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Investigating the Impact of *Moringa oleifera* Supplemented to Kenyan Breastfeeding Mothers on Maternal and Infant Health: A Cluster Randomized Single-Blinded Controlled Pilot Trial Protocol

*Jerusha Nyabiage Mogaka, BSN, MPH, †‡Patrick Mbullo Owuor, MA, PHD, †Silvia Odhiambo BS, §Carrie Waterman, PHD, ‖Michelle K. McGuire, PHD, ¶#George J. Fuchs, MD, and ¶Suzanna L. Attia, MD, MSCPH

Background: Undernutrition contributes to up to 45% of deaths globally in children <5 years, with an optimal time for intervention before 24 months of age. Breastmilk microbiome helps establish the infant intestinal microbiome and impacts infant intestinal and nutritional health. Inadequacies in breastmilk composition such as low vitamin A contribute to infant nutrient deficiencies. Changes in milk fatty acid composition (reduced saturated and increased unsaturated fatty acids) may reduce susceptibility to enteric infection and increase protective intestinal bacteria. *Moringa oleifera* leaves (moringa) provide high nutrient concentrations (including protein, iron, vitamin A) and increase milk production; this may enhance breastmilk quantity and quality and improve infant health.

Objective: To investigate the role of moringa supplementation to improve maternal and infant nutritional and intestinal health via changes in maternal milk quantity and quality.

Methods: Fifty mother-infant pairs exclusively breastfeeding will be enrolled in a single-blinded randomized controlled trial in Kombewa County Hospital and Chulaimbo SubCounty Hospital, Kisumu, Kenya.

Received January 5, 2022;accepted June 22, 2022.

From the *School of Nursing Science, University of Washington, Seattle, Washington; †Pamoja Community Based Organization, Kisumu, Kenya; ‡Department of Anthropology, Weinberg College of Arts and Sciences, Northwestern University, Chicago, Illinois; §Institute of Global Nutrition, University of California, Davis, Davis, California; ‖College of Agricultural and Life Sciences, Margaret Ritchie School of Family and Consumer Sciences, University of Idaho, Moscow, Idaho; ¶Department of Pediatrics, Division of Pediatric Gastroenterology, Hepatology, and Nutrition, University of Kentucky College of Medicine, Lexington, Kentucky; and #Department of Epidemiology and Department of Preventive Medicine and Environmental Health, University of Kentucky College of Public Health, Lexington, Kentucky.

Guarantor of the article/PI: Suzanna L. Attia

Correspondence: Suzanna L. Attia, MD, MScPH, Department of Pediatrics, Division of Pediatric Gastroenterology, Hepatology, and Nutrition, University of Kentucky College of Medicine 138 Leader Avenue, Lexington, Kentucky 40508. E-mail: suzanna.attia@uky.edu.

CW received NIH Fogarty International grant funding (FIC K01 no. TW 009987) to support this research. CW is a coinventor on the US patent application No. 14683730 titled: Extracts from Plants of the Moringaceae Family and Methods of Making. This does not alter our adherence to the journal's policies on sharing data and materials. The ramining authors have no conflicts of interest to report.

Clinical trial Registration: This clinical trial is registered at https://clinicaltrials.gov/ct2/show/NCT04587271 number NCT04587271.

This project is supported through a K01 award from NIH NCCIH/Fogarty International Center to Dr Carrie Waterman at the University of California, Davis, as well as Dr Suzanna Attia's funding through start-up funds from the University of Kentucky College of Medicine and the NIH BIRCWH (Building Interdisciplinary Research Careers in Women's Health) Scholar program.

JPGN Reports (2022) 3:3(e237)
ISSN: 2691-171X
DOI: 10.1097/PG9.0000000000000237

What is Known?

- Practical tools for the prevention or treatment of malnutrition remain elusive.
- Moringa is a drought-resistant and fast-growing tree whose leaves are high in complete protein, bioavailable iron, vitamin A precursors, and multiple other nutrients and can be dried, powdered, and stored for many years for future use.
- *Moringa oleifera* leaf powder (moringa) consumption at high doses has been shown to improve vitamin A and iron status and at low doses to increase human milk production and animal milk composition in limited clinical trials and animal studies.

What Is the Impact on Clinical Practice?

- The effect of high dose (20 g daily) moringa leaf powder supplementation on human milk output is unknown.
- The effect of moringa supplementation on human milk composition including milk microbiome and on the growth, nutrition, and microbiome composition of the breastfeeding infant is unknown; the effect on maternal and infant intestinal health is also unknown.
- Any positive impact from moringa leaf supplementation on breastfeeding mothers and their infants in a nutritionally at-risk population such as ours in western Kenya can be tested in a real-world setting where families grow, process, and consume their own moringa leaf powder.

Intervention: Dietary Supplementation of 20 g of *Moringa oleifera* leaf powder divided twice daily in corn porridge consumed daily for 3 months while control comparator will receive porridge daily for 3 months.

Outcomes: Change in infant growth and maternal milk output (primary); maternal and infant vitamin A and iron status, changes in infant and maternal intestinal health (secondary).

Participating Centers: Pamoja Community Based Organization, Kombewa Sub-County Hospital, and Chulaimbo Sub-County Hospital.

Keywords: malnutrition, breastfeeding, maternal and child health

INTRODUCTION

Childhood undernutrition (low weight for age, low height for age, low weight for height, or micronutrient deficiencies)

contributes up to 45% of deaths globally in children under 5 years and negatively impacts individual and societal health by impairment of long-term health, cognitive capacity, productivity, and wage-earning force (1). Undernutrition may begin *in utero* because of maternal undernutrition and micronutrient deficiencies (2). After birth, poor nutrient intake, frequent infectious disease, and poor intestinal health lead to ongoing impairments in growth and psychomotor and cognitive development. Intestinal inflammation and reduced gut integrity from chronic exposure to intestinal pathogens leads to Environmental Enteric Dysfunction or EED (aka "tropical sprue") (3). Large-scale research has increasingly established the role of intestinal inflammation and epithelial barrier dysfunction in stunting, reduced efficacy of oral vaccines, reduced absorption of nutrients, intestinal dysbiosis, and increased morbidity and mortality in childhood undernutrition (3–5).

Enhancing maternal and infant health in the first 1000 days of life from conception to 24 months is a crucial target to prevent childhood undernutrition (6,7). Inadequacies in breastmilk composition such as low vitamin A contribute to infant nutrient deficiencies (8), and decline in milk supply may lead to cessation of exclusive breastfeeding, among other reasons (9,10). Human milk is not sterile, and the breastmilk microbiome correlates to infant intestinal microbiome populations and may alter subsequent infant intestinal and nutritional health (11,12). Thus, enhancement of the quality and quantity of breastmilk is also an area of active research, especially in limited-resource settings (13,14), such as in Kenya where exclusive breastfeeding is estimated at 60% (15), with high rates of undernutrition in infants 0 to 3 months (10%–22%) (16–20).

Moringa oleifera (Moringa) is a drought-resistant, rapidly growing, nutrient-dense tree that can be grown and consumed at home (21). Moringa leaves have a high concentration of complete protein (30 g/100 g dried leaf), iron (97.9 mcg/g dried leaf), Vitamin A precursors (17.6–39.6 mg/100 g dried leaf), B vitamins, calcium, many other essential nutrients and fiber and can be dried for shelf-stable year-round consumption (22–24). As per our systematic review of moringa's effect on nutrition and lactation in humans and animals, we ascertained that moringa at high doses (14-30 g/day) but not lower doses (<10 g) improved iron and vitamin A status and growth in limited human populations, and 0.5 g a day increased human milk production in 1 high-quality study (25). Since completion of our review, Fungtammasan et al. have published a protocol of an RCT to evaluate changes in breastmilk volume as well (26). Moringa has also led in preclinical studies to reduced intestinal inflammation in rat models of colitis (27,28), and to improved breastmilk composition via increased antioxidants and reduced markers of oxidation including catalase, vitamin C, malondialdehyde as well as increased fat content and enhanced unsaturated fatty acid concentration in animal studies (29–31). Human studies assessing changes in breastmilk composition with moringa supplementation are not available. Limited preclinical studies suggest that moringa components may influence the intestinal microbiome, but the impact of leaf powder on the human intestinal microbiome is unknown (32–35). The human milk microbiome of the mother directly influences her infant's microbiome, and breastmilk compositional changes may lead to changes in intestinal health via alteration of the infant microbiome (11,36,37). Moringa leaf is safe with no anticipated adverse effects according to our review of nutritional interventions using moringa leaf supplementation and to other sources (25,38).

Rationale

This project will guide more extensive studies examining the role of moringa as a potentially effective, practical, and low-cost resource to reduce the impacts of maternal and child undernutrition.

Study Objectives

This study aims to determine if *Moringa oleifera* leaf powder supplementation (moringa) will improve maternal and childhood undernutrition through direct consumption and indirectly through enhanced maternal breastmilk volume and composition. The study will (1) determine if moringa supplementation improves infant and maternal growth, vitamin A, or iron status; (2) evaluate the effect of moringa on breastmilk output and composition; and (3) explore potential changes in maternal and infant intestinal health.

MATERIALS AND METHODS

Study Overview

Our study follows an acceptability trial in Kisumu, Kenya, of the intervention dosing of 20 g moringa leaf powder in corn porridge in our target population (unpublished data). We found that 20 g moringa was acceptable when divided into 10 g at a time mixed into 1 cup of corn porridge twice daily. This was made from corn flour and moringa leaf powder provided by us, prepared by us, and given to mothers (n = 10) with standardized ratings of taste, texture, smell, and acceptance to consume multiple servings. We will publish these details separately. We will then perform a single-blinded cluster randomized controlled trial enrolling 50 mother-infant pairs followed for 3 months receiving 10 g moringa leaf powder in corn porridge twice daily versus corn porridge alone twice daily.

Sample Size

Sample size calculations for this pilot study were limited because of the lack of available preliminary data on the potential impact of this intervention on our primary outcome of infant growth. The effect on growth is hypothesized to be because of increases in maternal milk output. Based on data from the highest quality study available of moringa's effect on milk output (39), we calculated sample size for 80% power at the 95% confidence level. According to this, 16 pairs total would be needed to see an effect as they observed by day 3 of supplementation with 0.5 g. Accounting for the possibility of attrition and the possibility for a much smaller effect on growth than on milk output directly, we have increased the sample size to the maximum estimated to fit within our study budget. This will be 50 mother-infant pairs (100 participants).

Location

Study activities will be conducted in western Kenya at the Chulaimbo Sub-County hospital and Kombewa County Hospital, which offer maternal and child health services to the Seme and Kisumu West Sub-Counties in Western Kenya. This region was chosen, as it encompasses the catchment region of study partner Pamoja Community Based Organization (CBO). This study has been registered at ClinicalTrials.gov (Clinical trials registration: NCT04587271). Ethical approval was obtained from the University of Kentucky Medical Institutional Review Board (UK IRB) in Kentucky and Amref Health Africa Ethics and Scientific Review Committee in Kenya. Written informed consent will be obtained from all participants before study procedures begin. Study enrollments and follow-up activities are anticipated to take up to 6 months.

Randomization, Recruitment, Enrollment, and Mitigation Strategies

Cluster randomization will be done at the facility level by a coin toss by the study staff. Eligible individuals will automatically be randomized to the control or intervention arm based on the enrollment site. Consecutive random sampling will be used when recruiting the mother-infant pairs until the sample size is achieved. Women with a single infant born at 36 weeks gestation or older, within 30

days old chronological age at enrollment, and intending to exclusively breastfeed their infant for at least 3 months will be invited to enroll with their infants in the study during their routine prenatal care visit at 2 subcounty hospitals of different subcounties within Kisumu. Utilizing 2 disparate subcounties and cluster randomization will reduce the possibility of participants in the intervention cluster sharing moringa with participants in the nonintervention cluster. Informed consent will be obtained from the mother. Women with contraindication for breastfeeding, receiving regular fortified food supplementation, consuming moringa regularly at the time of enrollment, or refusing to consume corn porridge with or without moringa after at least 3 days' attempt within the first month will be excluded. Infants unable to feed by mouth or with significant congenital disease will be excluded from the study. Written consent will be obtained from all eligible participants who agree to participate. Study staff will enroll the participants with informed consent in participant's preferred language (Luo, Swahili, or English), collect demographic and other characteristics from the mother and infant using a structured questionnaire, and take anthropometric measurements and laboratory sample collection of breastmilk (mother only), capillary blood (mother and infant), and fecal samples (mother and infant) from all participants (Table 1). We anticipate a dropout rate of 10% because of limited transportation during the COVID pandemic, the possibility of lost or nonworking cellphones of participants (the primary contact), and withdrawal by choice. We will enroll new participants only if the participants withdraw within the first 2 weeks of the study. After that we will not enroll new participants.

Intervention

In this region, taking multiple capsules or pills may lead to stigmatization with the suggestion of HIV positivity. We attempted to create a placebo powder but were limited by the challenges of replicating the bright green color and strong taste of moringa leaf powder without adding other factors that would also change nutritional outcomes. Our leading idea was microcrystalline died green with vegetable dye, but the process of obtaining such a placebo was both too expensive and too complicated for this pilot study. We welcome readers' ideas on an affordable and practical placebo for unencapsulated moringa leaf powder. Therefore, we chose to use an open-label method mixing moringa leaf powder into corn porridge, which is a commonly used food in our target region.

All participants will receive a standard volume corn flour and a standard measuring spoon with instructions on how much to prepare and consume daily as porridge mixed with water and salt or sugar. Moringa powder will be dispensed to the intervention group, who will be advised to consume 20 g moringa daily administered as 10 g in the same corn porridge twice a day for the whole study duration (3 months) based on our completed acceptability study. The control group will consume corn porridge without any additives. Moringa and corn flour will be replenished every month to ensure none is depleted for both study arms. We will instruct mothers on how to record their daily consumption of porridge using a pictograph and will weigh remaining moringa leaf powder and corn flour at each follow-up visit to assess adherence.

TABLE 1. Suggested enrollment, activities, and study visit schedule

Enrollment	1-month follow-up	2-month follow-up	3-month follow-up
Baseline questionnaire			Exit hemoglobin
Baseline hemoglobin			Blood and fecal collection
Blood and fecal collection			24-hour milk output
24-hour milk output			Breastmilk sample
Breastmilk sample			

Monthly anthropometrics.
Mothers: weight, mid-upper arm circumference; infants: weight, length.
Distribution of moringa leaf powder and corn flour monthly.
Review adherence, diarrhea, diagnosed illness, additional confounders monthly.

Blinding

Because of the difficulty in creating a blinded intervention as described earlier, we decided that we will maintain blinding of the primary investigator during the phase of data collection and through the analysis of primary outcomes (infant growth and 24-hour maternal milk output). Because of the prolonged nature of the analysis of secondary outcomes, the investigator will then be unblinded to which group receives control and which intervention.

Data Collection

A structured questionnaire will collect sociodemographic characteristics and obstetric and infant birth history. We will perform monthly anthropometric measurements for mother and infant (weight, height/length, mid-arm circumference). At enrollment and exit, we will collect 2 mL of whole blood for markers of iron and vitamin A status and inflammation (point-of-care hemoglobin by HemoCue Hb201, ferritin, serum retinol, soluble transferrin receptor, C-reactive protein, alpha-1-glycoprotein) and 8 g of fecal samples for microbiome and markers of environmental enteric dysfunction or inflammation (fecal calprotectin for mothers; infant fecal neopterin, myeloperoxidase, and alpha-1 antitrypsin for infants). We will collect 24-hour breastmilk output by manual pump and whole breast milk samples at enrollment and exit for analysis of milk volume, fat and fatty acid profile, vitamin A status, and microbiome. We will collect information monthly on confounders, including the incidence of diarrhea, daily moringa consumption, diagnosis of any illnesses, and iron and vitamin A supplementation during and after pregnancy and during the study period to mothers or infants.

We will assist mother to perform observed hand expression of the whole breast in the morning hours standardized to 7–11 AM collection time only from a healthy breast not emptied for 2 hours in a sterile and light-protected container. Breastmilk will then be gently swirled, approximately 15 mL aliquoted, and transported on dry ice to the lab for –80°C storage. We will collect anthropometric measurements using 2 study staff to perform recumbent length and weight in infants and to perform standing weight and height in mothers. The measurements will be repeated twice, and if deviation of more than 0.1 unit occurs, then a third measurement will be taken and the measurements averaged. Trained study staff will perform point-of-care hemoglobin testing using Hemocue Hb201 and capillary blood draw from infant heel and maternal finger. Capillary blood will be centrifuged to obtain serum and serum aliquoted and frozen at –80°C. We will collect fecal samples in a sterile container from mothers and infants, aliquot them, and freeze them at –80°C.

To facilitate the 24-hour breastmilk volume collection at home, we will provide mothers with the same brand of manual breast pump at enrollment and within a week of the exit visit after sterilizing them in boiling water for 5 minutes. We will instruct mothers on how to pump milk from both breasts before infant's usual feeding time from sunrise to sunrise, record the volume of milk in a pictographic manner in the parent diary, and feed the milk to their infant with a clean cup and provided spoon. We will recollect the breast pumps after the first (enrollment) measurement to sterilize them again before redistribution for the exit measurement following the same protocol. We will then instruct the mother on how to continue to disassemble, sterilize, and reassemble the breast pump and leave the pump with her for her ongoing use after the study ends.

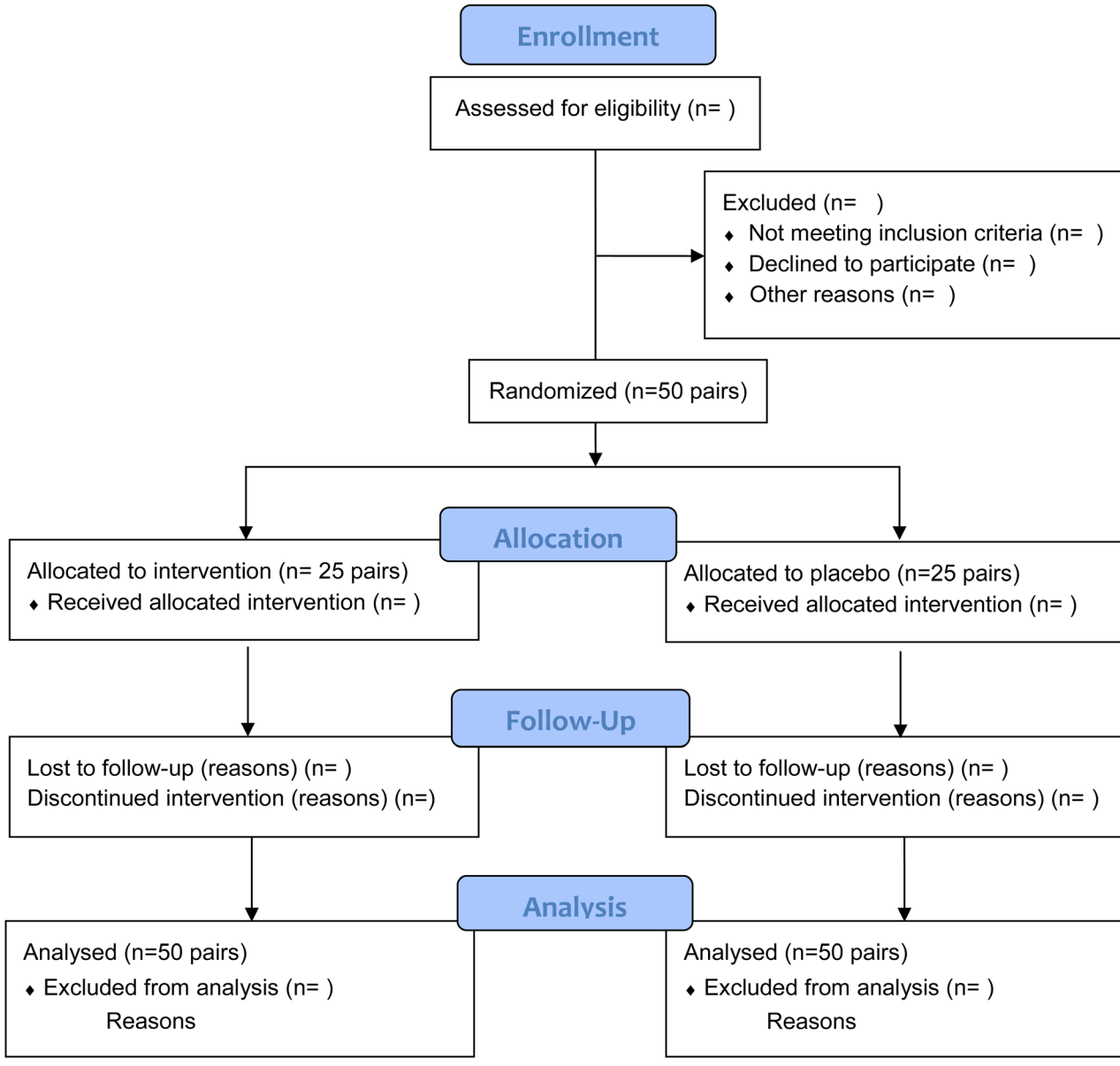

FIGURE 1. Consort flow chart.

Outcomes

The primary outcomes are infant growth (weight for length Z score, weight for age Z score, length for age Z score, insulin-like growth factor) and maternal milk output (24-hour pumped milk output). Secondary outcomes are infant and maternal micronutrient deficiencies (vitamin A: serum retinol-binding protein; iron status: hemoglobin and serum ferritin, soluble transferrin receptor), change in breast milk composition (microbiome, vitamin A/carotenoids, fat content, fatty acid profile), maternal growth (body mass index, mid-upper arm circumference), infant and maternal systemic inflammation (C-reactive protein, alpha-1-acid glycoprotein), and infant and maternal intestinal health (maternal and infant microbiome; maternal fecal calprotectin, infant fecal markers of environmental enteric dysfunction, maternal report of maternal, or infant diarrhea). Although various models for measurement of EED exist using both bloodwork or fecal testing (40,41), fecal markers neopterin, alpha-1-antitrypsin, and myeloperoxidase were chosen because of the ease of collecting ample stool versus the limitation of serum volume in capillary blood collection.

Additional Considerations

Infants with severe or moderate malnutrition or any study participants with significant illness or severe anemia (hemoglobin <7) will be referred to the medical center for additional care. Any adverse events will be reported to the University of Kentucky IRB and the Amref ESRC. Study results will be disseminated via publications in peer-reviewed journals and shared with the facilities where data collection occurred. We have full confidence in the feasibility of this study because of the long tradition of partner organization Pamoja CBO in this region and knowledge of the subcounty hospital system, lab resources, and experience in navigating this region and interacting with our target population. We have additionally carefully

revised our outcomes and duration of the study in relation to the budget available for this pilot study.

DATA ANALYSIS

Biosample Analysis

Markers of vitamin A status, iron status, and inflammation will be performed by the VitMin Lab using sandwich ELISA (Dr. Juergen Erhardt, http://www.nutrisurvey.net/vitmin). IGF-1, markers of EED, and fecal calprotectin will be performed at the University of Kentucky using commercially purchased assays. Lipid analysis will occur through the modified Folch method of lipid extraction including measurement of fat percentage followed by methylation of extracted lipids into fatty acid methyl esters via the sodium methoxide methylation protocol for running on a gas chromatograph to obtain the fatty acid profile (42–44). Vitamin A analysis will utilize reverse-phase HPLC to measure retinol and carotenoid content (45). Microbiome analysis will utilize DNA extraction, amplification by PCR, and sequencing of bacterial 16S ribosomal RNA genes for bacterial taxa identification (46,47). Fat composition fatty acid profile provide additional measures of nutrition and oxidation, and fatty acids may be correlated with microbiome populations and influence gut health (48–51).

Statistical Analysis

Because of the complexity of assessing microbiome data, biostatistical analysis will occur in collaboration with a biostatistician with extensive experience in microbiome analysis. Data analysis will be performed using SPSS (IBM Corp. 2021). Normal distribution will be assessed using Kolmogorov-Smirnov test and gg plots. Continuous variables will be presented as mean and median, whereas categorical variables as percentages/proportions. Statistical analysis will include univariate and multivariate analysis for primary and secondary outcomes assessed as a delta change from baseline per individual and group and compared with controls. We will use Pearson's correlation for relationships between continuous variables and Pearson chi-square and Fisher's exact for associations between binary variables or independent samples t-test for associations between binary and continuous variables. We will also utilize paired t-test as needed. We will create a multivariate model using logistic regression. Microbiome analysis will additionally utilize alpha and beta diversity indices to measure the variability in genus abundance and number of distinct genera as well as Pielou's evenness to assess uniformity of genus relative abundances. Significance will be assessed at the 95% confidence level. All enrolled participants will be analyzed regardless of loss to follow-up. Appropriate adjustments for multiple comparisons will be used for all statistical tests.

ACKNOWLEDGMENTS

SLA would like to acknowledge her mentors and Dr. Raphael Ondondo in assisting with refining the study design and protocol.

SLA designed the protocol with input from all authors. JM and SLA wrote the article. The authors accept the final draft submitted.

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