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Investigating probiotic yoghurt to reduce an aflatoxin B1 biomarker among school children in eastern Kenya: Preliminary study

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A B S T R A C T

Aflatoxin exposure remains a health problem in developing countries. The mean concentration of aflatoxin B1 in maize meal samples from eastern Kenya of 17.4 ppb verified that the food was contaminated. A probiotic yoghurt was created containing aflatoxin B1 binding *Streptococcus thermophilus*, *Lactobacillus rhamnosus* GR-1 and *Weissella cibaria* NN20 isolated from fermented kimere, a dough food product made from millet. Forty primary school children, with maize being a regular part of their diet, were randomly assigned to consume 200 mL yoghurt or control milk daily for 7 days, followed by a 7 day washout and another 7 day treatment. After both 7 day treatment periods, aflatoxin metabolite 1 concentration in urine samples was significantly lower than baseline in the probiotic group ($P > 0.01$), but increased in the milk group. The findings were confirmed using liquid chromatography-mass spectrometry (LC–MS). This suggests that locally produced probiotic yoghurt may reduce aflatoxin poisoning in Kenyan children.

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1. Introduction

Since 2004, about 477 aflatoxin poisoning cases associated with eating contaminated maize have been documented in eastern Kenya with a fatality rate of 40% (Daniel et al., 2011). Aflatoxins are secondary metabolites produced by *Aspergillus flavus* and *Aspergillus parasiticus* (Davis, Diener, & Eldridge, 1966; Ehrlich & Mack, 2014; Jiang et al., 2005) when environmental factors are favourable. Aflatoxins are hepatic toxins that can induce carcinoma and teratology problems, especially when consumed in small concentrations over a long period (Bennette, 1987).

Aflatoxin B1 (AFB1) enters the blood stream from the gut and is transported to the liver where upon metabolism, several end compounds are produced (Bennette, 1987). AFB1 is oxidised in the liver to the hydroxylated metabolite AFM1, which is found in urine, milk and blood (de Cassia Romero, Ferreira, dos Santos Dias, Calori-Domingues, & da Gloria, 2010). Studies have shown that aflatoxicosis can be fatal as well as causative of the aetiology of hepatocellular carcinoma (HCC) (Sun et al., 1999). Furthermore, the risk of HCC is increased 3.3-fold in detectable urinary AFM1 above 3.6 ng L^{-1} and in people with chronicle disease such as hepatitis B virus (Sun et al., 1999). A recent international report reiterated that around 500 million people living in sub-Saharan Africa, Latin America, and Asia are exposed to mycotoxins at concentrations known to increase mortality and morbidity, including stunting of growth and delaying development (Miller & Groopman, 2015). The fact that food producers in the US spend between $500 million and $1.5 billion each year managing fungal toxins, illustrates this is not only a problem for the developing world.

AFB1 is categorised as class one in food toxicological importance by the World Health Organisation. In favourable conditions, *A. flavus* spores germinate by attaching to food substrate and secreting enzymes that break down nutrients into simpler digestible compounds. During digestion, the *Aspergillus* produces secondary metabolites, including AFB1, ostensibly to give the fungi a competitive edge against other microorganisms (Brakhage, 2013). Studies have been carried out to find ways of containing aflatoxin contamination during growth and harvest; however, results showed that it is difficult, if not impossible, to eradicate AFB1 from cereals once contaminated (Hell & Mutegi, 2011). These have left

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consumers vulnerable to exposure. Legislated burning of contaminated cereals can lead to food insecurity, so maize is often not disposed of, and humans and animals continued to be heavily exposed (Okoth & Kola, 2012; Okoth et al., 2012; Ramesh, Sarathchandra, & Sureshkumar, 2013). Maize is not the only source of aflatoxin. Nuts and dried fruits have been reported to have caused aflatoxicosis after consumption (Zain, 2011). Studies have shown that vulnerability is more pronounced in the developing countries, including those in Sub Saharan Africa, due to multifaceted reasons including climatic conditions and poor farming methods (Bafana, 2015; de Vries & Hendrickse, 1988). When Kenya recorded 125 deaths due to aflatoxicosis (Azziz-Baumgartner et al., 2005; Daniel et al., 2011; Muture & Ogana, 2005), poor diagnostic methods lead to these cases being labelled as acute hepatic inflammation of unknown aetiology. In the eastern part of Kenya maize consumption per serving is approximately 100 g.

The rationale for our study was anchored on millet, formerly a staple food in eastern Kenya and consumed as fermented Kimere. The incidence of aflatoxicosis in humans was uncommon when the Kimere was consumed. As maize became the most important food crop in Kenya, people slowly reduced Kimere consumption in favour of it, coinciding with exponential increase in aflatoxicosis (Kirimi, 2012). Likewise, the change in lifestyle and uptake of a more Western diet has increased the rates of other diseases (Oniang'o, Mutuku, & Malaba, 2003). Thus, as probiotics bind AFB1 in the gut, reducing AFB1 absorption, this will limit the metabolism of AFB1 into AFM1 and reduce circular production of AFM1 detected in the urine.

The objective was to test strains from Kimere and a well-known probiotic *Lactobacillus* that is currently supplemented in yoghurt in impoverished Tanzanian and Kenyan communities, for their ability to bind AFB1 and reduce urinary levels of AFM1 in children consuming maize.

2. Materials and methods

2.1. Strains

The *Weissella cibaria* NN20 strain, isolated from Kimere in Eastern Kenya, confirmed to species level, and the well-documented probiotic *Lactobacillus rhamnosus* GR-1 were cultured in MRS agar under CO_2 (anaerobic container system, Gas Pak^{Tm} EZ, Dickson and company USA).

2.2. AFB1 binding by W. cibaria NN20 and L. rhamnosus GR-1 in vitro

To investigate the ability to sequester the toxin, 10^9 bacterial cells (*W. cibaria* NN20, *L. rhamnosus* GR-1 and also an *Escherichia coli* GR12 control known to poorly bind AFB1) were cultured for 24 h, and harvested by centrifugation (1900 × g for 10 min) then washed three times in phosphate buffered saline (PBS).

Thereafter, bacteria were incubated at 37 °C for 4 h in 96 micro wells plate with MRS broth (Oxoid Cm 0817) and standardised with simulated gastric juice and deliberately contaminated with a solution of AFB1 (Sigma Aldrich A6636-1mG) equivalent to 5 ppb final concentration of AFB1. After centrifugation, the pellet was decanted off with a 500 μL pipette, and 1.5 mL supernatant transferred into Eppendorf safe lock vials for aflatoxin binding analysis using high performance liquid chromatography technique (Wild, Jiang, Sabbioni, Chapot, & Montesano, 1990). The percentage of AFB1 bound to the cells was calculated as the difference between the total AFB1, (i.e., 5 ppb) and the amount of free AFB1 (supernatant). Thus the AFB1 bound in the pellet that was decanted off was not counted. Data from triplicate experiments were integrated and recorded using a Millennium chromatography manager Software 2010 (Waters, Milford, MA) as appropriate.

2.3. Testing the effect of simulated gastric juice

Simulated gastric juice was formulated (Corcoran, Stanton, Fitzgerald, & Ross, 2007) using glucose (3.5 g L^{-1}), NaCl (2.05 g L^{-1}), KH_2PO_4 (0.60 g L^{-1}), $CaCl_2$ (0.11 g L^{-1}), and KCl (0.37 g L^{-1}), adjusted to pH 3.0 using 1 M HCl, and autoclaved at 121 °C for 15 min. Porcine bile (0.05 g L^{-1}), lysozyme (0.1 g L^{-1}), and pepsin (13.3 mg L^{-1}) were added as stock solutions, prior to analysis. Protease peptone was omitted from the formulation as it may be a source of free amino acids, such as L-glutamate, which may be used to extrude protons from the cell, thus potentially enhancing bacterial survival. The juice was heated to 37 °C for 30 min and filtered through a 0.2 μm filter before use.

Following the same protocol as above, the optical densities (ODs) of bacteria after 4 h incubation were captured at 600 nm at 30 min intervals for 16 h, to test for viability in gastric juice.

2.4. Yoghurt preparation, treatments procedures and quality control

Yoghurt was produced by a qualified food technologist in an ISO and Good Manufacturing Practice certified facility in Embu, Kenya. A powder was prepared containing *W. cibaria* NN20, *L. rhamnosus* GR-1 and *Streptococcus thermophilus* at 10^9 colony forming units mL^{-1} per strain dried in skim milk. Fresh milk, sourced from local Kenyan farmers, was boiled for 20 min and cooled to 25 °C then tested for viable pathogens and microorganisms, and fast alcohol test. A mother culture was prepared by inoculating 1.5 g of the powdered bacteria into 3 L milk. Two 250 mL cups of mother culture were transferred aseptically into 20 L pasteurised milk, held overnight and finally stirred after physically confirming consistency of the gelled product.

2.5. Human study ethics

The human study was approved by the Kenyatta National Hospital and University of Nairobi Ethic Review Committee, certificate reference number P446/8/2013, The University of Western Ontario Ethics Review Board and registered in clinicalTrials.gov, Identifier NCT02041026.

The human pilot study was undertaken in children attending Ngenge Primary School, located 20 km south of Embu in Kenya. It is in Mbeere South, a semi-arid region with the majority of population depending on peasant farming. Maize forms the main source of energy and is complimented with vegetables and milk. Most (roughly 80%) of people around Ngenge primary school reside in a rural setting, higher than the Kenyan and sub-Saharan average (67.7%). Almost half of the population in Embu live below the Kenyan poverty line. Embu town has modest infrastructure and piped clean water. Written informed consent was provided by the guardian and individual written informed assent was provided by each child aged 6 years and above. The children had a basic understanding of the study purpose and study procedures. Fingerprints were used for guardians who were unable to sign or write their name. The consent and assent processes were documented through the use of consent and assent notes approved by the ethics review committees.

2.6. Sampling eligibility and recruitment

One hundred and twenty children from Ngenge primary school met the set criteria for inclusion, generated with the help of

teachers and guardians after an authority was sought from local administrative leaders and area Education Officers. Consenting, registration, enrolment and all individual study procedures for recruited subject took place at the school. The criterion for individual inclusion was children of age 6–10 years. The age bracket is within the range of population who highly depends on maize as their staple food. A list of all the children, male or female with equal chance of being included in the study, was generated with the aid of teachers in the primary school. After passing the entry criteria, the children were further selected randomly by toss of a coin. Children with chronic disease, overly severe malnutrition, lactose intolerance and mental disability were excluded. For purposes of clear consenting to participate, the goal of the study was clearly explained in local language to both the guardian and a child, thus children with mental disability were excluded. In total 21 boys and 19 girls participated in the study. The weight of each subject was taken to the nearest 0.1 kg before and at the end of the treatment. The protocol is outlined in Fig. 1.

After screening, children were further observed for two weeks to eliminate any chance of developing illnesses that would have excluded them. A qualified clinical officer helped in screening 40 participants. After selection, participants were separated into two groups of twenty, male and female. One group received 200 mL of either yoghurt prepared using probiotic bacteria or fresh milk each morning for one week, followed by one week with no treatment and week three again with treatment. The reason for the one-week break was to appease parental concerns that the week of therapy had no adverse effects or compliance problems.

Participants were monitored throughout the trial for fitness and well-being every day by a Government registered clinical officer. They were also monitored for 30 days after the completion of the trial.

Baseline urine samples were collected in the morning at the start of the trial and thereafter every seven days. All containers were coded and placed in plastic ziplock bags, and immediately placed in a −20 °C freezer at Ngenge Primary school.

2.7. Enzyme linked immunosorbent assay

Forty 200 g samples of maize were collected randomly from various homesteads and analysed for AFB1 along with urine samples from the subjects tested for AFM1. Every household provided a sample of maize from the collection that was being consumed by the family. In this part of Kenya, maize is purchased or harvested and stored in large consignments to be consumed for a stipulated period of time, in most cases two months (60 days). This can vary, for example with major drought or due to unavoidable reasons as reported by Lewis et al. (2005). The present study was undertaken during a bumper harvest of maize, thus the consignments tested for aflatoxin were from the same batch to those consumed for the 21 days of study. Before the sample collection the consignment was

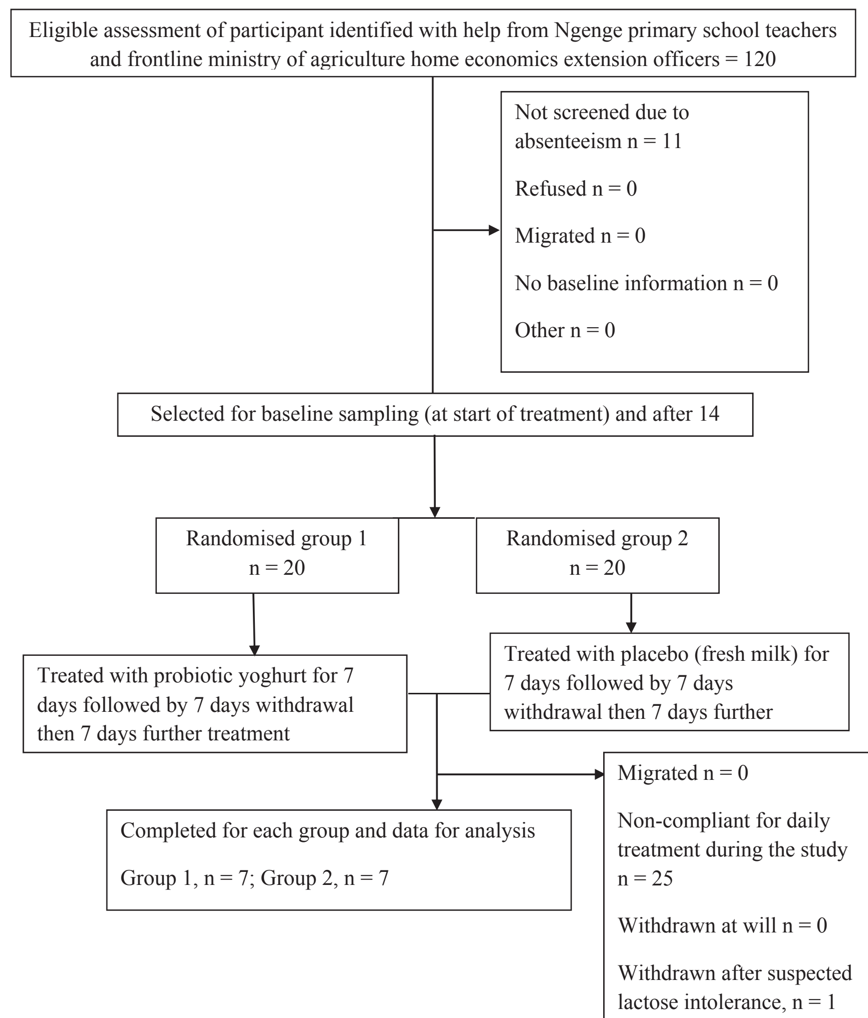

Fig. 1. Study protocol for sampling, treatment allocation and completion of the study; n = sample size, i.e., number of children selected.

first agitated to ensure homogeneity. The cobs were debrided and ground up the same day, then the liquid component was placed in sterile containers stored at −20 °C before thawing and processing.

Urine samples from the subjects were tested for AFM1, as described by Sabran, Jamaluddin, and Mutalib (2012) using an enzyme linked immunosorbent assay (ELISA) kit (Helica Biosystems Inc., Santa Ana, CA, USA) specific for urine in the case of the human samples, and an ELISA kit specific for AFB1 (Helica Biosystems Inc. cat log no 941BAFL01B1-96) was used in the case of maize samples, following the manufacturer's instructions. ELISA kits had aflatoxin-protein conjugate coated onto the microtitre plate. Samples or aflatoxin standards (0.1–20 ppb) were added to the micro wells followed by an aliquot of anti-aflatoxin antibody. The amount of antibody bound to the plate was detected by the addition of anti-rabbit IgG conjugated to alkaline phosphatase (ALP) followed by reaction with *p*-nitrophenyl phosphate to give coloured product. The resulting colour was then measured spectrophotometrically using microtitre plate reader (Neogen model) where the optical density of each micro well was read using a 450 nm filter. The limit of detection and upper limit of quantitation was 0.1–20 ppb. Samples of maize were collected randomly from various homesteads and analysed for AFB1.

2.8. Liquid chromatography–mass spectrometry

In total, 114 urine samples were sent under dry ice from Kenya to Canada, stored at −80 °C then thawed and prepared according to the methods of Warth, Sulyok, and Krska (2013). All the urine samples were analysed and results of detections recorded. Briefly, 200 μL urine was added to 800 μL of 1:9 Acetonitrile:H_2O. Samples were then centrifuge to pellet proteins, and 500 μL supernatant was transferred to LC–MS vials. Five microlitres of supernatant were injected into an Agilent 1290 Infinity HPLC coupled to a Q-ExactiveOrbitrap mass spectrometer (Thermo Fisher Scientific) with a HESI source in positive ionisation mode. Representative ions for each aflatoxin and their specific fragments were monitored as follows; AFB1 (313.07 → 241.05 *m/z*), AFB2 (315.09 → 259.06 *m/z*), AFG1 (329.07 → 243.06 *m/z*), AFM1 (329.07 → 273.07 *m/z*), AFG2 (331.08 → 245.08 *m/z*), AFB1-N7-guanine (480.11 *m/z*). Aflatoxins were quantified in Xcalibur by integrating the area under the curve for fragments ions mentioned above, with the exception of AFB1-N7-guanine, for which the parent M+H ion was used. Authentic standards were run with samples, with the exception of AFB1-N7-guanine, for which there was no standard available.

2.9. Statistical analysis

Data obtained were analysed by Statistical Package for the Social Sciences (SPSS) version 20 at 99 and 95% confidence interval where applicable; graphs were prepared using excel.

3. Results

An in vitro binding assay showed that the probiotic strains *W. cibaria* NN20 and *L. rhamnosus* GR-1 were resistant to gastric juice, and bound to aflatoxins (41.7 ± 2.3% and 37.3 ± 3.6%, respectively) while the *E. coli* GR12 control essentially did not (3.9 ± 1.5%).

We confirmed that AFB1 was present in maize and AFM1 in urine of school children in Embu County. Forty maize samples from the households of children in the study had significantly higher level of AFB1 concentration (17.4 ± 0.3 ppb) than the acceptable health limit of 5 ppb and all were positive for aflatoxin contamination, which ranged from 8.5 ppb to 22 ppb. There was no difference in consumption of maize by the yoghurt and milk groups, $P > 0.05$, which constituted 9.38 ± 3.8 units over 21 days per child. We did not assess the levels of aflatoxin in the milk, but the same milk was used to produce the yoghurt, so we would assume if any toxin was present it would equally affect both groups of children. While some regions in Kenya have cattle feeding on maize that may be contaminated by AFB1, the cows used in this study fed on grass.

Of the 14 children who fully complied with the protocol, 7 were receiving probiotic yoghurt and 7 were receiving milk; the mean age was 8 years 7 months old. The mean urinary levels of AFM1 were higher in the probiotic group at baseline, but not statistically different ($P > 0.05$). Mean child weight was also similar for both groups (22 kg; $P > 0.05$). Of those who did not comply fully with the study protocol, there was no evidence that adverse effects were the cause, and only one child withdrew due to lactose intolerance, although the guardian had failed to inform us of this issue upon enrolment.

Out of the urine samples tested, two in each group were negative at the beginning of the study (Table 1). The AFM1 urinary concentrations fell in the probiotic by 51% over the study duration, based upon ELISA ($P < 0.01$), despite cessation of yoghurt intake between days 7 and 14. On the other hand, the AFM1 concentrations increased by 39% in the control milk group, with a steady rise of 1.2 ppb each week. This is illustrated by graphing the tabulated data (Fig. 2). The effect was significantly different in favour of the probiotic treatment over milk ($P < 0.01$).

To further confirm aflatoxin was present, LC–MS was used on 114 samples. The number of samples with aflatoxin detected pretreatment was similar between groups (25% in probiotic versus 22% in controls), verifying exposure. The method was able to detect 5 ng mL^{-1} (ppb) in spiked samples. The trace amounts detected in samples were below quantifiable limit, possibly due to degradation with transportation. This may explain why LC–MS did not detect AFB1. Thus, the results are included only to confirm detection of the toxins not to compare with the ELISA data per se. For this reason, only the rate of detection for each aflatoxin was compared between groups (Table 2). The percentage of samples post-treatment with

Table 1
Mean AFM1 concentration in subject samples at various times of sample collection.[a]

Subject	Day				Subject	Day			
	0	7	14	21		0	7	14	21
Y1	12.3 ± 0.1	8.6 ± 0.66	5 ± 0.02	7.7 ± 0.13	M1	6.6 ± 0.02	6.4 ± 0.01	9.5 ± 0.05	15.2 ± 0.04
Y2	11.1 ± 0.07	6.8 ± 0.01	7 ± 0.03	5 ± 0.16	M2	6 ± 0.66	5.7 ± 0.3	6 ± 0.21	5.8 ± 0.5
Y3	0	0	0	11 ± 0.1	M3	5.9 ± 0.03	5.7 ± 0.1	5.9 ± 0.3	6 ± 0.31
Y4	5 ± 0.04	0	0	0	M4	12.1 ± 0.03	11.9 ± 0.2	12 ± 0.07	11.9 ± 0.16
Y5	11.2 ± 0.66	6 ± 0.02	2.9 ± 0.02	0	M5	0	6 ± 0.8	5.9 ± 0.1	4.5 ± 0.7
Y6	0	0	0	0	M6	6 ± 0.01	5.6 ± 0.03	6.9 ± 0.03	7.5 ± 0.01
Y7	6.9 ± 1	5 ± 0.01	5 ± 1	0	M7	0	0	0	0
Sum	46.5	26.4	19.9	23.7	Sum	36.6	41.3	46.2	50.9
Average	6.6 ± 0.12	3.8 ± 0.11	2.8 ± 0.3	3.4 ± 0.07	Average	9.2 ± 0.09	10.3 ± 0.27	11.6 ± 0.026	12.7 ± 0.25

[a] Total number of samples = 56; all concentrations were tested at ppb. Y represents subjects treated with probiotic yoghurt; M represents subject treated with fresh milk. The standard deviation of ELISA samples tested in duplicate was 0.01–0.66.

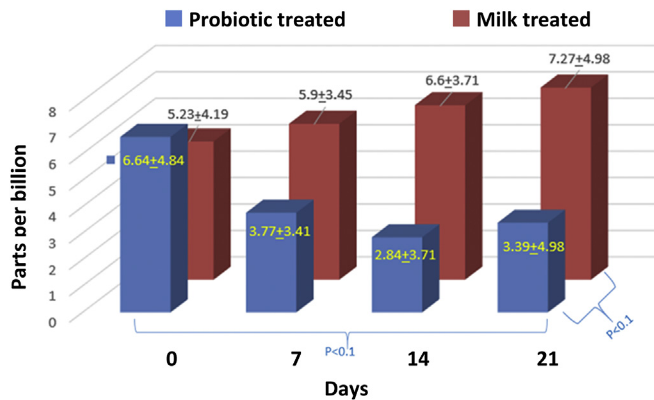

Fig. 2. Chart showing aflatoxin M1 ppb levels in urine at given times for 56 samples from 7 children treated with probiotics and 7 with milk.

Table 2
Percentage of urine samples with detectable aflatoxins as determined by LC–MS.[a]

Sample	Aflatoxin						
	AFM1	AFB1	AFB2	AFG1	AFG2	N7-guanine	Any
All samples	1.25	0	8.75	1.25	0	6.25	16.25
Probiotic	0	0	6.06	0	0	3.03	9.09
Control	2.13	0	10.64	2.13	0	8.51	21.28

[a] Samples taken pre-treatment were excluded from the analysis; a total of 114 samples were suitable for testing.

aflatoxin was 21% for controls and 9% for those consuming the probiotic yogurt.

4. Discussion

Maize is the primary staple food in Eastern part of Kenya. This pilot study showed that despite efforts to control the consumption of maize contaminated with AFB1 in Kenya, children continue to get exposed to this dangerous compound. To obtain the consumption rate, children were asked each day if they consumed maize on the previous day. We admit that a child could have interpreted consumption based on whole grain as this is consumed in one of the indigenous meal referred to as *githeri*-a mixture of cooked whole grain maize and beans. Maize in this region is consumed as ugali – dough made from maize flour, roasted maize, porridge, and flakes among other several maize products. Nevertheless, it was clear that maize was being consumed regularly and equally between the groups. During the 2004 aflatoxicosis epidemic in this region, consumption of contaminated maize was similar to the usage here (Muture & Ogana, 2005). The mean concentration of aflatoxins in maize samples was well above the maximum limits set by the European Union of 5 ppb for spices and 2 ppb for cereal, dried fruit and nuts (Codex, 2014), showing persistent contamination in this part of the world (http://www.sciencedirect.com/science/article/pii/S0168160508000305 Wagacha & Muthomi, 2008). Given the virulence of this toxin on the liver, and its potential lethality (Miller & Groopman, 2015), more needs to be done to address the issue.

The present intervention study showed a trend in favour of probiotic yoghurt to reduce intake of AFB1, assessed using ELISA and LC–MS, even though consumption was only for 14 days over a 21 day period. It is feasible that the probiotic lactic acid bacteria were retained in the gut for part or all of the 7 day 'washout' period, thus maintaining the effects.

Although the aim of the study was not to determine mechanisms of action, the fact that the two lactobacilli strains bind well to aflatoxins supports the hypothesis that this reduces the levels of toxins able to be adsorbed by the host. Based upon the research of others, this binding is mediated through peptidoglycan on the lactobacilli cell wall (Lahtinen, Haskard, Ouwehand, Salminen, & Ahokas, 2004).

The study succeeded in creating a local kitchen to produce the yoghurt. This empowered mothers, and led to them contributing to the community after the study, and acquiring a small salary from revenues generated. Such kitchens delivering probiotic yoghurt have previously been established in Tanzania, Uganda and Kenya (Kort et al., 2015; Reid, Gough, Enos, & Reid, 2013), and have been shown to provide a number of positive health and societal outcomes (Bisanz et al., 2014; Reid, 2010).

In summary, we provide evidence that children in Eastern Kenya remain exposed to aflatoxin poisoning, and that maize remains heavily contaminated despite government efforts. In vitro data and a pilot human study provide preliminary evidence that probiotic yoghurt consumption may help reduce toxin adsorption. This has implications for reducing the risk of liver cancer, since a Chinese study showed that a mixture of *L. rhamnosus* LC705 and *Propionibacterium freudenreichii* subsp. *shermanii* strains consumed twice daily for five weeks, reduced aflatoxin uptake (El-Nezami et al., 2006). The creation of a local kitchen demonstrates that the production and consumption of probiotic yoghurt is feasible in Eastern Kenya, and expansion to a wider region could offer a degree of protection against aflatoxins as well as confer other health benefits of the nutrient rich fermented milk.

5. Conclusions

The study concluded that the maize meal consumed by children in Ngenge, East Kenya is substantially contaminated with AFB1 (mean concentration of collected samples 17.4 ppb) in comparison to the international standards (5 ppb). A pilot intervention using locally produced probiotic yogurt indicated daily consumption of this food has the potential to lower AFB1 absorption in the human gut, and reduce the health burden of this toxin.

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