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Assessment of aflatoxin exposure, growth faltering and the gut microbiome among children in rural Guatemala: protocol for an observational prospective cohort and bioreactor simulations

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ABSTRACT

Introduction Aflatoxin B1 (AFB1) is a carcinogen produced by *Aspergillus flavus* and *Aspergillus parasiticus* which grow on maize. Given the high prevalence of child stunting (ie, impaired growth) and other nutritional disorders in low-income and middle-income countries, where maize is consumed, the role of aflatoxin exposure may be significant. Observational reports have demonstrated associations between aflatoxin exposure and impaired child growth; however, most have been cross-sectional and have not assessed seasonal variations in aflatoxin, food preparation and dynamic changes in growth. Biological mechanistic data on how aflatoxin may exert an impact on child growth is missing. This study incorporates a prospective cohort of children from rural Guatemala to assess (1) temporal associations between aflatoxin exposure and child growth and (2) possible mediation of the gut microbiome among aflatoxin exposure, inflammation and child growth.

Methods and analysis We will prospectively evaluate aflatoxin exposure and height-for-age difference trajectories for 18 months in a cohort of 185 children aged 6–9 months at enrolment. We will assess aflatoxin exposure levels and biomarkers of gut and systemic inflammation. We will examine the faecal microbiome of each child and identify key species and metabolic pathways for differing AFB1 exposure levels and child growth trajectories. In parallel, we will use bioreactors, inoculated with faeces, to investigate the response of the gut microbiome to varying levels of AFB1 exposure. We will monitor key microbial metabolites and AFB1 biotransformation products to study nutrient metabolism and the impact of the gut microbiome on aflatoxin detoxification/metabolism. Finally, we will use path analysis to summarise the effect of aflatoxin exposure and the gut microbiome on child growth.

Ethics and dissemination Ethics approval was obtained from Arizona State University Institutional Review Board (IRB; STUDY00016799) and Wuqu' Kawoq/Maya Health Alliance IRB (WK-2022-003). Findings will be disseminated in scientific presentations and peer-reviewed publications.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Aflatoxin B1 exposure is potentially associated with impaired child growth.
⇒ Aflatoxin B1 exposure is associated with alterations in the gut microbiome.
⇒ Mechanistic work demonstrating how aflatoxin might lead to a growth phenotype is needed.

WHAT THIS STUDY ADDS

⇒ This study will reveal mechanistic explanations for hypothesised links among aflatoxin B1 exposure, the gut microbiome and growth.
⇒ This study will provide insights into the development of therapeutics for aflatoxin-related child stunting.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ The outcome of this study will help support large-scale investments in aflatoxin remediation efforts on the part of public health organisations working to improve global child health.

INTRODUCTION

More than 40% of children under 5 years of age are at risk of not reaching their developmental potential, many due to the impact of stunting.[1] The rural Indigenous population in Guatemala has one of the highest rates of child stunting in the world. Interest in a possible role for aflatoxin in stunting has grown in recent years, made more compelling by the observation that many countries with high rates of stunting consume large amounts of maize and have documented aflatoxin in food sources.[2–7] However, most studies have been cross-sectional and have not assessed seasonal variations in aflatoxin, food preparation and dynamic changes in child growth. In addition, biological mechanistic data on

how aflatoxin may exert an impact on child growth are missing.

There have been many interventions to improve stunting in Guatemala, yet few have had tangible impact.[8] The gut microbiome has emerged as a key regulator of human health and nutrition, and a promising target for interventions.[9] Recently, our group demonstrated significant differences in children's gut microbiomes between those with high and low exposures to aflatoxins in Guatemala.[10] Our data also revealed potential shifts in diet-based aflatoxin exposure dependent on season and an association between diet-based aflatoxin exposure and child height-for-age.[11 12] To date, only a few animal studies have evaluated the impact of aflatoxin on the gut microbiome,[13–18] while direct investigation of the aflatoxin-exposed human gut microbiome is lacking. In addition to microbial changes induced by aflatoxin, gut microbiota can interact with aflatoxin through bioadsorption and biotransformation.[19–22] Aflatoxin can bind to extracellular structures on microorganisms (eg, *Lactobacillus* and *Saccharomyces*), which decreases its bioavailability.[19 22] Highly toxic aflatoxin such as aflatoxin B1 (AFB1) can also be metabolised by bacteria (eg, *Bacillus*, *Lactobacillus* and *Pseudomonas*) to less toxic or even non-toxic substances.[20] A close examination of aflatoxin degradation pathways and metabolites produced by human gut microbiota is also lacking.

We aim to assess temporal changes in diet, aflatoxin exposure and linear growth faltering in a prospective cohort of children from rural Guatemala, a country that has one of the highest rates of child stunting and aflatoxin exposure in the world. We will prospectively evaluate the association among AFB1 exposure, height-for-age growth trajectories, and the gut microbiome over 18 months for 185 children aged 6–9 months at enrolment. In addition, we will use bioreactors inoculated with faecal samples to evaluate the response of the gut microbiome to varying levels of AFB1 exposure, and the impact of the gut microbiome on aflatoxin detoxification/metabolism. We hypothesise that (1) aflatoxin consumption impacts child linear growth by altering the composition of the gut microbiome and inciting a systemic inflammatory response; (2) aflatoxin exposure alters luminal nutrient metabolism by the gut microbiome and (3) certain gut microorganisms metabolise aflatoxin and may be protective against aflatoxin exposure.

METHODS

Study setting and design

In this project, we will work in rural Guatemala in collaboration with Maya Health Alliance, the lead local institution, which facilitates primary care and research in service of the local Indigenous Maya population. Maya Health Alliance works alongside Indigenous communities to improve access to healthcare as well as leading clinical trial and observational studies on complementary feeding, stunting, dietary quality and early child development.[23–25]

This study includes field-based and lab-based components (figure 1). For the field-based component, children 6–9 months of age, only one child per household, will be enrolled in the study and followed for 18 months through 24–27 months of age. During the 18 months when children are enrolled in the study, households will be visited for data collection three times, at 9-month increments. Household visits will consist of surveys (eg, dietary intake), anthropometric measurements, sampling of household maize stores and foods, and collection of venous blood specimens and faecal samples.

Blood samples will be used to measure serum AFB1-lysine (AFB1-lys) adduct levels, C reactive protein (CRP) and erythrocyte sedimentation rate (ESR). Faecal samples will be used to analyse faecal calprotectin, gut microbiome composition and microbial metabolites such as short-chain fatty acids (SCFAs). Faecal samples will also be used in the lab to seed bioreactors which will then be dosed with varying levels of AFB1 (see the 'Bioreactor setup and sampling' section). Microbial metabolites (eg, SCFAs), AFB1 and its degradation products, microbiome structures (DNA) and functions (RNA) will be analysed.

Outcomes

The primary growth outcome will be height-for-age difference (HAD) scores, calculated as the difference between measured height-for-age and the median

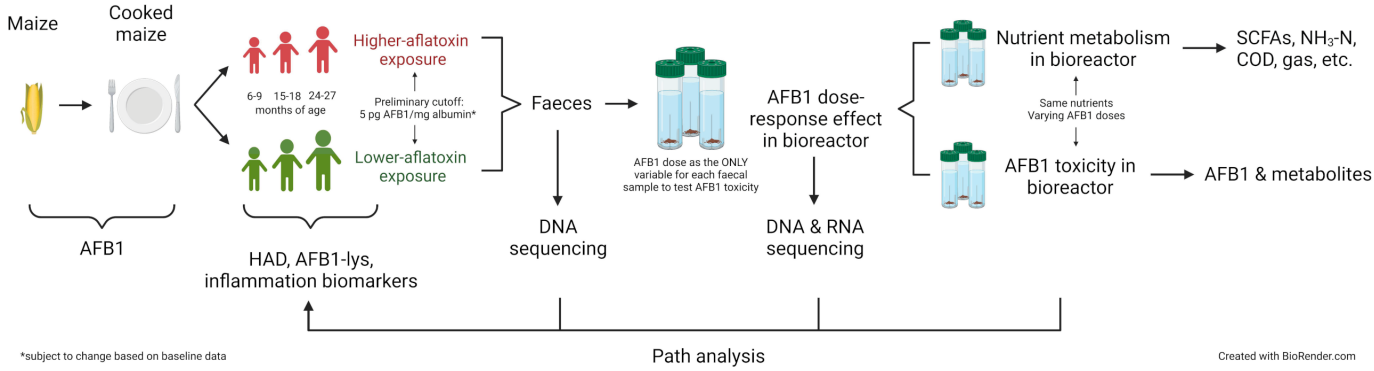

Figure 1 Overall project design and path analysis integration. AFB1, aflatoxin B1; HAD, height-for-age difference; AFB1-lys, aflatoxin B1-lysine; SCFAs, short-chain fatty acids; NH3-N, ammoniacal nitrogen; COD, chemical oxygen demand.

height-for-age from the WHO's Child Growth Standards reference population. The primary exposure outcome will be serum AFB1-lys, which will be used to estimate aflatoxin exposure. Children will be divided into 'high' and 'low' AFB1-lys groups using a potential cut-off of 5 pg AFB1/mg albumin (pending exposure data in the baseline assessments).[4 26–29] Secondary exposure outcomes will be markers of systemic and intestinal inflammation, including ESR, CRP, SCFAs and faecal calprotectin. These outcome measurements will be collected at three time points beginning at 6–9 months of age and will be collected every 9 months until the final time point at 24–27 months of age. In addition to these outcomes, we will evaluate the faecal microbiome as a mediator between exposures and outcomes. Other outcomes include the results from the bioreactor experiments, including AFB1-induced microbiome composition and function changes, microbial metabolite profiles and AFB1 degradation products.

Eligibility criteria

Participants will include children from Maya Health Alliance catchment areas located in the Departments of Chimaltenango, Sololá, Sacatepéquez and Suchitepéquez. Maya Health Alliance community health clinics and health centres will be the primary source for identifying potential children. In addition, the project will be promoted through community centres, churches, schools and community leader meetings.

Inclusion criteria are as follows:

- Infants who are 6–9 months of age at baseline.
- At least one caregiver willing to provide written informed consent and participate in study activities.
- Permanent residents of the communities or planned residence in the study area at least for the 24 months following enrolment.
- Singleton birth.

Exclusion criteria are as follows:

- Infants with moderate to severe acute malnutrition (weight-for-length z-score ≤−2).
- Infants with a chronic medical condition that affects growth and/or requires special care, present at baseline or diagnosed subsequently during the study observation period, such as:
  - Congenital heart disease.
  - Genetic conditions.
  - Kidney disease.
  - Neurological deficits.
  - Problems of cleft lip or palate.
- Infants whose caregivers have cognitive or other impairments that prevent them from providing informed consent or reliable information.
- Concurrent participation in any other clinical trial.

Sample size

Our planned sample size is 185 children. This is based on assumptions of an SD for HAD of 3.5 cm and an intrasubject correlation coefficient of 0.8 for repeated measures, based on recent Guatemalan Demographic Health Survey Data[30] and Maya Health Alliance observational data.[31–33] With these assumptions, a sample of 154 children will allow us to detect a minimum difference in HAD slopes of 1.0 cm between high and low AFB1-lys groups (above or below 5 pg AFB1/mg albumin) with 80% power, at an alpha level of 0.05. The total sample of 185 participants includes an increase of 20% to account for possible dropouts or lost to follow-up and will also allow for detection of the same 1 cm difference in slopes if our SDs are smaller than expected (as low as 2.5).

Recruitment

For the recruitment process, study staff will identify potential participants and inform caregivers of the study using a recruitment script, and directly answer questions or concerns about the study. This will either be conducted by phone, in routine healthcare settings or via home visits. Interested caregivers will be screened for eligibility via a rapid screening including general information on the child's demographic characteristics and inclusion criteria. Caregivers of children that meet the inclusion criteria after the rapid screening will review the informed consent form the same day or in a rescheduled home visit. Staff conducting recruitment and informed consent activities will be bilingual (Spanish and Kaqchikel, or other Mayan languages, as appropriate), and will provide information in the caregiver's preferred language.

Field data and sample collection

The study will involve three household visits at 9-month intervals. During these visits, the team will conduct surveys and anthropometric measurements, collect samples of maize, and collect venous blood specimens from the children (less than 2 mL/kg body weight). In addition, the team will place a sterile diaper on the child at the start of each visit and collect faeces on defecation. The maize samples will be tested for AFB1 concentrations, and a probable daily intake (PDI) score will be estimated via cooking practices, maize consumption recall and child body weight. The team will conduct parallel non-consecutive 24-hour dietary recalls using a locally validated method. Breastfeeding practices will be recorded, although previous data suggest that breast milk is not a clinically significant source of aflatoxin exposure in the population being studied and similar settings.[2] Blood samples will be collected by a trained nurse phlebotomist. Faecal samples will be collected in raw form and in glycerol and stored at −80°C until further processing. In addition to the planned testing, blood and faecal samples will be archived for future testing related to areas of specific interest to the study aims of this research project.

Bioreactor setup and sampling

We will use bioreactors to investigate the AFB1-gut microbiome interactions and use these results to elucidate microbiome-related observations in the cohort study.[34–36] We will select 20 children from the lower-aflatoxin

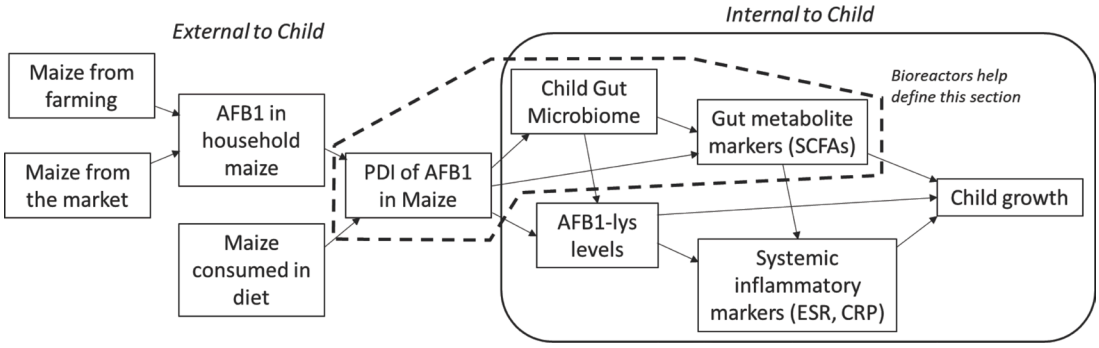

Figure 2 Diagram of hypothesised pathways in aflatoxin-child growth model. AFB1, aflatoxin B1; PDI, probable daily intake; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; SCFAs, short-chain fatty acids.

exposure group and 20 from the higher-exposure group based on their AFB1-lys levels, HAD scores and faecal microbiome composition (figure 1). We will collect their faeces in glycerol at two time points and combine those from the same age and same group as the inocula for bioreactors (ie, four inocula in total). We will feed the bioreactors with maize starch (the primary carbohydrate source in the maize-based diet and the matrix that AFB1 is associated with), other necessary nutrients and three doses of AFB1 that represent the maximum, minimum and average amount of AFB1 ingested by the children. We will operate the reactors in a fill-and-draw mode to simulate transit and retention time in the colon. We will collect liquid samples periodically for metabolite analyses (eg, SCFAs, AFB1 degradation products), and microorganisms for microbiome composition (DNA) and function (RNA) analyses.

Statistical methods

We will use the statistically appropriate correlation-based methods to evaluate associations between the hypothesised pathways (figure 2). We will assess growth trajectory differences between higher and lower aflatoxin exposure groups (as defined above) using a longitudinal mixed model.[37] We will control for the effect of a set of potential confounding factors and covariates (eg, diet, age, sex) collected in the household survey. Furthermore, to describe the mediating, moderating, direct and indirect effects among the microbiome, aflatoxin exposure, child growth outcomes and other factors, we will use path analysis and latent growth models.[38 39]

Data management and confidentiality

Study personnel will be trained on standard operating procedures for recruitment, enrolment and data collection tasks. Data quality will be ensured using native data field definition functions in digital data capture software and ongoing quality control measures such as database review and random audits of in-field operations. Each subject will be assigned a unique study ID number and this number will be the only link between their name and research data. Identifiable data will be retained by Maya Health Alliance for at least 3 years from the date of study completion or primary outcomes are published, whichever is later. Deidentified datasets will be transferred to Arizona State University and may also be deposited in public data repositories at the time of publication. No identifiable data will be released publicly.

Data monitoring, harms and auditing

The rate of subject accrual and compliance with inclusion/exclusion criteria will be monitored monthly during the recruitment phase. The study may be stopped early if there is regional or national political instability or difficulty with recruitment or retention that significantly impacts the ability to evaluate the study endpoints. The study has minimal risk to subjects. The anticipated risks include lost productivity or interference with domestic routines for the caregivers of enrolled children, risk of psychological stress or stigma for caregivers discussing possible delays in child development, risk of accidental disclosure of personal or confidential data, and risk of pain or infection associated with blood draws. To address these risks, standard operating procedures have been developed, including a specific operating procedure governing behavioural distraction techniques and limited phlebotomy attempts for children. The study is not expected to have any significant adverse events, but any perceived adverse events or complaints from participating communities or caregivers will be reported to the institutional review board (IRB) and granting authorities. Staff will be trained to report adverse events following established protocol. Staff will provide counselling to caregivers when laboratory results are returned and assist with linkages to clinical care when indicated.

Patient and public involvement

Patients or the public were not involved in the design of our research protocol. However, prior to initiating field work, community meetings will be held to solicit feedback on planned research activities, and results obtained from the study will be communicated back to the community in regular community meetings.

Informed consent

Study staff members will explain the study and obtain written informed consent from the caregiver/legal guardian of the child participating in the study. Informed

consent will be administered in Spanish or the Mayan language of the caregiver's choosing. Children participating in the study are less than 24 months old and are not capable of providing assent. Once signed informed consent is given, the study staff member will provide the caregiver will be provided with a signed copy of the informed consent (online supplemental file 1).

Dissemination policy

Laboratory results will be returned to participating caregivers by study staff. Results will be explained in detail and linkages to clinical care facilitated when indicated. We will educate the public and the scientific community by publishing in peer-reviewed scientific journals, presenting our findings at microbiome-related, toxicology-related and environmental engineering-related conferences and webinars, and by conducting town-hall style meetings with participating communities.

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Contributors All authors contributed to the study design, sample size calculations and writing of the protocol for this study. QC, HG and LEV-G prepared the first draft of the paper. All authors contributed to revisions of the manuscript and contributed to the revision of the final manuscript. All authors have read and approved the final manuscript.

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Competing interests No, there are no competing interests.

Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Patient consent for publication Not applicable.

Ethics approval The study protocol has been approved by the Maya Health Alliance IRB (WK-2022-003) in Guatemala and the Arizona State University IRB (STUDY00016799) in the USA.

Provenance and peer review Not commissioned; internally peer reviewed.

Data availability statement No data are available.

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