Feed the Future Innovation Lab for Collaborative Research on Grain Legumes

PROJECT TECHNICAL DESCRIPTION

COVER PAGE

Code and Title of Legume Innovation Lab Project:
Legumes and growth

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Project Period:	Total Funding for 45 month Project	Total non-federal cost share commitment by U.S. institution(s)
January 1, 2014 – September 29, 2017	$3,000,000	$166,743

HCs where project activities will be implemented:	HC institutions to be sub-contracted (abbreviated names):	Percent of total project funding budgeted for each HC institution to be subcontracted
Malawi	College of Medicine, University of Malawi	53.56%

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SUMMARY PAGE (must print on one page)

Code and Title of Legume Innovation Lab Project:
Legumes and growth

Name and Institutional Affiliation of the U.S. Lead Principal Investigator:
Mark Manary MD, Helene Roberson Professor of Pediatrics, Washington University School of Medicine in St. Louis

Abstract (Limit: 1800 characters including spaces—about 200-250 words):
Interventions that decrease the burden of childhood malnutrition are urgently needed, as millions of children die annually due to undernutrition and hundreds millions more are stunted. Environmental enteropathy (EE), a pervasive chronic subclinical inflammatory condition among children when complementary foods are introduced, places them at high risk for stunting, malabsorption, and poor oral vaccine efficacy. Here we propose two randomized, controlled clinical trials to determine if common beans or cowpeas improve growth, ameliorate EE, and alter the intestinal microbiome during this high-risk period. The first study involves 6-11 month old children who will receive common beans, cowpeas, or standard local complementary foods for 6 months. Anthropometry will be compared among the three groups. EE will be assessed using a urine dual-sugar absorption test and by quantifying human intestinal mRNA for inflammatory messages, and the intestinal microbiota characterized by deep sequencing of fecal DNA to enumerate the host microbial populations and their metabolic capacity. The second randomized, controlled trial will enroll 12-35 month old children and follow them for 12 months; each subject will receive dietary interventions, either legume-based or control. Anthropometric, host inflammatory and gut microbiota analyses will be conducted similar to the first study. The studies will also facilitate the training of 2 doctoral-candidate nutrition students from the University of Malawi and 4 food science master's level students from the national agricultural university. By amalgamating the power of the clinical trial and advanced biological analyses, we will elucidate the potential of legumes to have a major impact of child health in sub-Saharan Africa.

Summary Checklist ***(select as many as appropriate)***

	Project involves the use of proprietary transgenes or the generation of genetically modified organisms (GMOs)
XX	Project involves human subjects and requires approval
	Project involves animal use and requires approval
	Project involves the use of agricultural pesticides and requires a Pesticide Evaluation and Safe Use Action Plan
XX	Project involves M.S. or Ph.D. degree training of HC personnel at a U.S. university (How many?) 2

TECHNICAL APPLICATION (maximum of fifteen pages, excluding the budget and budget narrative)

A. Technical Approach

1. Introduction and Problem Statement/Justification

Approximately 45% of all deaths worldwide among children under the age of five, i.e.,3.1 million deaths annually, are directly or indirectly related to undernutrition [1]. Additionally, stunting permanently affects an additional 165 million children worldwide, and reduces the affected individual's physical, immunological and cognitive capacity throughout his/her lifetime. Stunting is estimated to account for 21% of all disability adjusted life years (DALYs) in children. Both stunting and wasting are causally related to the dietary intake and gut health in children less than 3 years of age. USAID's Feed the Future (FTF) program aims to reduce the burden of these scourges.

In developing, impoverished settings, a nearly-ubiquitous gut inflammatory condition known as environmental enteropathy (EE, formerly known as tropical enteropathy) develops early in life. While subclinical, EE predisposes children to more clinically manifest forms of malnutrition: wasting and stunting. EE is characterized by T-cell infiltration of the intestinal mucosa leading to a chronic inflammatory state with increased intestinal permeability, translocation of gut microbes, micro- and macronutrient malabsorption, poor weight gain, stunted physical and cognitive development, frequent enteric infections, and decreased response to enteric vaccines [2]. While a precise etiology of EE has not been identified, EE is epidemiologically associated with unsanitary living conditions [3]. Given the significant contribution of malnutrition to childhood morbidity and mortality, meaningful progress on reducing EE is needed to establish a lasting foundation for progress against global hunger, which FTF endeavors to do [4, 5].

EE often develops within the first three years of life, a high-risk period marked also by the transitions from exclusive breastfeeding to mixed feeding with complementary foods to the complete reliance on adult foods for sustenance [4]. In traditional sub-Saharan African societies, complementary foods are dominated by monotonous, protein-poor and micronutrient-poor starches such as maize, cassava, and sorghum. Alternative, yet culturally acceptable, complementary foods that can provide a better and more palatable balance of nutrients may promote a decrease in EE and improved growth. Legumes provide just such an opportunity, as their protein content is significantly higher than cereals, and they are rich in dietary fiber, starch, minerals, vitamins, and antioxidants. Common beans and cowpeas, for example, have 3-4 fold more protein per gram as corn. The zinc content in legumes is also relatively high and might further decrease the progression of EE, as we have demonstrated recently in a prospective randomized trial [6]. Legumes make an excellent complementary food for children weaning from exclusive breastfeeding and with appropriate preparation are quite digestible and well-tolerated [7]. Successful legume-maize blends have, in fact, already been developed in the past and demonstrated favorable acceptability profiles in children less than one year of age; they were also nutritionally sound as a weaning supplement [8].

Additionally, interventions with anti-inflammatory effects might improve gut health, in view of the fact that EE is a chronic inflammatory condition, both in the lamina propria of the intestinal tract and systemically due to translocation of enteric bacteria and their products across the compromised

mucosal border [9]. Simply attacking the bacterial confounders of EE alone is unlikely to be sufficient, given past failures to improve EE via the use of probiotics [10] or antibiotics [11]. A growing body of evidence [12-15] suggests that a diet enriched in legumes decreases in markers of inflammation as well as being correlated to illnesses with inflammatory components such as colorectal cancer and cardiovascular disease [16].

Beans have been cultivated for more than 700 years and were harvested in Africa long before the colonial era. They might thus serve as major sources of protein in southeast Africa if proven to be of benefit in these young children. Indeed, the Great Lakes region of Africa has the highest per capita bean consumption in the world, demonstrating the cultural acceptability of such legumes in our target population [17]. However, the consumption of legumes among young children in rural Malawi remains extremely low due to the preference for maize and other carbohydrate-rich staples [18]. Legumes could therefore serve as a complementary food in this high-risk population, with key measurable endpoints and biomarkers, including markers of EE and growth parameters.

Ample evidence from molecular and animal models supports a mechanistic explanation whereby legumes have direct anti-inflammatory effects on the intestines of children suffering from EE. For example, a meal of Swedish brown beans (*Phaseolus vulgaris* var. *nanus*) decreases the inflammatory markers IL-6 and IL-18 in human subjects [19]. Additionally, the non-digestible portion of common beans down-regulates signaling pathways that lead to inflammation in a mouse model of colon cancer [20]. Intriguingly, there is likely an essential microbial component to the down-regulation of this inflammatory cascade: when fermented by normal gut flora, the non-digestible fraction from common bean produces short-chain fatty acids that inhibit colon cancer HT-29 cell growth and modulates protein expression associated with apoptosis, cell cycle arrest, and proliferation. These effects were also demonstrated morphologically [21]. The non-digestible fraction of common also protects against chemically-induced crypt inflammation in rat colon [22, 23].

Cowpea (*Vigna unguiculata*) is also attractive for study, as it grows well in the African context, is culturally accepted, and is a hardy, drought-tolerant, crop. Cowpea also has significant anti-inflammatory effects, mediated by specific phenolic profiles and antioxidant activity [24]. These phenolic compounds are also active following cooking and simulated enzymatic digestion [25].

The evolving intestinal microbiome in African children also warrants study, particularly in the context of an intervention trial with legumes. Although no specific microbial populations or disruptions have been linked to EE [26], evidence suggests that a disruption in the relative populations among the four dominant bacterial phyla (*Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria*) are correlated with gut mucosal breakdown in inflammatory bowel disease (IBD) [27, 28]. Recent work in patients with celiac disease, which has histopathologic similarities to EE, demonstrates that a similar dysbiosis of the microbiota is related to severity of illness. Specifically, patients with more severe gastrointestinal symptoms in both celiac disease and IBD had higher numbers of *Proteobacteria* and decreased numbers of *Firmicutes* in their small intestines [29]. We have demonstrated recently that empiric antimicrobial interventions in severely malnourished children improves nutritional recovery [30] and specific disturbances in the maturation of young children's intestinal microbiota are linked to severe malnutrition [31]. Hence, it is critical to understand the

effect that specific food-based interventions have on the commensal gut organisms and their metabolic capacity [32].

Human and animal studies of the effect of legumes on the intestinal microbiome are limited. Rats fed diets rich in legumes have higher quantities of *Bifidobacterium* and lower counts of *Enterobacter* and *Bacteroides* in their intestinal tract [33]. A recent study comparing the gut microbiota in children from rural Burkina Faso who consumed a diet rich in legumes with European children showed a relative lack of potentially pathogenic Enterobacteriaceae in the African children, conceivably protecting these children from severe gut inflammation and bacterial translocation [34].

We propose to perform our studies in rural Malawi, a nation that is central to the FTF initiative, and home to a population already familiar with beans and legumes. Malawi is a small, impoverished country in the Great Lakes region of southeastern Africa; 13% of Malawian children are underweight, 47% are stunted, and 4% are wasted [35]. We have found EE in more than 80% of the children studied in ongoing projects [6, 10, 11]. Using rigorous clinical trial methods and the most advanced biomedical techniques, we propose to interrogate the effects on childhood growth, gut health, and the intestinal microbiome caused by the systematic introduction of cowpeas and common beans into the complementary diet of vulnerable Malawian children.

The knowledge to be gained aligns closely with FTF objectives and operational implementation of any nutritionally beneficial findings will directly improve the health of women in these societies, given the very close link between child survival and gender equity. Importantly, we include a significant collaboration with local educational institutions and training of students in food science, clinical trials implementation, and laboratory methods as an essential capacity building component of the studies. The research team will directly engage with USAID Mission in Malawi and we anticipate the results of this work will be applicable to other FTF target countries.

2. Objectives

This project will conduct two randomized, controlled clinical trials to investigate the effect of legume consumption on infant and toddler growth and gut health. These studies are aligned with the **FTF Objective** of developing sustainable nutritional interventions to decrease poverty and hunger. The **specific scientific aims** to be tested are:

Aim 1: Evaluate changes in childhood anthropometry (height-for-age and weight-for-height z scores), biomarkers of EE (lactulose:rhamnose and a panel of human mRNA messages correlated with EE) and the characteristics of the microbiome (population taxonomy from phyla to genus, and the collective metabolic capacity expressed as Kyoto Encyclopedia of Genes and Genomes (KEGG) categories) after inclusion of either cowpeas or common beans as an integral component of complementary feeding for 6-11 month-old rural Malawian children.

Aim 2: Evaluate changes in child growth (height-for-age and weight-for-height z scores), biomarkers of EE (lactulose:rhamnose and a panel of human mRNA messages correlated with EE) and the characteristics of the microbiome (population taxonomy from phyla to genus, and the collective metabolic capacity expressed as Kyoto Encyclopedia of Genes and Genomes, KEGG, categories) after

adding either cowpeas or common beans to the diet of 12-35 month-old rural Malawian children.

Both **Aims** are prompted by the overarching ***hypothesis*** that *children provided with a legume supplement will have greater linear growth and an improvement in biomarkers of EE, compared to those who receive standard food supplements.* The Aims will be accomplished by conducting a randomized controlled clinical trial.

Furthermore, an **exploratory mechanistic analysis** of changes in the developing intestinal microbiome among both age cohorts and all three intervention cohorts (cowpeas, common beans, standard feeding) of children will be conducted to inform an understanding of the role of the microbiota in early childhood growth and gut health.

The **specific capacity building aim** of the project will be to engage Malawian graduate students from diverse backgrounds and provide them with an intensive experience in food science, public health, clinical studies, data analysis and laboratory methods in order to prepare them for independent research and teaching careers.

3. Approaches and Methods

Each study will randomize infants and toddlers at high risk for EE and stunting to a sustained intervention of cowpea, common bean, or standard maize supplements and assess the outcomes of interest every 3 months. The **outcomes** will include anthropometric measurements, clinical symptoms, biomarkers of EE and gut inflammation, and population characteristics of the microbiota.

Aim 1: Evaluate changes in childhood anthropometry (height-for-age and weight-for-height z scores), biomarkers of EE (lactulose:rhamnose and a panel of human mRNA messages correlated with EE) and the characteristics of the microbiome (population taxonomy from phyla to genus, and the collective metabolic capacity expressed as Kyoto Encyclopedia of Genes and Genomes (KEGG) categories) after inclusion of either cowpeas or common beans as an integral component of complementary feeding for 6-11 month-old rural Malawian children.

Sample size. The primary outcomes of interest will be change in height-for-age Z score and improvement in a biomarker of environmental enteropathy, the lactulose:rhamnose (L:R) test. To detect a difference in change in length of 1.1 cm, which corresponds to a change in height-for-age Z score (HAZ) of 0.45 units at 12 months of age, 79 children are needed in each group. This sample size was calculated for a two-tailed test using G*Power 3.17 [36] at a significance level of 0.05 and 80% power.

We also desire to detect a (medium) effect size of 0.5 in the L:R test after the legume intervention, again with 80% power at a significance level of 0.05. Given these parameters, if the L:R results were to follow a normal distribution thereby allowing the use of Student's t-test to compare the differences between independent means, 64 children would be needed in each group. If the L:R results are not normally distributed and require non-parametric analysis with the Wilcoxon-Mann-Whitney test, then up to 74 children might be needed per group. In recent studies, we have experienced approximately a 5% rate of insufficient urine specimens [6]. We therefore very

conservatively estimate that this rate might increase to 15-20%. At the same time, this study will have more intensive follow-up and community engagement in order to trace dropouts and will also require only 2 hours of urine collection rather than 4 hours due to the use of rhamnose instead of mannitol as the monosaccharide, thereby decreasing the likelihood that a urine sample will be insufficient. Thus, we believe that enrolling 300 children with a 15-20% rate of failure to obtain sufficient urine (due to dropouts and specimen collection failures) will yield complete analysis for at least 240 children, or 80 in each group. Identifying differences in L:R smaller than these limits are not likely to be clinically significant.

Should 100 children indeed be enrolled and retained in each intervention arm, post hoc analysis indicates that the power achieved for the HAZ criteria is 89% and for the L:R criteria is 94% (if normally distributed) and 93% (non-normal distribution).

Meaningful lasting improvements in cognitive and physical stunting are not measurable until some years later as these children grow and we have thus not taken them into account.

Microbiome analyses will be performed on a subset of 50 children at 2 time points each; the sample size calculations for these tests are made on a recently developed program, which accounts for the depth of sequencing and the microbial diversity of the population [31, 37, 38].

Study population. Approximately 300 healthy children aged 6-11 months in villages surrounding Mitondo in the Chikwawa District of southern Malawi will be randomized to receive a legume-based complementary food made from cowpeas, common beans or an isoenergetic amount of corn flour, a traditional Malawian complementary food. These villages are very similar in that the residents are subsistence farmers growing maize on small plots, live in mud huts with thatch roofs, and use boreholes or nearby streams as their water source.

These infants will be recruited between the ages of 5.5 and 6.5 months, and their participation will last for 6 months. Enrollment will be ongoing, and extend over a 12 month period and involve health surveillance assistants, midwives, and other local health staff and village leaders to maximize outreach into the community. Given our extensive prior experience working in this community and our excellent working relationship with the Ministry of Health and District Health Officers in this area, we are optimistic about community engagement and subject retention.

Eligible infants will be screened by the research supervisors and physicians from our team. Specific exclusion criteria will be severe or moderate acute malnutrition, severe developmental delay or congenital malformations (including congenital heart disease) or any other known chronic disorder. After a thorough, tiered informed consent process presented to the community and parents, written as well as oral consent will be sought from the primary caretaker, who is almost always the mother or another matriarchal figure. Attempts will be made to engage any paternal figures in the household in the consenting process as well in order to maximize compliance with the study interventions and decrease attrition. Any caretakers reluctant to participate will not be encouraged to do so, and any participant desiring to leave the study after enrollment will be allowed to do so without coercion. This method of informed consent has been used successfully by the research team in the past, and been endorsed by the University of Malawi College of Medicine Research and Ethics

Committee and the Washington University Human Research Protection Office.

Food interventions. Recipes will be developed by the research team in conjunction with MSc students in the Department of Food Science and Technology on the Bunda Campus of the Lilongwe University of Agriculture and Natural Resources (LUANAR). LUANAR, formerly known as the Bunda College of Agriculture, is the principal institution of higher education for agriculture, development studies, nutrition, natural resource management, and food sciences in Malawi. The Washington University research team has developed over 50 recipes in prior studies that have been accepted by the Malawian general population. Candidate recipes will undergo acceptability testing in 6-11-month-old Malawian infants over a 2-week period to select those to be used in the study. About 3-4 recipes will be selected for each of the target legumes (cowpea and common bean) to offer diversity and choice to the caretakers, as they will be asked to feed the food to their child daily for 6 months. The energy content of the complementary food will be in accordance with WHO specifications, 200 kcal/d for children 6-9 months old and 300 kcal/d for children 9-11 months old [39]. The materials that will be supplied to mothers will have undergone pre-processed, such that the families need only to add hot water and stir the content to prepare the complementary food. Mothers will be taught how to prepare these recipes in a manner compatible and feasible with available local resources and customs. Compliance will be assessed through home visits every 4-6 weeks and detection of amylase-resistant starches in the infant's stools indicative of legume consumption.

Study participation. Each child will return for biweekly follow-up, including assessments of potential adverse events, anthropometry, and screening for acute infectious morbidity (fever, cough, and diarrhea). The village health workers and local health centers will be engaged throughout this process, particularly for referral of children identified as suffering from an acute actionable illness. Dietary intake will be assessed every 6 weeks by a nurse-administered standardized food frequency questionnaire [18, 40]. Children will return 12 and 24 weeks after starting the intervention for post-intervention assessments, including dual sugar absorption tests, stool collection, and anthropometric measurements.

These sites will be supervised on a weekly basis by the Malawian graduate student, after appropriate mentoring and education by the PIs. Our research group's team of research nurses and local staff will be involved, as we have done in prior studies. We are well-suited to this task, having had extensive experience in all of the aspects of data collection described in this proposal in our earlier efforts in this population. Given our history in these villages and our excellent working relationships with the community leaders, we anticipate a high likelihood of success in the enrollment and conduct of these studies.

Anthropometry. Weight will be assessed to the nearest 5 g using standard digital scales (seca 334, Chino, CA) and height will be measured to the nearest 0.1 cm using a rigid height board (seca 417, Chino, CA). Mid-upper-arm circumference (MUAC) will be measured on each child's left upper arm using standard flexible plastic insert tapes to the nearest 0.1 cm (TALC, St. Albans, UK). Each child will also be assessed for kwashiorkor by pressing with the pad of the thumb on the dorsum of the foot and observing for pitting edema. Any child with a weight-for-height Z-score (WHZ) less than -3, MUAC less than 11.5, or pitting edema will be classified as severely malnourished; children with WHZ between -2 and -3 or with MUAC between 11.5 and 12.5 will be classified as moderately

malnourished [41]. Malnourished children will be excluded from enrollment in the study and instead be treated with ready-to-use lipid-nutrient spreads per usual protocols until they recover [42].

Assessments of EE. Before the food interventions are begun, infants will undergo a dual-sugar permeability test, namely, the L:R test. The L:R test is based on the observation that monosaccharides, whether metabolized by the body for energy or not, are normally absorbed across the surface of a healthy small intestine, while disaccharides are largely not absorbed. In EE, there is decreased absorption of the monosaccharide because of reduced intestinal surface area, while at the same time inflamed epithelial, resulting in increased permeability via non-intact paracellular junctions, permit passive diffusion of disaccharides into the body (67) . Through the oral administration of a non-metabolized monosaccharide and disaccharide, both of which are subsequently excreted unchanged in the urine, the relative absorption of these two compounds can be quantified in urine and a non-invasive assessment of gut integrity can be made.

The L:R test involves the ingestion of a solution of a fixed quantity of these safe, non-metabolized sugars in a water solution by the child, followed by a complete 2-hour urine collection. The total quantity of urine is recorded and an aliquot is flash frozen in the field and saved for later analysis in the laboratory where the quantities of the sugars are measured. An inhibitor of bacterial growth is used as a preservative in the urine to prevent bacterial degradation of the sugars. The sugars are detected in the urine by a high pressure liquid chromatography in the laboratories of Robert Shulman, a USDA collaborator [43]. Other sugars have been used in dual sugar permeability testing, most commonly lactulose and mannitol, but a recent comparative investigation has found that the L:R test is the most reliable in low diuresis conditions, making it most practical for such a large study with such young infants [44].

Fresh stool samples will also be collected from the infants for fecal human mRNA measurement and microbiome assessment. Stools will be transferred to clean cryovials using sterile instruments and flash frozen in liquid nitrogen. The method of quantification of human mRNA was developed by the PI and has been used successfully in many thousands of samples [45]. Nucleic acids are first extracted using a NucliSens EasyMAG kit (BioMérieux), which is a bead-based RNA-specific method. A digital droplet polymerase chain reaction is used to reliably detect low copy numbers of human mRNA. Digital drop PCR is unique in that after the PCR is set up in the aqueous phase, the reaction is emulsified in oil, creating 20,000 aqueous bubbles that are counted by laser for positive fluorescence of the probe of interest. The quantities of mRNA in a given sample are normalized to GAPDH, a housekeeping gene present in abundant quantities. Aggregate results from each isolation technique demonstrate the utility of at least 7 probes correlated with EE in this population: defensin α5, super oxide dismutase-2, chemokine ligand 20, REG1B, leucine aminopeptidase-3, keratin 20, and CD69.

Specimens will be initially processed on site or in Blantyre at the University of Malawi College of Medicine if appropriate facilities are available, then subsequently transported back to the United States where EE specimens will be analyzed as we have previously reported [6, 11, 45-47].

Microbiome analysis. Bacterial genomic DNA will be prepared separately from fecal samples obtained from study children. To prepare the stool samples for sequencing, genomic DNA will be extracted using standard protocols (http://www.hmpdacc.org/tools_protocols/tools_protocols.php).

V1-V3 variable regions of bacterial 16S rRNA genes will be amplified by PCR and the resulting amplicons will be subjected to high throughput sequencing. We have mostly used a Roche 454 sequencer to generate about 5000 reads per sample. Currently, we are transition ---from the 454 platform to Illumina Miseq platform for targeted 16s rRNA sequencing, which provides the same or greater reading depth but at lower unit cost (depending on bar-coding opportunities with which to split runs into multiple single specimen analysis opportunities). Deep sequencing permits the recognition of rare members of the microbiota that may be shared or that may discriminate these human populations. 16S rRNA sequences will be processed through the pipeline developed by human microbiome projects at The Genome Institute at Washington University. In brief, quality trimming, denosing and chimera removal will be performed to yield high quality reads [48]. Reads passing the above filtering steps will be classified from the phylum to the genus level using an automated implementation of the Ribosomal Database Project's Classifier [49]. Species-level bacterial phylotypes will be defined as organisms sharing at least 97% nucleotide sequence identity in the V1-V3 regions of their 16S rRNA genes using the Mothur software package [50].

Shotgun sequencing will be performed for all samples to examine the taxa that are prominently represented in the infant microbiome but inefficiently captured by PCR primers designed to target other variable regions. Whole genome sequencing libraries will be prepared following a standard protocol from Illumina. Ten GB 100 bp pair-end (PE) reads per sample will be sequenced on the Illumina HiSeq platform. Reads passing quality filtering and human contamination removal will be classified to species level based on nucleotide sequence alignment to reference genomes using the RTG map software (Real Time Genomics, San Bruno, CA). Read mapping results will be transformed to values representing the coverage of each reference genome using refcov software (http://gmt.genome.wustl.edu/gmt-refcov). The coverage values are then scaled to a million aligned reads to calculate Depth of Coverage Per Million reads (DCPM) values. Taxa-based analysis will be the same as the 16S rRNA genes approach described above.

Data Management and Analyses. Clinical data, such as anthropometry, demography, and morbidity data, will be collected on standardized forms by field workers. Field workers will be trained and validated with the questionnaires and measurements they collect prior to data collection. Anthropometric z-scores will be calculated from the 2006 WHO Multicentre Growth Reference Survey [51]. Data will be double-entered into a password-protected Microsoft Access database by clerks blinded to food assignments. All discrepancies will be resolved by examination of the original data sheets and discussion with the relevant field workers. Once all values are entered and discrepancies resolved, the data set will be locked.

Comparisons will be made between cowpea and control separately from common bean and control. Fisher's exact test will be used for discrete parameters, and Student's t-test will be used for continuous parameters. A difference that has a $P < 0.05$ will be considered statistically significant. These statistical methods will also be used for all clinical data and EE measures, which are continuous measures of EE status.

The microbiome data analyses and interpretation require specialized tools, to which we have close access at Washington University. Read-based metabolic profiling of the microbial communities is performed by first using whole genome sequencing reads from each sample to probe the KEGG gene

database using Mblastx (MultiCoreWare, St. Louis, MO) and then search results are run through the metabolic pathway pipeline the Genome Institute developed as part of the Human Microbiome Project, to obtain enzyme and pathway abundance and coverage from metagenomic communities. Differentially expressed enzymes and pathways between groups are identified using LEfSe (http://huttenhower.sph.harvard.edu/lefse). The same exploratory analyses using Principal Component Analysis (PCoA) will be performed to identify cluster patterns based on the gene content and metabolic pathway abundance between case and control groups.

All microbiome data will be rarefied to ensure the sample comparisons are at the same read depth. Ordination methods will facilitate the pattern analysis of the high dimensional microbiome data by reducing its dimensionality to 2-3 dimensions, such that the original distance between the samples is preserved to a large degree. In particular, PCoA with Bray-Curtis dissimilarity will be used to identify the sample cluster pattern based on the microbial community structures.

A robust statistical approach that is widely used in ecology and metagenomics, Permutational Multivariate Analysis of Variance (perMANOVA in R), will be used to test the significance of differences at the whole bacterial community level between two or more groups. Dissimilarity matrices will be generated based on the taxon abundance table, and will be treated as the dependent variable in the model. We will use metastats, a software developed in the Human Microbiome Project, to detect differentially abundant taxa between case and control groups [52]. The statistical tests will be corrected for false discovery rates using the q-values approach, an approach that has been effectively applied in metagenomic studies for identifying the taxa of interest. Indices such as richness (the total different number of taxa) and Shannon diversity (the number of taxa present as well as the relative abundances) that categorize the complexity of the bacteria community will be computed by using R software (http://www.r-project.org). Student's t-test will be used to compare the diversity differences between groups.

Specific Aim 2: Evaluate changes in child growth (height-for-age and weight-for-height z scores), biomarkers of EE (lactulose:rhamnose and a panel of human mRNA messages correlated with EE) and the characteristics of the microbiome (population taxonomy from phyla to genus, and the collective metabolic capacity expressed as Kyoto Encyclopedia of Genes and Genomes, KEGG, categories) after adding either cowpeas or common beans to the diet of 12-35 month-old rural Malawian children.

Sample size. Similar to the trial described in Aim 1, we plan to enroll 300 children for this study.

Study population. Children aged 12-35-months-old in the villages surrounding Nthole in the Chikwawa District of southern Malawi will be randomized to receive either cowpeas or common beans or an isoenergetic quantities of maize, the traditional Malawian staple, for 12 months. Children will be excluded if they have moderate or severe malnutrition, overt chronic illness such as cerebral palsy or congenital heart disease, or their caretaker declares an intention to leave the geographic area during the 12 study months.

Food interventions. For this older population of children, approximately 10 recipes that include either cowpeas or common beans will developed by collaborators at LUANAR. These recipes will be

practical in terms of the processing and cooking requirements, and tested for palatability and acceptability by children similar to the study population in a 2-week trial. The amounts provided will be such that they constitute 15% of the dietary intake (calculated by energy). An appropriate overage amount will be chosen to account for family sharing. Because these children are typically fed the same foods that the rest of the family consumes, provision of rations of unprocessed legumes will need to be provided for the rest of the household as well to ensure that the study child receives an adequate aliquot of the intervention food for consumption.

Study participation. Inclusion and exclusion criteria, screening methods, anthropometry, and the consent process will be similar to those employed in Aim 1. Dietary intake will be assessed every 6 weeks by a food frequency questionnaire. Follow-up in this study will also occur biweekly with children returning 12, 24, 36, and 48 weeks after starting the intervention for follow-up specimen collection. Compliance will be assessed and intervention food intake will be measured via scheduled home visits.

Assessments of EE and characterization of the microbiome and its metabolic capacity. These analyses will be accomplished using identical specimen collection techniques and laboratory methodology as described in Aim 1.

4. Collaboration with Host Country Institutions

This project will be a collaboration between Washington University in St. Louis, the University of Malawi College of Medicine, and LUANAR, the principal agricultural university in Malawi.

The two senior investigators from the US also hold faculty appointments at the College of Medicine, and their collaboration with the two Malawian senior investigators ensures a balance of scientific interests and cultural sensitivity. The College of Medicine is the principal authority on preventive and curative health in Malawi, with the Ministry of Health reliant on the College for innovation and guidance in matters of health and nutrition. By basing the project within the College of Medicine, we are assured that the findings will be viewed by the Ministry as relevant to the local context and representative of the subsistence farmers that FTF aims to assist. By engaging students and faculty at LUANAR, the development of appropriate recipes for our chosen legume varieties will also be culturally sensitive and feasible in the village setting, and the interventions that are successful are more likely to be implemented for the long term. These crops are already easily and widely cultivated in Malawi and will not post burdensome agronomic challenges. Moving forward, the agricultural experts at LUANAR will also be able to provide guidance on intercropping with common cereals and other farming practices that will provide a practical path to implementation of the intervention on a broad scale by subsistence farmers if the data ultimately support such use. Their expertise and advice on sustainable farming methods will also be sought so as to minimize the environmental degradation that may occur with a transition in crops that are farmed.

In a practical manner, the Malawian PIs will maintain day-to-day responsibility over the project and budgeting, including the supervision and mentoring of the graduate students supported by the project. In addition to the PIs, the research team currently consists of five research nurses, three ancillary staff, and a business manager, all of whom are Malawians and are essential to ensuring that

the interventions and methods used are appropriate to local context. This team will be expanded to include more local expertise in agronomy, given the focus of the studies described here.

The processing of blood, urine, and stool specimens will take place at the College of Medicine and will also serve to employ a student or recent graduate of the College's Medical Laboratory Technology program. As is our custom, laboratory equipment needed for the study including investments such as centrifuges will be geographically located in the labs at the College and will be available for sharing with local researchers who otherwise would not have access to such equipment. This equipment then becomes the property of the College following completion of our studies. At the same time, during the several months per year that Dr. Trehan is physically in Malawi, he will continue to spend a significant portion of his time (typically 30%) teaching students and registrars at the College and providing direct patient care, as he has done during prior studies. These efforts will serve to further develop local capacity and future researchers with an interest in nutrition and food security.

5. Coordination with other International Nutrition and Grain Legume Research Programs

The co-principal investigator in Malawi is Vernon Kabambe at LUANAR. He is the foremost legume researcher in Malawi, and is an active faculty at his university. He is the project's primary conduit to any other grain legume research programs in Malawi. At our regular project meetings, he will attend and offer insight as to into how our project can complement other efforts.

Ken Maleta is a lead member of the Investigation of Lipid Nutrient Supplements (iLiNS) project, a large Bill and Melinda Gates Foundation-supported effort in Malawi. He provides a direct link between this legume project and any other international nutrition programs in Malawi. We have already agreed to share equipment with the iLiNS project, reducing our costs.

We will present our results at international nutrition and food research meetings focused on FTF themes, which will allow us an opportunity to synergize with other projects.

6. Outputs

The most important anticipated output from this project is an understanding of whether or not the targeted introduction of legumes (specifically, cowpeas and common beans) into the early weaning diets of children can promote normal growth and limit the occurrence of EE. If so, these interventions could then be anticipated to have a host of positive health outcomes in these high-risk children, including improving height and weight gain, decreasing the incidence of acute diarrheal illnesses, and improving the effectiveness of oral vaccines. This would be a novel, sustainable, and feasible agricultural and dietary intervention for this pressing problem in global child health.

As with our previous research, the major method of dissemination of our findings, whether positive or negative, will be through peer-reviewed publication in major medical journals. Manuscripts describing the findings from each Aim and describing each component under study (EE, growth, microbiome, legume acceptability) will all be shared through this medium. We can also anticipate that the process and results of developing novel legume recipes for use in this context will be of

interest in the global food science community. Given the importance of interactive feedback and peer engagement in the scientific process, we anticipate that preliminary results will also be shared at relevant scientific meetings. Close involvement of colleagues and interested parties at FTF, USAID, the Legume Innovation Lab, and the Malawi Ministry of Health will also be maintained and include periodic presentations to these groups. Finally,, and most importantly, periodic (at least semi-annual) interactive updates to colleagues at LUANAR and the College of Medicine will be made, including in public forums, for example at the College's annual Research Dissemination Conference.

The major anticipated milestones of the project include:

Year 1:

- IRB approval from the University of Malawi and Washington University
- Production of a Manual of Operation
- Recruitment of staff
- Development of legume recipes for both specific aims with LUANAR colleagues
- Acceptability testing of legume recipes in infants and children
- Identification and training of Malawian graduate student for Aim 1
- Community mobilization and engagement in Mitondo for Aim 1

Year 2:

- Continuous enrollment, randomization, intervention delivery, and specimen collection in Mitondo for Aim 1
- Identification and training of Malawian graduate student for Aim 2
- Community mobilization and engagement in Nthole for Aim 2

Year 3:

- Conclusion of follow-up visits for children enrolled in Mitondo for Aim 1
- Specimen processing and data analysis for Aim 1
- Continuous enrollment, randomization, intervention delivery, and specimen collection in Nthole for Aim 2

Year 4:

- Conclusion of follow-up visits for children enrolled in Nthole for Aim 2
- Specimen processing and data analysis for Aim 2
- Manuscript preparation and submission
- Evaluation of future directions and implications of findings with key local and international stakeholders

B. Capacity Building of Partner Host Country Institutions

Capacity building for operational research of the highest quality is important in translating results into the betterment of the population. Even if the results of the proposed study are striking and convincing, a series of operational implementation studies would be needed to actually improve the health and well-being of Malawians. We therefore strongly believe LUANAR and the University of

Malawi must be ready to lead subsequent combined health, nutrition, and agricultural studies and projects. This capacity building starts with employing the highest quality and most practical study design in the current project. The local team implementing the clinical trial will be trained in the principles and practice of "Good Clinical Practice". The field team leader, Chrissie Thakwalakwa, will complete her PhD as a part of this project. She has already completed the coursework for a PhD in Public Health Nutrition, and this research will help satisfy requirements to complete her thesis.

A key component of our capacity building plan will be through LUANAR. The collaborating agronomist identified at LUANAR is also the Program Manager for the Soil Health Consortium of Malawi. The main role of the consortium is to encourage stakeholders to disseminate knowledge on Integrated Soil Fertility Management (ISFM), which includes legume rotation. The consortium holds ISFM symposia, travel workshops, and annual meetings, producing technical and policy briefs after these various consultations. Relevant National Agriculture Research Societies (NARS) and Consultative Group on International Agriculture Research (CGIAR) institutions, the National Legume Platform, the Seed Traders Association, and the fertilizer associations are all members. Since he routinely attends NARS and CGIAR planning meetings, the research program and outputs from this proposed project will thus be shared with partners in all of these platforms, helping fill gaps in their knowledge base and also to help identify areas for synergy and collaboration.

The project will support Masters-level training of up to 4 students who will undertake their dissertation work within the project. These students will work on developing and testing applicable recipes for the interventions and will be enrolled in the Master of Food Science program at LUANAR. Two Malawian doctoral students, one for each Aim, will be identified and trained in clinical research design and in the theory and practice of malnutrition and nutritional interventions. They will enroll in the graduate program at the College of Medicine. This will include didactic coursework in Malawi, online, and with mentored one-on-one instruction with the PIs. To complement their field experience, these students will then spend 12 months in the classroom and laboratory in St. Louis following the end of the clinical phases of the study. There they will be mentored in specimen preparation, DNA extraction, PCR amplification, and analysis of the EE mRNA specimens and/or in the liquid chromatography methods used to quantify urinary sugars for the L:R test. They will also receive support for any coursework they choose to take at Washington University in St. Louis to complement their studies in Malawi. Coursework that we can anticipate being relevant include skills courses such as Outcomes and Clinical Research, Statistics, Scientific Writing and Grantsmanship, and Ethical Issues, as well as knowledge-based courses such as Medical Botany, International Public Health, Anthropology and Public Health, and International Perspectives on Child Development, and the Global Hunger and Undernutrition transdisciplinary problem solving course. In addition, a number of seminars are offered through Washington University's Institute for Public Health and the Mentored Training Program in Clinical Investigation, in which both United States PI's serve as members and faculty advisors. The degree granting institution will be the University of Malawi. Some basic investment in laboratory outfitting for stool processing will be included in the grant, as well as training for laboratory technicians. This proposal thus includes capacity building on a variety of levels, so that a competent field team for clinical trials is intact in Malawi, with capacity built among scientists, nurses, laboratory technicians, and infrastructure.

C. Alignment with USAID Feed the Future Goals and Strategic Research Objectives

1. Alignment: The US Government's FTF Objectives include supporting a *multifaceted approach to nutrition and sustainably reducing global poverty and hunger*. Local crop-based approaches, such as legumes, provide the opportunity to be sustainable as legumes are already grown throughout the developing world. This proposal is strategically aligned with these goals. EE is estimated to cause about one third of the child stunting seen worldwide and the causes of EE are multifactorial. A dietary intervention utilizing a ubiquitous, nutritious foodstuff, legumes, is therefore an attractive and durable intervention.

2. Gender Equity: The focus of this project is on improving child health, which, in rural sub-Saharan Africa, remains in practical terms primarily a women's issue. Thus, the research results will be of direct interest to a majority of African women with young children. Farming and food selection are primarily the purview of women in sub-Saharan Africa as well. If the intervention of increased legume consumption is shown to reduce gut inflammation, this would ultimately need to be implemented by women. Studies to evaluate the effectiveness of legume supplementation in decreasing the high rates of stunting and EE in children have the potential to contribute to the empowerment of women to optimize the selection of which crops are planted for their families' consumption. Furthermore, given the heavy investment in time and resources to keep numerous children alive in these difficult rural settings, and given the body of evidence that correlates improvements in child mortality to improvements in women's empowerment and autonomy in family planning, improvements in child survival will serve to improve women's health by this mechanism as well [53]. Ultimately, in the long term, improvements in child mortality and morbidity may lead to decreased fertility and increased opportunities for women's education and other contributions to gender equity. Gender equity is present in the research team as well, as already the majority of the team consists of Malawian women, including a senior investigator. The 6 trainees that this project will support offer an additional opportunity to promote gender equity.

3. USAID Mission Engagement: The PI met with Cybill Sigler and John Edgar from the FTF team at the USAID mission in Lilongwe, Malawi in January 2014. This project was described to them, and they were delighted to learn that FTF research might be centered in Malawi. They expressed interest in taking advantage of the "associate" role and providing funding for a pilot study if the findings from this work warrant subsequent investigation. They expressed interest in describing the project up for the local USAID publication, and we promised to remain in close communication.

D. Past Experience

Our research group has conducted dozens of groundbreaking studies over the last 20+ years into the pathophysiology, diagnosis, and treatment of acute and chronic malnutrition, both in inpatients and outpatients. Most significantly, the PI conducted the first clinical trials that demonstrated that a peanut-based ready-to-use therapeutic food (RUTF) was an effective, reliable intervention for the treatment of severe acute malnutrition (SAM) [54, 55]. This work was then extended to include the first trials to demonstrate that locally-produced RUTF was as effective as imported, more expensive, commercially-produced RUTF [56]. This demonstration of the effectiveness of locally-produced foods

then led to the construction and continued operation of RUTF factories in Malawi, Sierra Leone, and soon Ghana, demonstrating the local capacity-building nature of the research [57]. In sum, the development and continued evolution of community-based management of acute malnutrition (CMAM) [42, 58, 59] is in large part to the clinical studies conducted in Malawian villages similar to the ones proposed here. We have also developed and tested in large clinical trials novel therapies for moderate acute malnutrition [60, 61], the results of which have also been implemented into operational settings in other vulnerable populations.

Our research group continues to conduct high-quality, high-impact clinical trials advancing the state of knowledge in the treatment of SAM [30, 62], moderate acute malnutrition MAM [61, 63, 64] and the intestinal microbiome's role in health [37] and disease [31]. The microbiome work continues to benefit from the collaboration with basic science faculty at Washington University, an institutional pioneer in the rapidly growing science of the commensal microbiome [32]. Recent internal developments at Washington University also have moved many staff and facilities from The Genome Institute under the umbrella of our Department of Pediatrics, allowing for even closer collaboration, bringing Professor Phil Tarr into a collaborator role on this application. The Genome Institute offers expertise in translating reads to genes and transcripts, and led the NIH Human Microbiome Project [65].

In addition to this broad array of field studies in malnutrition, our group's experience with EE specifically is long-standing and well-established [6, 11, 66-69]. The group has expertise in the design and conduct of EE studies using the standard methods for the measurement of EE with dual-sugar absorption tests [70], as well as being active in developing newer methods involving fecal biomarkers of enteropathy [45-47].

Dr. Manary and Ms. Thakwalakwa maintain faculty appointments in the Department of Community Health at the University of Malawi College of Medicine; Dr. Trehan maintains an appointment in the Department of Paediatrics and Child Health; and Dr. Maleta serves full-time as the Principal of the College of Medicine and a Professor in Community Health. As active participants in the education and research enterprise at the College of Medicine, they are all committed to local capacity building and nurturing future generations of researchers focusing on poverty and malnutrition.

Finally, Dr. Manary and Dr. Trehan are respected members of the biomedical research community at Washington University, and have access to services and experts in the realm of microbiome analyses and complex genetic associations. In this project the deep sequencing will be done by the Genome Institute, the lead organization in the recent Human Microbiome Project. With that comes the skills and insight of the finest sequencers and bioinformaticians in the world. Yanjaio Zhou is the bioinformatician who will be dedicated to our project.

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