF LACTIC ACID FERMENTATION IN REDUCING AFLATOXIN B1 IN TANZANIA MAIZE-BASED GRUEL

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ABSTRACT

Aflatoxins are toxic by-products of fungi, with harmful effects on human and animal health. Although maize is known to be highly susceptible to aflatoxin contamination, and a staple in many African countries, there is still lack of methods to mitigate the effects. The effect of lactic acid fermentation on reduction of aflatoxin B_1 in Tanzania maize-based gruel (*togwa*) by four monocultures (*Lactobacillus plantarum*, *Pediococcus pentosaceus, Lactobacillus casei* and *Lactobacillus fermentum*), natural fermentation and back-slopping at 30°C for up to 24 h was investigated. Monocultures removed 45–55% of aflatoxin B_1 while natural fermentation and back-slopping removed 56% and 68% of aflatoxin B_1, respectively. Thus, lactic acid fermentation could be a part of a comprehensive mycotoxicosis prevention strategy in the commonly consumed maize-based gruels. Consumers could benefit from enhanced food safety through consumption of gruel less contaminated with mycotoxins and might also benefit from the probiotic effects of lactic acid bacteria. In the scenario where lactic acid bacteria starter culture access and handling could prove challenging, especially to households and small-scale food processors in developing countries, the use of back-slopping in gruel fermentation might be advocated for in order to reduce aflatoxin B_1.

Key words: aflatoxins, lactic acid fermentation, maize gruel, *togwa*, food safety, mycotoxins, East Africa

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INTRODUCTION

Although maize is known to be highly susceptible to aflatoxin contamination, there is lack of adequate technical and economic resources in developing countries to ensure proper implementation of existing regulations to reduce aflatoxin exposure in the food supply. Many biotic and abiotic factors could contribute to fungal infection in maize, including extreme weather conditions, insect infestation, soil factors, agricultural practices and storage conditions [1, 2]. Additionally, unprecedented climate changes provide the right conditions where fungi proliferate into colonies and produce high levels of mycotoxins [3]. As such, the production environment and handling practices increase the risk of aflatoxin contamination especially in developing countries, thus presenting serious health problems to both humans and animals [2].

The danger to humans is even more exacerbated because maize is one of the cereal staples and components of complementary foods, such as gruel in many sub-Saharan African countries, thus increasing the risk of exposing children to significantly high levels of aflatoxins in their diets at an early stage [4]. Exposure to aflatoxin in children may cause stunted growth and in severe cases, culminate in liver failure and even death. Unfortunately, many rural and urban communities in developing countries may not be aware of this [5].

Organizations and institutions, for example, the International Agency for Research on Cancer and the United States Environmental Protection Agency, have classified aflatoxin B_1 as a Group 1 carcinogen, that is, carcinogenic to humans [6]. Thus, they have proposed a number of prevention strategies to reduce the risks posed by aflatoxin B_1, particularly for developing countries. However, the full benefits of their efforts to local communities have not been realized due to lack of political will and financial commitments from host governments.

Traditional lactic acid fermentation, one of the oldest practices of food preparation, could be a practical and novel mitigation strategy for reduction of mycotoxins in cereal-based foods. It is a low-cost method of food preservation that improves the nutritional value, organoleptic properties and digestibility of food [7]. Traditional fermentation is widely used in many African countries to make fermented maize gruel, such as *togwa* in Tanzania, which is consumed as a complementary food by children and as a non-alcoholic beverage by adults [8]. The gruel, usually home-made from maize, cassava, sorghum, millet or a combination of these cereals is normally not formally packaged and is typically consumed by an entire family or community during special occasions. Thus, consuming gruel made from maize highly contaminated with aflatoxin could result in significant adverse health and economic impacts.

Lactic acid fermentation may confer preservative and detoxifying effects on food and feeds. The ability of some lactic acid bacteria strains to repress mycotoxins by producing low-molecular-weight metabolites and/or binding of the toxin to bacteria cell walls has been reported [9]. Lactic acid bacteria can absorb mycotoxins either by attaching them to their cell wall components or by active internalization and accumulation [10]. During cell rupture, it is postulated that lactic acid bacteria can release molecules that potentially

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inhibit mould growth and, therefore, lead to a lower accumulation of their mycotoxins [11]. Some lactic acid bacteria have been identified with the strain-specific ability to reduce mycotoxins, especially aflatoxins, with great efficiency [12]. For example, an *in vitro* study on probiotic strains such as *Lactobacillus rhamnosus* GG and *Lactobacillus rhamnosus* LC-705 demonstrated that they were very effective in removing aflatoxin B_1, with more than 80% of the toxin removed [13]. Thus, it is expected that fermentation could be one of the strategies used to reduce health risks associated with exposure to aflatoxins in maize gruel. Therefore, the aim of this study was to determine the effect of lactic acid fermentation on the reduction of aflatoxin B_1 in a bid to protect the safety and livelihood of consumers.

MATERIALS AND METHODS

Preparation of starter cultures

Strains of lactic acid bacteria, namely *Lactobacillus plantarum* B 4496, (LP) *Lactobacillus casei* subsp. *casei* B 1922 (LC), *Lactobacillus fermentum* B 1840 (LF) and *Pediococcus pentosaceus* B 14009 (PP), were obtained from the NRRL Culture Collection Centre at the National Center for Agricultural Utilization Research, Agricultural Research Service, United States Department of Agriculture, Peoria, Illinois, USA. The strains had generally regarded as safe (GRAS) status, and were similar to those previously isolated from lactic acid fermented gruel, *togwa* (Tanzanian traditional lactic acid fermented gruel). The inocula were prepared according to the procedure described by Mugula *et al*. [7]. The lyophilized strains were activated by inoculation into MRS broth (Becton) in screw-capped test tubes and incubated at 30°C for 24 h (Becton Dickinson incubator, Cockeysville, MD, USA). The lactic acid bacteria were single-colony isolated by streaking on MRS agar (Fischer) plates which were incubated at 30°C for 48 h. A colony was picked from each plate, grown in MRS broth, and then centrifuged at 655×g for 15 min (Bench top centrifuge, Kubota 2010, Tokyo, Japan). The bacterial cell pellet was then washed in peptone physiological salt solution, centrifuged as above, and re-suspended in physiological salt solution. The procedure resulted in a culture preparation containing 10^7 colony forming units per milliliter (examined as viable count on MRS agar). The remaining lactic acid bacteria were stored in an ultra-freezer at –80°C (GFL, Burgwedel, Germany) in sterile cryo-tubes containing MRS broth with 10% (v/v) glycerol and acid-washed glass beads until required.

Preparation of samples

Maize grains collected in Morogoro Municipality, Tanzania were ground using a Neogen Grinder #9401 (Neogen, USA) and the flour was sieved (50-mesh size) (Retsch, USA) and stored in cool and dry container. Gruel was prepared according to the procedure described by Mugula *et al.* [7]. Maize flour slurry (1:9 w/v) was boiled (Belling, UK), while stirring continuously, for 20 min to gruel and cooled to around 30°C. Naturally fermented samples were prepared by supplementing the gruel with either millet malt alone or with millet malt followed by back-slopping with 1:9 (v/v) of native *togwa* and 100 ml quantities in 250 ml screw-capped bottles were incubated at 30°C. For controlled fermentation samples, the malt was added when the gruel was at 55–58°C and then left to cool for 30 min. They were then autoclaved at 121°C for 15 min and cooled to 30°C prior to inoculation.

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Spiking and inoculation of samples

For natural fermentation and back-slopping, 100 ml of gruel was spiked with 800 ppb solution of aflatoxin B_1 (1 ml of sample per 8 ppb of aflatoxin B_1). For controlled fermentation, 100 ml of the sterile gruel was spiked with 800 ppb aflatoxin B_1 solution followed by inoculation with 1 ml of lactic acid bacteria culture. The inoculated samples were thoroughly mixed (VortexGene-2, Model G-560E, Scientific Industries, Behemia, New York) to make a homogenous mix. Triplicates of the spiked samples were prepared as follows: (i) gruel spiked with aflatoxin B_1 and fermented naturally (NF); (ii) gruel spiked with aflatoxin B_1 and fermented by back-slopping (inoculation of raw materials with a residue from a previous successfully fermented batch) (BS) and (iii) gruel spiked with aflatoxin B_1, fermented with various lactic acid bacteria cultures. Each tube was aseptically screw-capped, thoroughly mixed and allowed to ferment in an incubator set at 30°C for 24 h. Aflatoxin B_1, pH and lactic acid concentrations were monitored at 0, 4 and 24 h. All measurements were taken in triplicate for three replications.

Aflatoxin B_1 determination

Aflatoxin B_1 in fermented gruel samples was extracted by using 65% ethanol (v/v). The samples were thoroughly mixed for 3 min and allowed to settle. Aliquots of the solvent extracts were decanted and filtered using Whatman #1 filter paper. The concentration of aflatoxin B_1 in the extracts was determined by using a reverse phase high performance liquid chromatography (RP-HPLC) according to the procedure described by El-Nezami *et al*. [14] with minor modifications. The extract was evaporated to dryness with an evaporator system and dissolved with 100 µl of 65% ethanol. The RP-HPLC system (Applied Biosystem, CA, USA) consisted of a pump solvent delivery system, a programmable fluorescence detector and an ODS Hypersil (100 x 4 mm) column C18. The sample injection volume was set to 70 µl. Aflatoxin B_1 was eluted with water/ethanol/acetonitrile (7/1.5/1.5; vl/v/v) as the mobile phase. The flow rate was 1 ml/min. The detection wavelengths for excitation and emission were set at 360 and 420 nm, respectively. The retention time of aflatoxin B_1 was approximately 6 min. The percentage of aflatoxin B_1 removed from fermented gruel was calculated by the following formula:

100% x (1-aflatoxin B_1 peak area of sample/ aflatoxin B_1 peak area of 5µg/ml control)

Aflatoxin B_1 controls at the corresponding incubation period were used.

pH determination

The pH was determined with a pH meter (Model HI 9124, Hanna Instrument Inc., Romania) equipped with a glass electrode. The pH meter was calibrated against standard buffer solutions (Sigma Aldrich, USA) at pH 4.0, 7.0 and 10.0.

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Lactic acid content determination

Lactic acid concentration was determined according to the method developed by Taylor [15]. The sample was filtered using Whatman #1 filter paper and the filtrate diluted (1:100) with de-ionized water. The filtrate (1 ml) was placed in a tube (15 ml Corning plastic centrifuge tube) and 6 ml of concentrated sulfuric acid was added. The tubes were allowed to stand in boiling water for 5 min and cooled to room temperature in a water bath. To each tube, 100 µl copper sulfate (Sigma Aldrich, USA) was added immediately, followed by 200µl of the p-hydroxydiphenyl reagent and the contents were thoroughly mixed to ensure even distribution of the insoluble reagent. The tubes were kept at room temperature for about 30 min and the absorbance was measured using a spectrophotometer at a wavelength of 340 nm (Thermo Fisher Scientific GENESYS 20). Lactic acid concentration was derived by using a standard curve.

Statistical analysis

The data obtained were subjected to analysis of variance (SAS 9.2 software) and mean differences determined by Tukey's Honest Significant Difference ($P<0.05$).

RESULTS

Aflatoxin B_1 in fermented gruel

The ability of lactic acid bacteria to reduce mycotoxin levels in foods during fermentation has been reported by various investigators [16]. The lactic acid bacteria cultures used in this study reduced aflatoxin B_1 levels in fermented maize gruel as indicated in Table 1 and Figure 1. It was observed that the levels of aflatoxin B_1 decreased with fermentation time. In the first four hours of fermentation, the reduction of levels of aflatoxin B_1 was the least (8%) in naturally fermented (NF) samples, while it was more than 25% in samples fermented by back-slopping and the monocultures. All bacterial cultures showed a significant ability ($p<0.05$) to remove aflatoxin B_1 after 24 h of fermentation. Natural fermentation removed more than 50% of the initial aflatoxin B_1 concentration while all the *Lactobacillus* strains removed 52–55% of the aflatoxin B_1 and *Pediococcus pentosaceus* removed the least (45%) after 24 h of fermentation. Since bacterial activity on substrates differs considerably, this suggested that bacterial effect on aflatoxin B_1 reduction during fermentation processes could be strain specific and, therefore, there is need for further exploration with other different lactic-acid-producing bacterial strains. Some researchers also reported that a number of lactic acid bacteria vary in their aflatoxin B_1 detoxification rate, and some strains had higher percentage of aflatoxin B_1 reduction than others [17].

Back-slopping, a popular traditional processing procedure used in gruel fermentation, reduced more aflatoxin B_1 than the pure monocultures. Back-slopping reduced 68% of aflatoxin B1 in gruel fermented for 24 h, and this was significantly higher ($p<0.05$) than that of monocultures tested (Figure1). Furthermore, back-slopping was even superior to natural fermentation in reducing aflatoxin B_1 in samples after 24 h. It could also be argued that the synergistic effect of mixed bacterial strains on aflatoxin B_1 reduction was effective in the back-slopped sample which results in high toxic reduction compared to pure culture of single strains.

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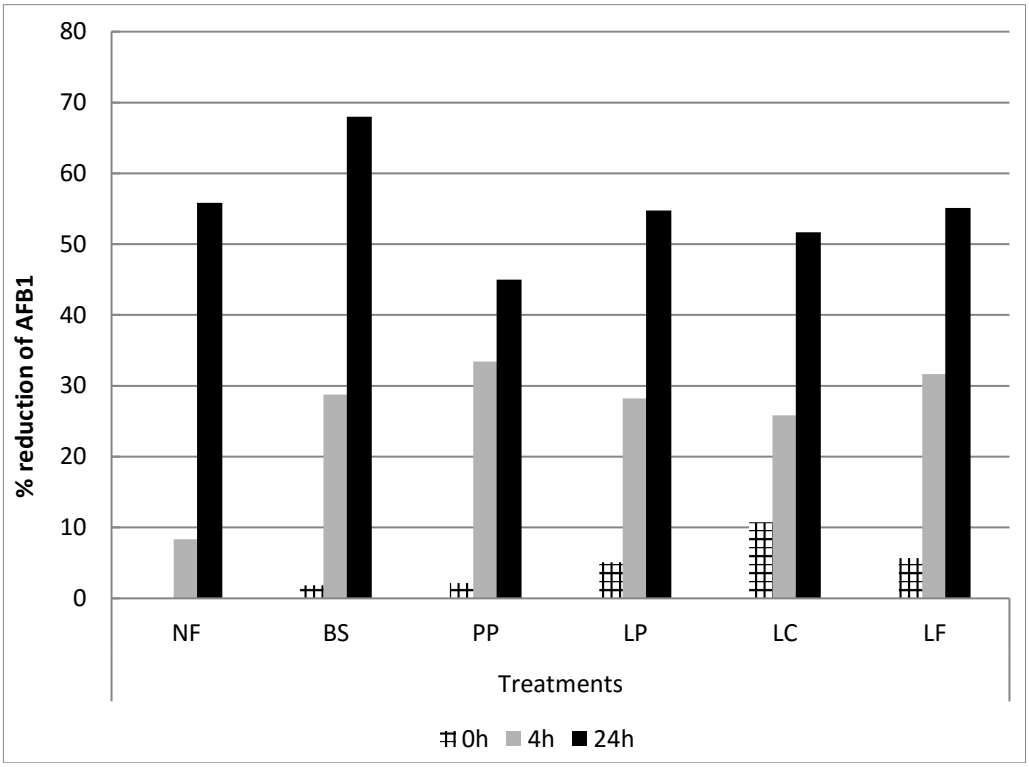

Figure 1: Percentage aflatoxin B_1 reduction. Values plotted are means of triplicate analysis. Natural fermentation (NF); back-slopped (BS); *Pediococcus pentosaceus* (PP); *Lactobacillus plantarum* (LP); *Lactobacillus casei* (LC); *Lactobacillus fermentum* (LF)

Effect of pH and lactic acid on aflatoxin reduction

Bacterial production of lactic acid and lowering of the pH of fermented foods may result in active inhibition of spoilage and pathogenic bacteria. This inhibition is not only due to lactic acid production but also to production of fermentation end-products such as diacetyl, acetaldehyde and acetic acid, which may accumulate over time to inhibitory levels. However, lowering pH through production of lactic acid by lactic acid bacteria may also be connected to reduction of aflatoxin B_1 in fermented foods. In this study, a proportional relationship was established between the reduction in pH values and the corresponding reduction of aflatoxin B_1 in fermented maize-based gruel samples, that is, the lower the pH value, the greater the decrease of aflatoxin B_1 content in the gruel samples tested (Figures 2 and 3).

It was observed that the rate of reduction of aflatoxin B_1 in maize-based fermented gruel at the end of the fermentation period varied with the type of bacterial strain (Figure 2). Back-slopping (BS) had the most marked reduction of aflatoxin B_1 in the pH range of 3.4–3.6. Within the same pH range, *L. plantarum* and *L. fermentum* removed 55% of aflatoxin B_1, while *P. pentosaceus* removed 45% of aflatoxin B_1 after 24 h of fermentation.

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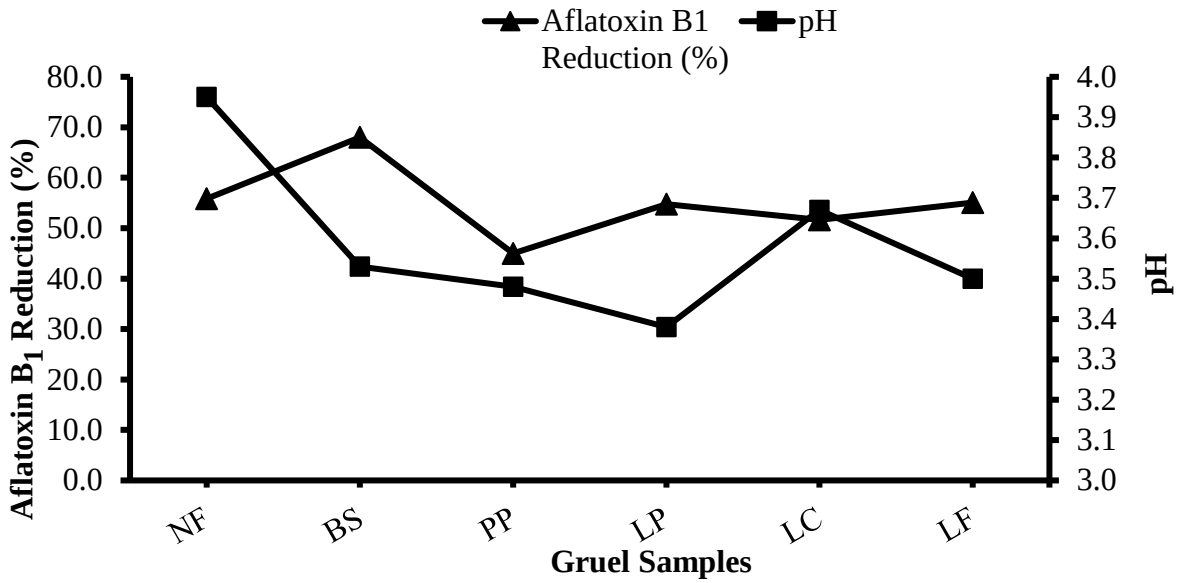

Figure 2: Effect of pH on aflatoxin B_1 reduction during lactic acid fermentation of maize-based gruel at 24 h. Values plotted are means of triplicate analysis. Natural fermentation (NF); back-slopped (BS); *Pediococcus pentosaceus* (PP); *Lactobacillus plantarum* (LP); *Lactobacillus casei* (LC) and *Lactobacillus fermentum* (LF)

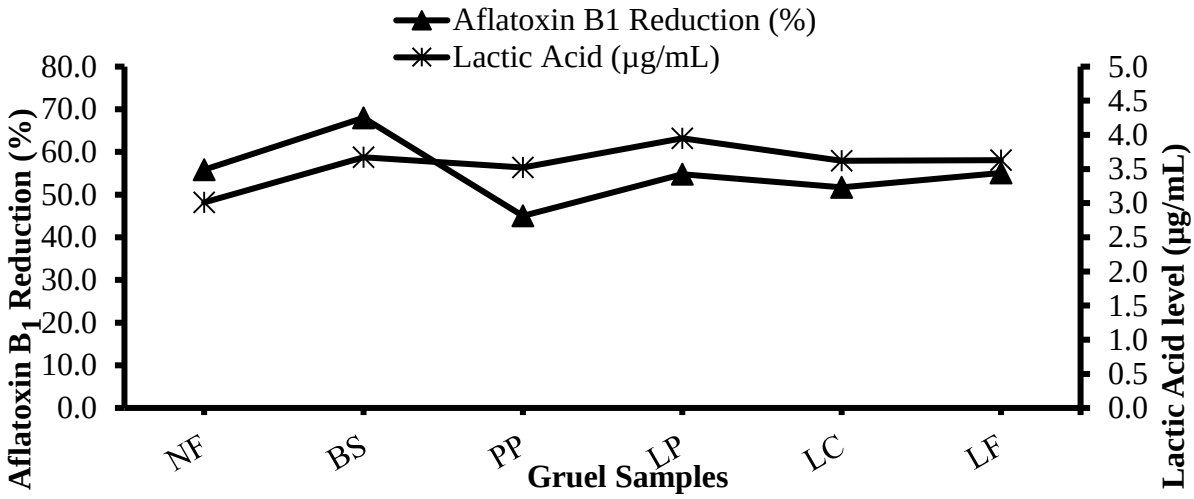

Figure 3: Effect of lactic acid on aflatoxin B_1 reduction during lactic acid fermentation of maize-based gruel at 24 h. Values plotted are means of triplicate analysis. Natural fermentation (NF); back-slopped (BS); *Pediococcus pentosaceus* (PP); *Lactobacillus plantarum* (LP); *Lactobacillus casei* (LC) and *Lactobacillus fermentum* (LF)

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DISCUSSION

In Tanzania and many developing countries, maize is typically ground into flour for various uses and thus must be processed before consumption. Therefore, intervention strategies must also encompass and promote economically feasible processing methods, such as fermentation, which have great potential of reducing mycotoxins before being ingested.

The popularity of lactic acid fermented human foods may continue to grow in part due to claimed therapeutic properties and pleasant organoleptic properties. Lactic acid bacteria are also popular in food systems due to their GRAS status. In addition, lactic acid bacteria strains are known to bind aflatoxins and other mycotoxins to their surface [18]. Thus, they have the ability to reduce the bioavailability of these toxins in foods.

In this study, all the *Lactobacillus* species tested were found to remove between 45–55% of aflatoxin B_1 in maize-based gruel fermented for 24 h at 30°C. This indicated that single strains could be used to remove single compounds, such as aflatoxin B_1, from maize-based gruel. Different single bacterial strains have previously been reported to bind these toxins in significantly different amounts [19]. Other studies have also reported a wide range of genus, species and strain specific binding capacities of several lactic acid bacteria strains [12]. These results indicated that back-slopping had the greatest efficacy in the reduction of aflatoxin B_1 in fermented maize-based gruel.

As bacteria metabolize substrates during growth, they produce several by-products, including lactic acid, which reduce the pH of the product and modify its sensory properties. In this study, a proportional relationship between ability of lactic acid bacteria to reduce pH, by predominantly producing lactic acid, and the corresponding reduction of aflatoxin B_1 in maize-based fermented gruel was observed.

These observations concur with those reported by others that most reduction of toxin content occurred at reduced pH values [20]. It has been suggested that pH may contribute to reducing the content of mycotoxins by transforming mycotoxins to their less toxic compounds [16]. In this study, however, researchers could not determine with certainty that the observed aflatoxin B_1 removal was only influenced by pH reduction, since other factors such as higher bacteria populations after 24 h of fermentation could also have been involved. In addition, inhibition of aflatoxin B_1 accumulation could be related to production of low-molecular-weight metabolites produced by the lactic acid bacteria at the exponential growth phase [21].

However, pH reduction or lactic acid production may not be solely responsible for removal or inhibition of aflatoxin B_1 in foods. Some authors had previously reported inhibitory effects conferred by different metabolites other than organic acids [22]. It would, therefore, be interesting to establish the association between the levels of other metabolites produced by different lactic acid bacteria strains and removal or binding of aflatoxin B_1 during the fermentation of gruel.

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Table1: Effect of lactic acid fermentation on reduction of aflatoxin B_1 in maize gruel

Culture	Concentration of aflatoxin B_1 in gruel[1] (ppb)		
	0 h	4 h	24 h
Natural fermentation (NF)	40.0±0.00[a]	36.7±1.20[a]	17.7±0.33[c]
Back-slopping (BS)[2]	39.3±0.26[a]	28.5±0.28[b]	12.8±0.41[d]
Pediococcus pentosaceus (PP)	39.1±0.59[a]	26.6±0.31[b]	18.4±0.30[c]
Lactobacillus plantarum (LP)	38.0 ±0.03[a]	28.7±0.17[b]	18.1±0.15[c]
Lactobacillus casei (LC)	35.7±1.19[a]	29.7±0.33[b]	19.3±0.24[c]
Lactobacillus fermentum (LF)	37.7±0.26[a]	27.3±0.33[b]	18.0±0.08[c]

[1]Values are means of three replicate measurements
[2]Back-slopped with 10% (v/v) previously fermented gruel, (BS)
Means in the same column with the same superscript are not significantly different ($p<0.05$)

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