THESIS

RICE BRAN SUPPLEMENTATION MODULATES ENVIRONMENTAL ENTERIC DYSFUNCTION MARKERS AND SERUM METABOLITES IN WEANING NICARAGUAN INFANTS.

Submitted by

Luis Enrique Zambrana Gutiérrez

Department of Environmental and Radiological Health Sciences

In partial fulfillment of the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Summer 2018

Master's Committee:

Advisor: Elizabeth Ryan

Marie Legare
Tiffany Weir

ABSTRACT

RICE BRAN SUPPLEMENTATION MODULATES ENVIRONMENTAL ENTERIC DYSFUNCTION MARKERS AND SERUM METABOLITES IN WEANING NICARAGUAN INFANTS.

Rice bran (RB) is an agricultural byproduct from whole grain rice processing. It is an accessible, underutilized food ingredient that merits global health research attention to improve nutritional security, reduce childhood malnutrition, and mitigate environmental enteric dysfunction (EED). The objective was to analyze the effects of dietary RB supplementation on growth, EED biomarkers, and serum metabolites in healthy, weaning infants from six-to-twelve months old residing in León, Nicaragua. Effects of dietary RB supplementation on growth and EED biomarkers were examined after a six-month feeding period. Five-month-old infants (n=71) were screened for eligibility and 62 infants were randomized, for a prospective clinical trial (NCT02615886). The randomization was done within the health sector where each child belongs and by sex to either be allocated to RB dietary group or control group without RB consumption.

Weight and length measurements and stool samples were collected at 6 (baseline), 8 and 12 months of age. Blood was collected at 12 months only. Stool and serum EED biomarkers were compared between study groups. Two-sample t-tests were used to compare weight and length between the two groups, and a non-parametric Wilcoxon Rank-Sum test was used to test differences for EED biomarkers. Targeted and non-targeted serum metabolite profiling was completed by using liquid chromatography tandem-mass spectroscopy. The RB group had significantly increased length-for-age Z-score (LAZ) from 6 to 8 months, and 6 to 12 months

compared to control ($p<0.01$). RB participants showed decreased intestinal permeability and inflammation in the stool marker Alpha-1-Antitrypsin ($p=0.02$) and beneficial effects on gut function in the serum Glucagon-like-peptide-2 ($p<0.04$). Fifty-four serum metabolites were significantly different following RB supplementations versus control. These results support multiple favorable outcomes from RB supplementation in weaning infants. Findings from this pilot study support that RB intake during weaning is safe, promotes healthy intestinal functions, and enhances growth outcomes.

ACKNOWLEDGEMENTS

This study was supported by the Grand Challenges Explorations in Global Health award from the Bill and Melinda Gates Foundation (OPP1043255) and the Fulbright Faculty Development scholarship award. The authors would like to thank Claudia Cortez, Silvia Altamirano, Ridder Guido and Remigio Carvajal for their assistance in data collection, as well as the participating families for their contributions to this study.

The authors' responsibilities were as follows – SV and EPR: designed research and maintained study oversight; ECB and LZ: conducted research and sample collection; IZ, SM, JP, CP: analyzed stool and serum samples; AH and LZ data analysis; LZ, ECB, and EPR wrote paper; EPR had primary responsibility for the final product. All authors read and approved the final manuscript. The authors declare that they have no conflicts of interest with the publication of this work.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PUBLICATIONS AND PRESENTATIONS

Publications

2018 Luis E. Zambrana ECB, Iman Zarei, Starin McKeen, Hend Ibrahim, Johann Perez, Claudia Perez, Sylvia Becker-Dreps, Lijuan Yuan, Samuel Vilchez, & Elizabeth P. Ryan. Rice bran supplementation modulates environmental enteric dysfunction markers and serum metabolites: a randomized-controlled trial in Nicaraguan weaning infants. 2018. (Under editorial review)

Poster presentations

2018 19th Annual CVMBS Research Day. Colorado State University, Fort Collins, CO, January 20, 2018. Effects of Rice Bran on the gut microbial community in 6-12-month-old infants in Nicaragua: A pilot randomized controlled trial and analysis.

2017 Graduate Student Showcase at Lory Student Center. Colorado State University, Fort Collins, CO, November 9, 2017. Rice Bran supplementation effects on enteric dysfunction biomarkers and gut microbial communities in 6-12-month-old in Nicaraguan infants: A pilot randomized controlled trial.

2017 CCTSI Summit Colorado Clinical and Translational Science Institute, Denver, August 16,2017. Rice Bran supplementation effects on enteric dysfunction biomarkers and gut microbial communities in 6-12-month-old in Nicaraguan infants: A pilot randomized controlled trial

2017 18th Annual CVMBS Research Day. Colorado State University, Fort Collins, CO, January 28, 2018. Dietary rice bran supplementation supports growth and impacts environmental enteric dysfunction markers: a randomized-controlled trial in Nicaraguan weaning infants.

CHAPTER 1- INTRODUCTION

The high prevalence of malnutrition in low and middle-income countries has negative consequences on growth of children during the first five years of life (1, 2). There is an increased risk of death among children under 5 years of age due to underweight, stunting, or wasting conditions (2, 3). Risk factors for undernutrition may include, but are not limited to: low birth weight, inadequate breastfeeding, improper complementary feeding, and recurrent infections (3, 4). Diarrheal diseases are also one of the primary causes of undernutrition in children under five years (1, 3, 4).

Environmental enteric dysfunction (EED) is an acquired subclinical condition of the small intestine where children and adults among low-and middle-income countries are the most affected (5-7). Even though there is no evidence for a specific cause for EED, research shows that chronic exposure to enteric pathogens from the environment early in life is a major contributor (8). EED in children reflects altered gastrointestinal function such as mucosal inflammation, intestinal malabsorption and increased intestinal permeability, which lead to protein loss (6, 7). In spite of different dietary supplementations with isolated nutrients (e.g. vitamin A, Zn, Fe) during weaning to increase the total energy intake, supplementations have been not shown to be successful and did not improve growth outcomes in children with EED (9-11).

Negative impacts of EED on children involve failure in a number of gut mucosal immune mechanisms that contribute to normal growth and development (12-14). In Nicaragua, the prevalence of stunting in children is 22% according to a report by UNICEF and WHO (15, 16).

Rice bran (RB) is a novel food ingredient with important macro and micronutrients that has been shown to promote innate resistance against enteric viral and bacterial pathogens that cause diarrhea (17-19). It is a globally accessible food ingredient with a distinct stoichiometry of phytochemicals and prebiotics that induce non-specific gut mucosal immune responses (17, 18, 20, 21). A recent study showed that macronutrients and secondary metabolites are present in RB with human health-promoting properties such as antioxidant, anti-inflammatory, antimicrobial, and chemopreventive (22). Multiple mechanisms of mucosal immune induction have been identified which support that increased dietary RB intake reduces host susceptibility to enteric infections via enhanced gut mucosal immunity (23). The current study evaluated feasibility and tolerance of RB consumption and assessed reduction on gut inflammation [EED biomarkers] after 6 months of RB consumption in weaning infants residing in Léon, Nicaragua. As a secondary objective this study assessed changes in the growth [weight-for-age Z-score (WAZ), length-for age Z-score (LAZ) and weight-for-length Z score (WLZ)] on these infants after RB supplementation compared to controls and identified metabolic changes in serum at 12 months of age.

CHAPTER 2- MATERIAL AND METHODS

2.1 Study design

A 6-month, parallel, randomized-controlled dietary intervention was conducted in a cohort of weaning infants residing in León, Nicaragua. Infants were recruited from public health rosters provided by the local Health Ministry from Perla Maria and Sutiava health sectors.

To be eligible, infants were between 4-6 months of age. However, infants could not have had a diarrheal episode between above mentioned ages to avoid any confounders that would lead to a misinterpretation of the RB effect, as diarrhea is very common in this setting (>300,000 cases of diarrhea per year and 70% of those are in children below 5 years of age). Exclusion includes known allergies or history of immunocompromising conditions, prior hospitalizations, and antibiotic or prophylactic treatment within 1 month prior to participation was required.

Additionally, all eligible participants had to have received all doses of the rotavirus vaccine per regular administration through the Immunization Program in Nicaragua (24). RB intervention started when infants were 6 months of age because the Nicaraguan Ministry of Health (MINSA) promotes exclusive breastfeeding during the first six months of life and strongly recommends to not interfere with this feeding practice.

The Institutional Review Boards at Colorado State University, Universidad Nacional Autónoma de Nicaragua – León, University of North Carolina at Chapel Hill, and Virginia Polytechnic Institute and State University approved this study (protocol #s 14-5233H, Acta No. 129, 14-2501, and 00000657, respectively). Written informed consent was obtained from the infant's parent or responsible guardian prior to any data collection. Once infant participants were 6 months of age and met the eligibility criteria, they were randomized within each health sector

and by sex to either RB dietary intervention or control group that was not provided RB. RB and Control group had normal feeding practices with the exception that RB group consumed RB and the control group no consumed RB. The intervention occurred between March 2015 and October 2015 (NCT02615886).

2.2 Rice bran packaging for consumption

The United States Department of Agriculture-Agricultural Research Service (USDA-ARS) Dale Bumpers National Rice Research Center provided RB that was polished from the U.S. variety, Calrose. RB is prone to fat oxidation that can cause the bran to go rancid, thus heat-stabilization was performed by heating the bran at 100^{O}C for five minutes to inactivate the lipase/lipoxygenase enzymes that cause rancidity (25). The heat-stabilized RB was then sifted to remove any additional debris (rice husk, rice grain). Packaging of the RB was completed by Western Innovations, Inc. (Denver, CO) where 22 kg of RB was weighed into 1g increments, separated into water-proof sachets, and heat-sealed to ensure the RB would not be contaminated.

Fourteen sachets (1g/sachet) were packes into a 4" x 3" x 2" box that was labeled for study participants and included nutrient information. These boxes were stored in a cool 8C, dark, dry place until they were provided to study participants.

2.3 Study intervention

The study team (doctor, nurse and study coordinator) provided a 2-week supply of heat-stabilized RB at each routine home visit and instructed the participant's parent or guardian to add the daily amount of RB to the participant's food. At 6 months of age, participants in the RB group consumed 1g of RB/day (1 sachet). Between the ages of 7-9 months, participants consumed 2g of RB/day (2 sachets). At 10 months of age, participants consumed 3g of RB/day (3 sachets). The amount increased to 4g of RB/day (4 sachets) and 5g of RB/day (5 sachets) at 11

and 12 months of age, respectively. The RB was added to appropriate weaning foods, such as rice cereal, yogurt, fruit and natural juices, vegetables, and soups. At the beginning of the intervention (six months of age), infant´s parents or guardians were instructed and monitored daily for one week by study personnel in order to assess that guardians knew how to administer and record the amount of RB consumed, and then were subsequently followed every week. RB intervention compliance was calculated from the records that had the amount of RB consumed each day circled in increments of none (0%), half (50%), or all (100%). The study team also collected any unused boxes or sachets during these visits, and the rest of daily diet was recorded on every visit using a questionnaire.

Participants in the control group did not receive any RB during the 6-month study duration but were visited every 2 weeks to assess for diarrheal episodes. The study doctor, nurse, and study coordinator together visited each participant every 2 weeks during the 6-month intervention to assess any diarrheal episodes that occurred in the preceding 2 weeks. If a participant had an episode of diarrhea, the study team would collect a stool sample, and collect information that included the onset date, how long the episode lasted, numbers of bowel movements within 24 hours, any associated signs and symptoms (e.g. nausea, vomiting, fever), if any other family members had diarrhea, and if any treatment was provided (e.g. antibiotics, rehydration).

Study visits for data and samples collected occurred when participants were 6 months, 8 months, and 12 months old. These included anthropometric measures (weight and length), stool, and blood (only at 12 months) collections. Infant participants were measured for length and weight via a portable Stadiometer and weighing balance for children. Length was collected to the nearest centimeter and weight to the nearest 0.1 kg. Anthropometric measures were

calculated for LAZ, WAZ, and WLZ scores following the World Health Organizations (WHO) child growth standards using the WHO Anthro software (version 3.2.2) (26). Stool was collected directly from soiled diapers. Freshly collected stool was diluted 20-fold and homogenized in a sterile pre-reduced anaerobic saline - 0.1 M potassium phosphate buffer (pH 7.2) containing 20% glycerol (vol/vol). Four aliquot suspensions were prepared in 15 mL falcon tubes, transported on dry ice to the UNAN-León-Center of Infectious Diseases Laboratories within 1 hour and then immediately transferred to a -80ºC freezer until analysis. Blood was collected via venipuncture in a yellow top tube (HumaTube Serum Gel-C/A 73030, Human Diagnostics Worldwide, Germany) at the 12-month visit only. Blood was centrifuged to separate for serum and stored in a -80^{O}C freezer until analysis.

Additionally, in the Laboratories of the Center of Infectious Diseases a total of 9 diarrheal episodes (5 from RB and 4 from Controls) were analyzed for enteric pathogens as previously reported (27). All biospecimen samples were stored at -80 ºC at UNAN-Leon and then shipped to Colorado State University on dry ice, where they were relocated into a -80ºC freezer prior to analysis.

Each visit a study questionnaire was completed by the participant's family member. (e.g. mother, father, or grandparent) to assess for duration of breastfeeding, types of and timing of introductions to complementary foods, as well as antibiotic use. The breastfeeding questions included whether or not the child was receiving breast milk, and/or had the child been receiving formula (28). The complementary feeding history included a list of eleven common Nicaraguan foods that are introduced to infants during weaning. The infant´s parent or guardian recorded how often the infant consumed each of the eleven foods. The questionnaire also recorded if a participant had received treatment with antibiotics since the last visit, the reason for taking the

antibiotic, the name of the antibiotic, as well as the length of time the participant had been taking the antibiotic. A household survey was also completed at the beginning of the trial to collect data on the mother's education level, drinking water source, household flooring type, and animals present in the household. Analysis of breastfeeding and formula feeding patterns, complementary feeding practices, as well as associations with nutritional status for both groups at 6-months old (i.e. baseline) were reported previously (28).

2.4 Stool and serum analysis for EED markers

Stool biomarkers were selected to report gut inflammation and epithelial integrity as indicators of EED. These included neopterin (NEO), myeloperoxidase (MPO), calprotectin, (CAL) and alpha-1 antitrypsin (AAT) (29). Suspended stool samples from 6, 8, and 12-month collections were centrifuged at 3,000 RPM to remove debris following agitation, and the remaining supernatant was used for Enzyme-Linked-Immunosorbant-Assay (ELISA) determination of EED biomarker concentrations. Laboratory analysis protocols included in commercial kits were followed. Concentrations of CAL were determined at a 1:360 final dilution factor (Eagle Biosciences- Nashua, NH. Ref: CAL35-K01). Samples were diluted to 1:100 for determination of NEO concentrations (GenWay Biotech Inc- San Diego, CA, USA). MPO concentrations were determined at a 1:500 dilution factor (Immundiagnostik AG- Bensheim, Germany). Samples were diluted to 1:12,500 for determination of AAT concentrations (Immuchrom GMBH- Heppenheim, Germany), and dilution factors accounted for stool suspension ratios (20-fold). Final concentrations were determined from averages of replicate assays and duplicate optical density readings and interpolated using Graphpad 6.0 according to standards measured on each 96 well plate.

Serum at 12 months of age was analyzed for alpha 1-acid glycoprotein (AGP), C-reactive protein (CRP), and glucagon-like peptide-2 (GLP-2) (30). All samples were thawed completely on ice and mixed well prior to analysis. Serum was diluted according to the commercial kit's instructions for ELISA determination of EED biomarker concentrations. Concentrations of AGP were determined at a 10,000-fold final dilution (R&D Systems, Minneapolis, MN, USA).

Samples were diluted to 3000–fold for determination of CRP concentrations (ThermoFisher Scientific, Waltham, MA, USA). GLP-2 concentrations were determined directly without any dilution step (EMD Millipore Corporation, Billerica, MA, USA). Final concentrations were determined from averages of replicate assays and duplicate optical density readings and interpolated using Graphpad 6.0 according to standards measured on each 96 well plate.

2.5 Ionomics analysis for serum

Serum was analyzed for elemental concentrations via inductively coupled plasma mass spectrometry (ICP-MS) at the Proteomics & Metabolomics Facility at Colorado State University.

For sample preparation, 150µL of serum was added to a 13x100mm culture tube and mixed with 643µL of 70% nitric acid (BDH Aristar® Plus) followed by 30µL of internal standard solution (10ppm each of Sc, Ga, Y, In, and Bi). Samples were left overnight to digest at room temperature and were then heated in a sand bath for 2 hours at 120^{O}C. After samples cooled, 100µL of hydrogen peroxide (J.T. Baker, 30% Ultrex® II Ultrapure reagent) was added to each sample and was heated again in a sand bath for one hour at 120^{O}C and then allowed to cool to room temperature. Solution was transferred to a 15mL centrifuge tube and diluted to 15mL using pure water. Samples had an internal standard concentration of 20ppb in 3% nitric acid.

Elemental concentrations were measured using an Elan DRC (dynamic reaction cell) II mass spectrometer (PerkinElmer) connected to a Seaspray^{TM} MEINHARD nebulizer and a quartz cyclonic spray chamber. Samples were introduced using an ASX-520 autosampler (CETAC Technologies). Li, Be, B, Na, P, S, Mg, K, Ca, W, Fe, and Pb were measured in standard mode. Cd, Se, and As were measured in DRC mode using oxygen as the reactive gas.

Before analysis, the nebulizer gas flow and lens voltage were optimized for maximum indium signal intensity (75,597 counts per second), 0.86 and 8.25, respectively. A daily performance check was also run which ensured that the instrument was operating properly and obtained a CeO^{+}:Ce^{+} of 0.026 and a Ba^{++}:Ba of 0.013. A calibration curve was obtained by analyzing 7 dilutions of a multi-element stock solution made from a mixture of single-element stock standards (Inorganic Ventures). To correct for instrument drift, a quality control solution (pooled serum sample prepared by mixing 1mL of each digested individual sample) was run every 10th sample.

Data was processed, whereby each element was subjected to internal standard corrections and subsequently drift corrected (31). Corrections were chosen based on minimizing the coefficient of variance for the quality control samples. After drift correction, samples were corrected for the dilution factor. Limits of detection and limits of quantification were calculated 3 times or 10 times the standard deviation of the blank divided by the slope of the calibration curve, respectively (32). Final concentrations are given in ppb (μg/L). Measured calculations below the limits of quantification were assigned to the limits of quantification value for that element.

2.6 Serum metabolomics

Serum was sent to Metabolon Inc. (Durham, NC, USA) for non-targeted metabolite profiling via ultrahigh-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS). All samples were accessioned into the Metabolon Library Information Management Systems (LIMS) and stored in -80°C until metabolome analysis. They were prepared using the automated MicroLab Star® system (Hamilton Company, Switzerland). Eight to ten recovery standards were added prior to the first step in the extraction process for quality control purposes.

Extraction was performed using 80% ice-cold methanol under vigorous shaking for 2 min (Glen Mills GenoGrinder 2000) followed by centrifugation to remove protein, dissociate small molecules bound to protein or trapped in the precipitated protein matrix.

The resulting extract was divided into five fractions: two for analysis by two separate reverse phase UPLC-MS/MS methods with positive ion mode electrospray ionization, 1 for analysis by reverse phase UPLC-MS/MS methods with negative ion mode electrospray ionization, 1 for hydrophilic interaction liquid chromatography UPLC-MS/MS with negative ion mode electrospray ionization, and 1 sample for backup. All samples were placed briefly on Concentration Evaporator (TurboVap® Zymark) to remove organic solvent.

UPLC-MS/MS methods utilized a Waters ACQUITY ultra-performance liquid chromatography and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution.

Raw data was extracted, peak-identified and processed for quality control using Metabolon's hardware and software, rescaled to set the median equal to 1.

2.7 Metabolic pathway visualizations

To visualize networks of metabolic pathways from serum metabolites, the relative abundance of each metabolite was evaluated in a pathway analysis software and metabolite classification system (Metabolync^{TM} plug-in for Cytoscape, Version 2.8.3). Pathway enrichment scores were calculated as previously described (33). Pathway specific network views (Cytoscape) are presented for sub-metabolic pathways. Each metabolite is represented as a node, extending from a central metabolic pathway.

2.8 Statistical analysis

Z scores (LAZ, WAZ, and WLZ) for each child were calculated using a WHO anthropometric calculator (26) and statistical analyses for anthropometric measures (length, weight, LAZ, WAZ, and WLZ), stool and serum EED biomarkers, and serum elemental concentrations were completed using SAS 9.4 (Cary, NC, USA). Normality was evaluated by visual inspection. For anthropometric variables, two-sample t-tests were used to compare means for the 2 treatment groups (RB and control) separately at birth and 6 months (prior to start of treatment). A repeated measures analysis was performed for each response variable separately using SAS Proc Mixed.

Specifically, treatment (RB or control), age (8 or 12 months), and treatment-age interaction were included in the model as fixed effects. Baseline (6 month) weight (or length) was included as a covariate. The participant was included as a random effect to account for repeated measures. At each age, treatment groups were compared using contrasts of the model.

For EED biomarkers and serum elemental concentration variables, the non-parametric Wilcoxon Rank Sum test was used to test for differences between the treatment groups. For stool markers (which were measured at 6, 8 and 12 months) separate comparisons were done at

each time point. Benjamini-Hochberg multiple testing adjustment was applied separately for the marker and MS data.

For serum metabolites, Welch's two-sample t-test was used to analyze statistical significance between groups' serum metabolites at 12-months of age, after participating in the 6-month dietary trial. A p-value of ≤ 0.05 was used for statistical significance. An estimated false discovery rate (q-value) was calculated to take into account the multiple comparisons that are typical of metabolomic-based studies. Pearson correlation analysis was completed to examine the relationship between serum EED biomarkers and metabolites.

CHAPTER 3- RESULTS

3.1 CONSORT flow of participants

A total of sixty-two, healthy, 4-month-old infants were recruited and randomized to one of two intervention groups. Participants were followed between four and six months old to ensure they continued to meet inclusion criteria before starting the intervention. A total of twelve infants were withdrawn during this time period due to antibiotic use (n=6), diarrhea episode (n=4), and hospitalization (n=2). Twenty-four children in the control group and twenty-three children in the RB group successfully completed the 6-month dietary intervention. A total of three participants were withdrawn after the intervention started due to noncompliance (i.e not providing study samples or not regularly consuming RB). One of the withdrawn RB participants experienced vomiting after consuming the RB supplementation and was reported as an unanticipated problem to our Institutional Review Boards. The CONSORT flow of participants is shown in Figure 1.

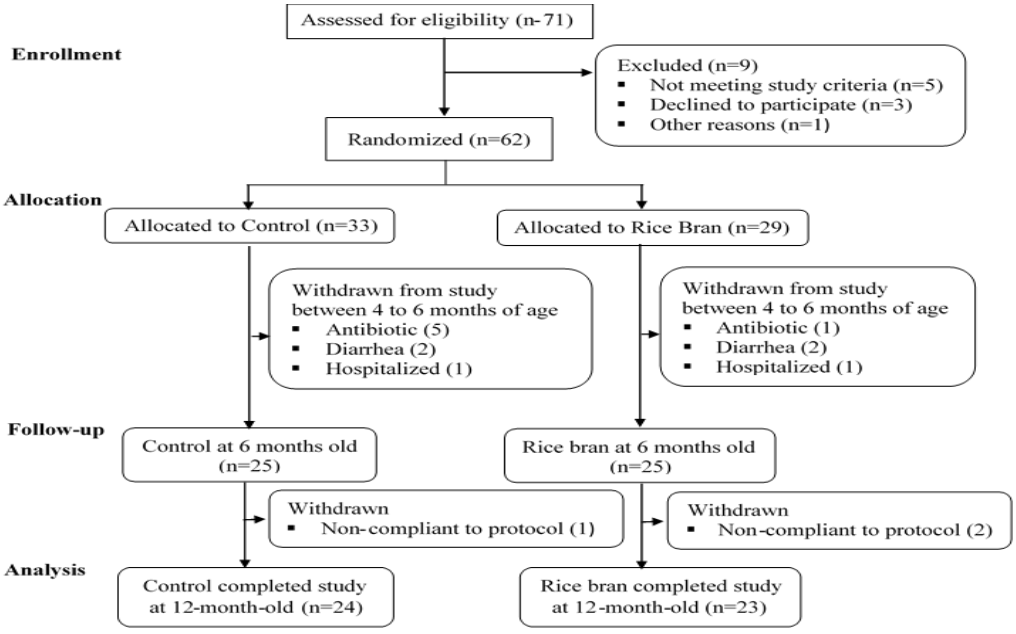

Figure 1. Study recruitment and participation based on CONSORT Statement guidelines.

3.2 Study participant characteristics

Baseline characteristics for all participants are shown in Table 1. No significant differences were observed between sex and geographic location of health post (p=0.67). For breastfeeding status, 96% of infants in the control group and 83% in the RB group were consuming breast milk at six months old. Table 1 illustrates a difference in the birth weight and length, whereby the control group infants were slightly heavier and longer than the RB group at birth, with significance for weight (p=0.05). At 6 months of age, when the dietary intervention started, no significant difference between weight and length was observed (p=0.58 and p=0.88, respectively). Dietary compliance was averaged for consuming RB and during the 6-month intervention (90%). No adverse events were reported during this period.

Table 1. Baseline participant characteristics for study cohort

Variable	Control (n=24)	Rice bran (n=23)	p-value[1]
Sex (%)			
Male	14 (58.0)	12 (52.0)	0.671
Female	10 (42.0)	11 (48.0)	
Health post (%)			
Perla Maria	14 (58.0)	12 (52.0)	0.671
Sutiava	10 (42.0)	11 (48.0)	
Breastfeeding Status (%)			
6 months	23 (95.8)	19 (82.6)	0.142
Weight[2] (kg)			
Birth	3.17 ±0.39	2.94 ±0.38	0.050
6 months	8.09 ±1.10	7.93 ±0.89	0.583
Length[2] (cm)			
Birth	50.67 ±1.93	49.55 ±3.03	0.147
6 months	66.38 ±2.1	66.26 ±2.9	0.878
Birth Z-scores[2]			
WAZ	-0.30 (0.85)	-0.83 (0.89)	**0.049**
WLZ	-1.54 (1.41)	-2.08 (1.64)	0.258
LAZ	0.88 (0.93)	0.37 (1.60)	0.204

[1]Two-sample t-test were used to compare means for the two groups separately at birth and 6 months.
[2]Mean ± standard deviation. LAZ= length for age Z score; WAZ= Weight for age Z score; WHZ= Weight for length Z score

3.3 Anthropometric measures

Figure 2 illustrates anthropometric indices (LAZ, WAZ, WLZ), at birth, 6, 8 and 12 months of age. Figure 2A shows significant differences in LAZ at 8 and 12 months ($p<0.01$). We observed an increase in LAZ over time in infants that consumed RB compared to control, (8 months control -0.130; RB 1.185 and 12 months control -0.730; RB 0.356).

WAZ scores were significantly different at birth between groups, where the control group had higher birth weight compared to RB group (Mean control -0.304; RB -0.826 $p=0.05$). As shown in Figure 2B, we did not observe any significant differences during the intervention for WAZ at 6, 8, and 12-month-old time points (Mean 6-month control 0.327; RB 0.266 p-value 0.841, 8-month control 0.218; RB 0.111 p-value 0.679, and 12-month control -0.031; RB 0.108 p-value 0.197). WLZ scores over time are shown in Figure 2C. No differences at 12 months of age were observed between RB group and control. At 8 months of age, children from the control group had a significantly higher WLZ compared to the RB group (control 0.440, RB -0.541; p-value <0.01).

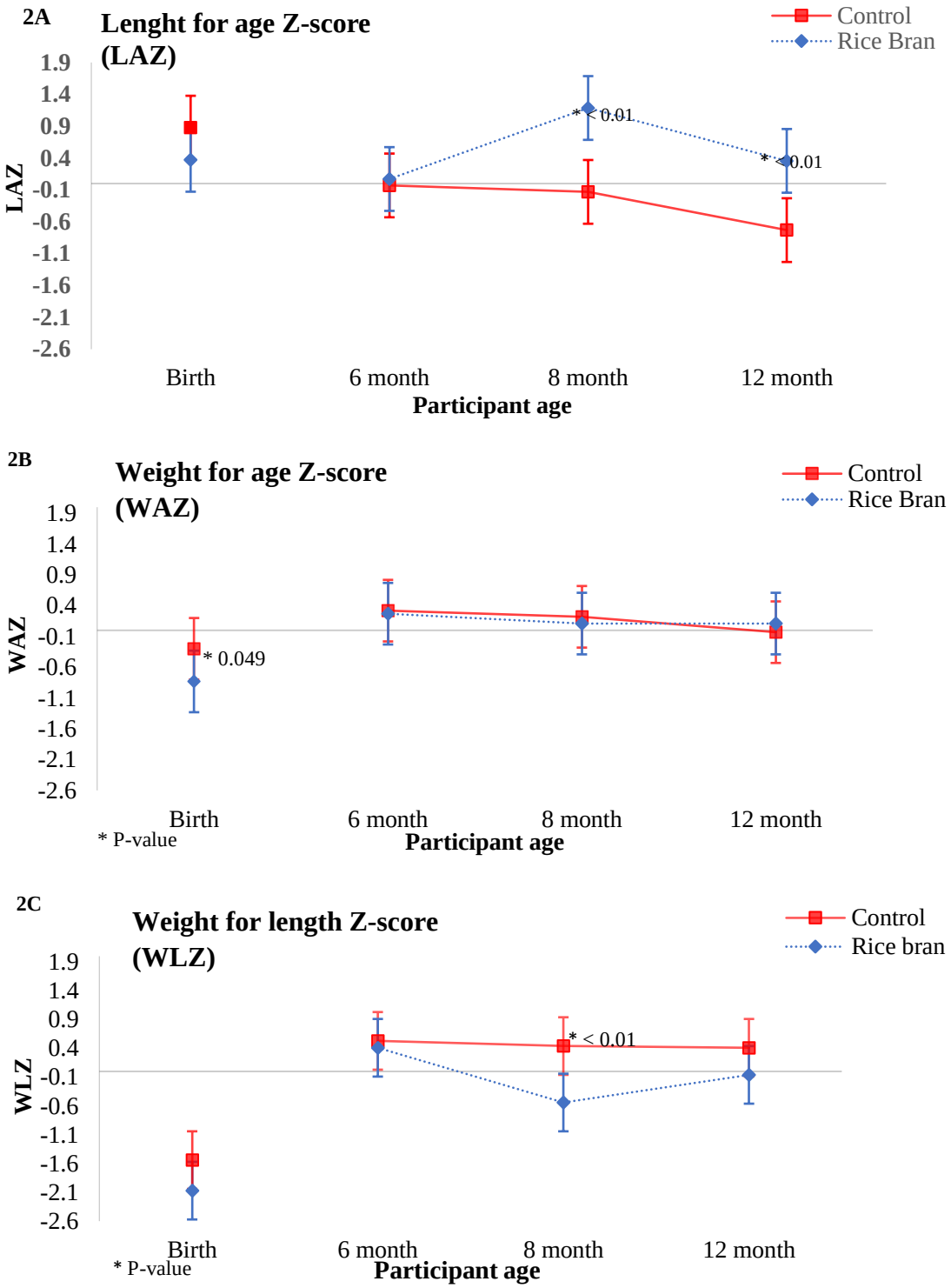

Figure 2. Rice bran and control infants at birth, 6, 8 and 12 months. A. Length-for-age Z score (LAZ). Significant LAZ ($p<0.05$) at 8 and 12 months in the RB group. B. Weight-for-age Z score (WAZ). Significant WAZ at birth in the control group. And C. Weight-for-length Z score (WLZ). Significant WLZ at 8 months in the control group.

3.4 Diarrheal episodes among infants from 6 to 12 months of age

Overall, 9 episodes of diarrhea were reported among the infants between months 6 and 8: 5 episodes (21.7 %) in the RB group, and 4 episodes (16.7 %) in the control group. Diarrhea episodes were associated with a maximum of 6 stools per 24-hour period on average. Vomiting was present among 55.5% of episodes and fever among 44.4%. Of all diarrheal episodes reported, 100.0% received oral rehydration solution.

Enteric pathogens were detected among 66.7% of the stool samples from infants that experienced a diarrheal episode. The most commonly detected organisms among diarrheal episodes were rotavirus 33.3% (2 out of 5 episodes RB and 1 out of 4 episodes Controls), Enteropathogenic *E. coli* 22.2% (0 out of 5 RB and 2 out of 4 Controls) and Adenovirus 11.1% (1 out of 5 RB and 0 out of 4 Controls).

3.5 Environmental Enteric Dysfunctional biomarkers

Table 2 illustrates stool and serum mean concentrations for each EED biomarker. For stool biomarkers, the RB participants showed decreased AAT at 8 months (p=0.09) and a significant decrease was also illustrated at 12-months (p= 0.02) compared to the control group.

No differences were detected in NEO, MPO and CAL between treatment groups.

For serum biomarkers, the RB participants had significant increased GLP-2 concentrations at 12 months compared to control (p= 0.04); CRP and AGP did not show significant differences between the RB and the control group.

Table 2. Environmental enteric dysfunction (EED) biomarkers in stool and serum at 6, 8, and 12 months of age.

EED Biomarker	Control Group[1] (n=24)	Rice Bran Group[1] (n=23)	p-value[2]
Stool (month)			
Neopterin (nmol/L)			
6	150.83 (182.25)	208.65 (131.75)	0.382
8	222.28 (241.08)	144.79 (196.89)	0.287
12	137.50 (285.25)	182.42 (230.41)	0.900
Myeloperoxidase (ng/ml)			
6	277.00 (374.55)	237.54 (376.52)	0.371
8	331.12 (312.26)	266.35 (236.37)	0.544
12	182.03 (324.85)	158.55 (376.73)	0.419
Calprotectin (µg/g)			
6	32.25 (108.68)	88.03 (281.08)	0.205
8	24.66 (87.46)	58.19 (213.25)	0.572
12	23.99 (74.36)	50.47 (133.94)	0.274
Alpha-1 Antitrypsin (ng/ml)			
6	130.08 (177.67)	109.52 (217.41)	0.616
8	152.01 (115.41)	73.47 (122.42)	0.086
12	130.90 (129.81)	70.78 (87.85)	**0.022**
Serum (12 month)			
C-reactive protein (mg/L)	2.32 (3.52)	2.06 (2.06)	0.964
Alpha 1-acid glycoprotein (mg/ml)	0.734 (0.84)	1.16 (0.96)	0.082
Glucagon-like peptide-2 (pg/ml)	592.50 (223.59)	743.53 (380.54)	**0.037**

[1]Median (IQR)
[2]Non-parametric Wilcoxon Rank Sum test was used to test for differences between the treatment groups

3.6 Micronutrients and heavy metals concentration at 12 months of age.

Serum ionomics in Table 3 showed no significant differences between study groups. Although RB supplementation did not influence serum elemental composition, some micronutrients had a slight increase in the RB group, such as calcium, iron, manganese, potassium, sodium and sulfur; and some heavy metals decreased in serum in the RB group such as arsenic, barium and lead.

Table 3. Serum concentrations of micronutrients and heavy metals at 12 months of age.

Element	Control (n=24)		Rice Bran (n=23)		
Micronutrient (ppb)	**Median**	**IQR**	**Median**	**IQR**	**P-value[1]**
Calcium (Ca)	84246.10	15089.54	89131.61	16534.64	0.301
Cobalt (Co)	1.54	0.46	1.5	0.83	0.860
Copper (Cu)	1188.59	436.40	1180.99	226.55	0.628
Iron (Fe)	1387.97	940.02	1525.09	1012.45	0.741
Lithium (Li)	18.18	4.47	18.76	6.21	0.800
Magnesium (Mg)	18826.19	3826.64	19043.85	4417.56	0.826
Manganese (Mn)	10.6	12.58	12.95	13.02	0.379
Molybdenum (Mo)	4.18	0.75	4.04	1.18	0.435
Phosphorus (P)	100173.93	23601.99	106780.58	16598.49	0.391
Potassium (K)	165909.06	27683.48	172316.12	23069.4	0.929
Selenium (Se)	71.13	18.13	70.97	30.18	0.322
Sodium (Na)	2618278.09	235985.53	2722915.47	278141	0.322
Strontium (Sr)	38.6	10.03	43.87	10.51	0.312
Sulfur (S)	907297.39	195377.78	971260.61	142961	0.344
Zinc (Zn)	845.96	195.42	845.25	238.94	0.758
Heavy Metal (ppb)					
Arsenic (As)	15.07	2.39	14.69	1.66	0.416
Barium (Ba)	64.45	7.33	61.1	12.50	0.461
Cadmium (Cd)	3.82	1.44	3.88	2.85	0.886
Lead (Pb)	5.95	1.08	5.76	0.93	0.531
Nickel (Ni)	5.16	1.79	5.18	0.90	0.644
Other (ppb)					
Aluminum (Al)	11332.14	1025.34	10994.14	2694.09	0.482
Tungsten (W)	0.54	0	0.54	0.2	0.550
Vanadium (V)	82.71	33.73	74.99	44.17	0.613

[1]Non-parametric Wilcoxon Rank Sum test

3.7 Rice bran consumption influences serum metabolome in Nicaraguan infants.

Serum metabolite analysis of children at 12 months of age resulted in the detection of 1081 biochemicals, of which 772 compounds were of known identity and 309 compounds of unknown structural identity. Table 4 shows 39 metabolites with significant fold differences between children consuming RB compared to control. There were also fifteen significant metabolites between groups that were classified as unknowns (data not shown). Significant fold differences occurred for 15 amino acids, 3 peptides, 13 lipids, 4 nucleotides, and 4 plant/food components of children consuming RB compared to control. Amino acid metabolites of

significant nutritional importance and increased with RB intake were from lysine (1.91-fold, N acetyllysine), tryptophan (1.18-fold tryptophan, and serotonin 1.81-fold), proline (1.31-fold, prolylhydroxyproline), and methionine (1.27-fold) metabolic pathways. However, the tryptophan metabolite "Indole-propionate" in the RB group was significant decreased (0.69-fold) compared to control group. Within the broad chemical class of lipids, we observed differences amongst phosphatidylcholine, sphingolipid, and secondary bile acids, with RB and control group bidirectional fold differences in the fatty acid metabolism pathway. Significantly increased metabolites from RB intake included di-homo-linolenoyl-choline (1.64-fold) and oleoylcholine (1.50-fold) in the fatty acid metabolism. A full list of significant serum metabolites between RB and control group are listed in Table 4, many of which were reported to exist in the RB food metabolome (22).

Figure 3 visualizes the spectrum of serum metabolites and metabolic pathways affected by RB consumption via metabolite pathway network analysis. Figure 3A focuses on amino acids, with a significant pathway enrichment score of 4.7 for the tryptophan metabolic pathway, and a 5.7 for polyamine metabolism. Lipids (fatty acid metabolism/acylcholine) represent another significant metabolic pathway affected by RB consumption with a pathway enrichment score of 5.7 (Figure 3B).

Significant changes in the GLP-2 EED biomarker between two groups support the effects of RB consumption with a positive correlation amongst the significantly modulated serum metabolites 1-methylurate, 5-(galactosylhydroxy)-L-lysine, N2-acetyllysine and Prolylhydroxyproline (Figure 4).

Table 4. Serum metabolites significantly modulated by rice bran supplementation compared to control at 12 months of age.

Chemical class	Metabolic Pathway	Metabolite[1]	HMDB[2]	Fold Difference[3]	p-value
Amino Acids	Glycine, Serine and Threonine Metabolism	Sarcosine	HMDB00271	1.46↑	0.0006
	Lysine Metabolism	N2-acetyllysine	HMDB00446	1.91↑	0.0114
		5-(galactosylhydroxy)-L-lysine	-	1.17↑	0.0153
	Tryptophan Metabolism	Tryptophan	HMDB00929	1.18↑	0.0281
		N-acetylkynurenine (2)	-	2.80↑	0.0009
		Serotonin	HMDB00259	1.81↑	0.0219
		Indolepropionate	HMDB02302	0.69↓	0.0343
		5-bromotryptophan	-	1.20↑	0.0391
	Leucine, Isoleucine and Valine Metabolism	3-hydroxyisobutyrate	HMDB00336	1.52↑	0.0084
	Methionine, Cysteine, SAM and Taurine Metabolism	Methionine	HMDB00696	1.27↑	0.0081
	Urea cycle; Arginine and Proline Metabolism	N-acetylarginine	HMDB04620	1.41↑	0.0021
		N-acetylcitrulline	HMDB00856	2.10↑	0.0011
		Prolylhydroxyproline	HMDB06695	1.31↑	0.0019
	Polyamine Metabolism	N1, N12-diacetylspermine	HMDB02172	0.66↓	0.0365
		4-acetamidobutanoate	HMDB03681	1.23↑	0.0461
Peptides	Gamma-glutamyl Amino Acid	Gamma-glutamylmethionine	HMDB29155	1.44↑	0.0329
		Gamma-glutamylthreonine	HMDB29159	1.47↑	0.0120
	Polypeptide	HWESASXX*	-	2.14↑	0.0462
	Medium Chain Fatty Acid	Heptanoate (7:0)	HMDB00666	0.73↓	0.0331
	Fatty Acid, Dicarboxylate	Glutarate (pentanedioate)	HMDB00661	2.00↑	0.0316
		Undecanedioate	HMDB00888	0.77↓	0.0022
	Fatty Acid Metabolism	Oleoylcholine	-	1.50↑	0.0173

Lipids	(Acyl Choline)	Dihomo-linolenoyl-choline	-	1.64↑	0.0102
	Endocannabinoid	N-oleoylserine	-	1.21↑	0.0394
	Phosphatidylcholine (PC)	1-palmitoyl-2-palmitoleoyl-GPC (16:0/16:1)	HMDB07969	1.30↑	0.0469
		1-palmitoyl-2-oleoyl-GPC (16:0/18:1)	HMDB07972	1.14↑	0.0227
	Diacylglycerol	Linoleoyl-linolenoyl-glycerol (18:2/18:3) [2]	HMDB07250	0.53↓	0.0089
	Sphingolipid Metabolism	N-behenoyl-sphingadienine (d18:2/22:0)	-	0.64↓	0.0291
	Androgenic Steroids	Epiandrosterone sulfate	-	0.55↓	0.0275
		Androstenediol (3α, 17α) monosulfate (3)	-	0.59↓	0.0378
	Secondary Bile Acid Metabolism	Glycodeoxycholate sulfate	-	0.40↓	0.0182
Nucleotide	Purine Metabolism, Adenine containing	Adenine	HMDB00034	1.56↑	0.0170
		N1-methyladenosine	HMDB03331	1.21↑	0.0209
	Pyrimidine Metabolism, Uracil containing	Pseudouridine	HMDB00767	1.18↑	0.0248
		5-methyluridine (ribothymidine)	HMDB00884	1.18↑	0.0285
Xenobiotics	Xanthine Metabolism	Caffeine	HMDB01847	1.85↑	0.0321
		1-methylurate	HMDB03099	1.69↑	0.0233
	Food Component/Plant	Umbelliferone sulfate	-	0.21↓	0.0156
		Eugenol sulfate	-	0.21↓	0.0172

[1] Table displays metabolites with statistically-significant differences between RB and control group in serum.
[2] HMDB refers to the Human Metabolome Database. Access numbers are provided for each metabolite identified in the database.
[3]For each metabolite, fold difference was calculated by dividing the scaled relative abundance of rice bran vs control.

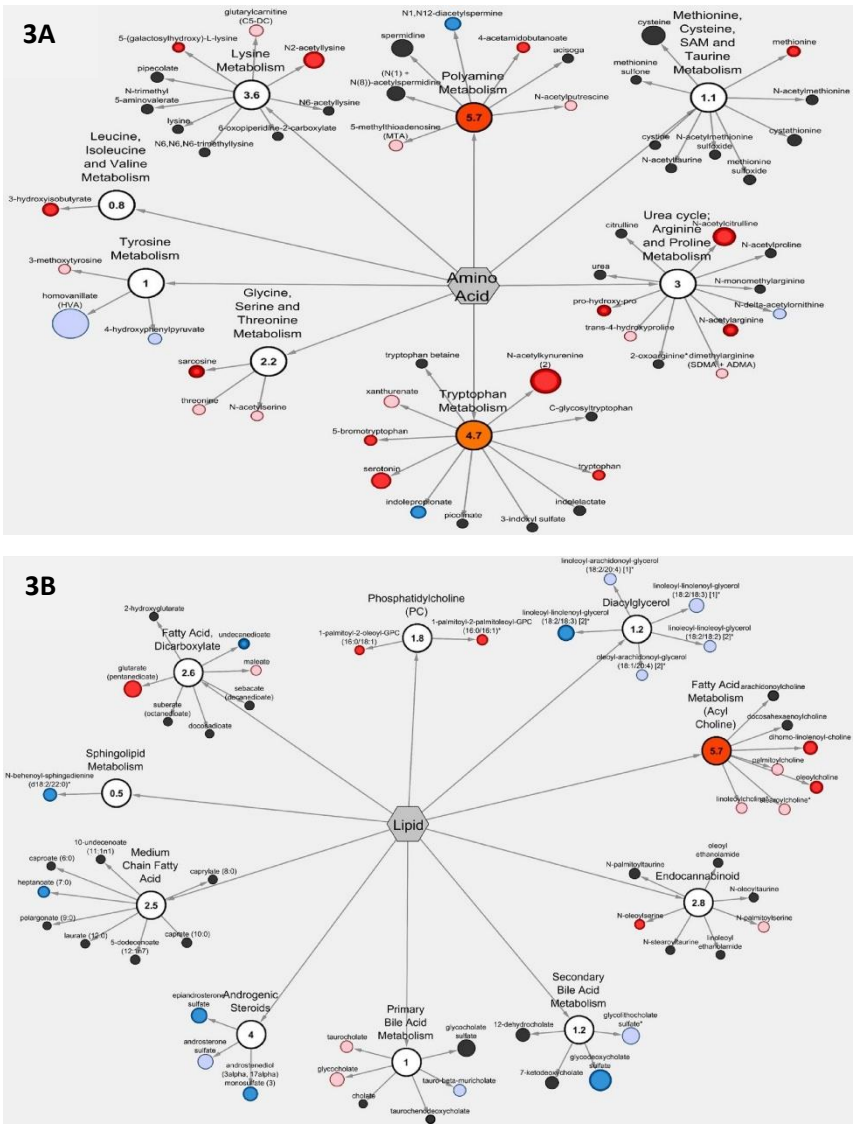

Figure 3. Cytoscape network analysis of serum lipid and amino acid metabolites in RB group at 12 months compared to control group. A. Pathway specific network visualization for serum amino acid metabolites. B. Pathway specific network visualization for serum lipid metabolites. Score of >1.0 indicated that the metabolic pathway contained more metabolites with statistically significant differences between groups at 12 months compared to other pathways in the overall analysis. Red node indicates significant increased difference ($p \leq 0.05$) between the RB and the control group, with metabolite ratio of ≥1. Light red node indicates narrowly missed statistical increase cutoff for significance $0.05<p<0.10$, between RB and control group. Blue node indicates significant decrease difference ($p \leq 0.05$) between the RB and the control group, with metabolite ratio of <1, Light blue node indicates narrowly missed statistical decrease cutoff for significance $0.05<p<0.10$, between RB and control group, and black node indicates not significantly differences between RB and control group comparison. Each metabolite is represented as a node extending from a central sub-metabolic pathway node. The central hexagon is the super metabolic pathway.

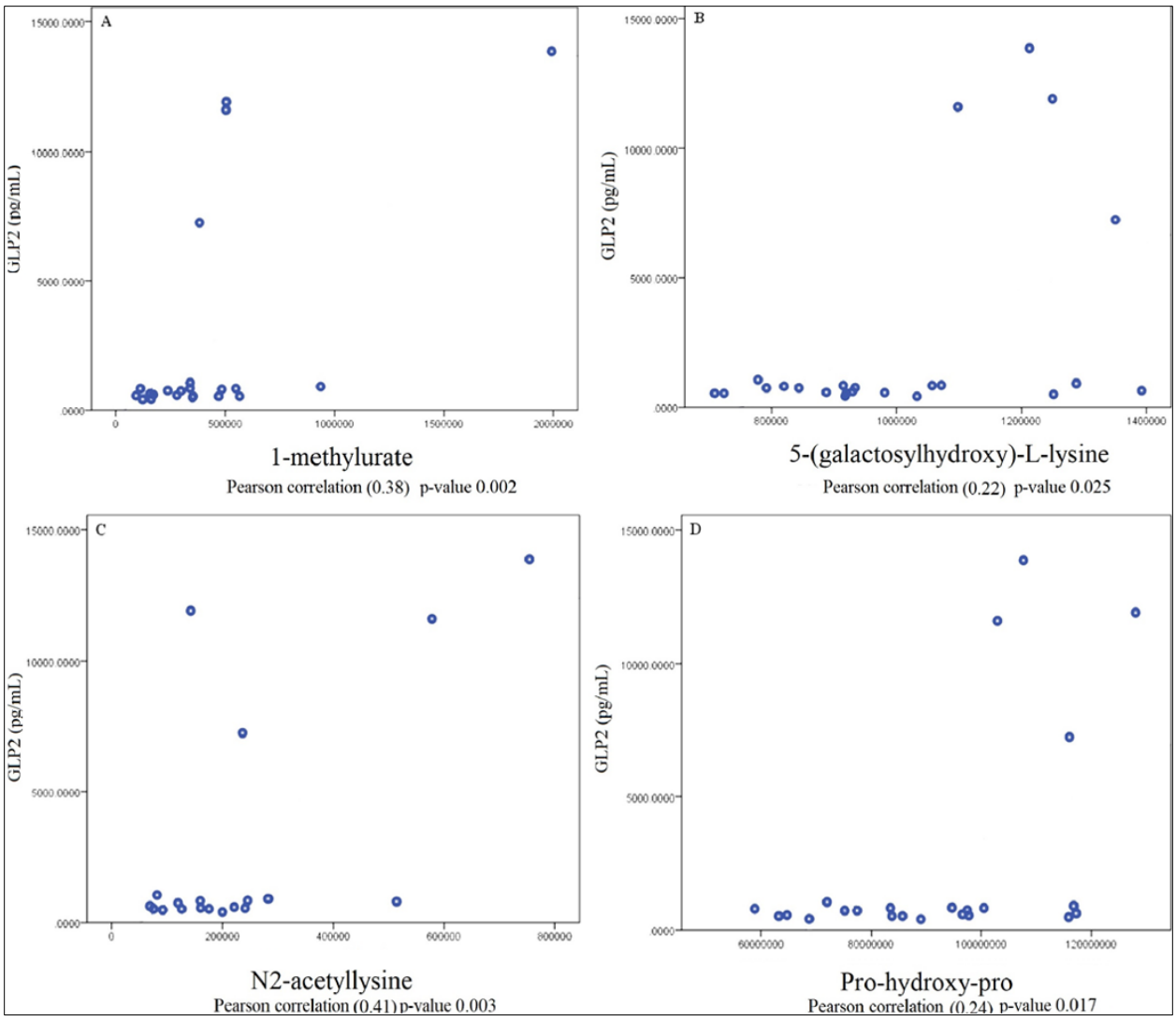

Figure 4. Significant correlations between serum GLP-2 (EED biomarker) and serum metabolites in infants consuming RB at 12 months. OrigScale relative abundance for each metabolite. A. 1-methylurate (Pearson's correlation of 0.385); B. 5-(galactosylhydroxy)-L-lysine (Pearson's correlation of 0.22); C. N2-acetyllysine (Pearson's correlation of 0.41) and D. Prolyhydroxyproline (Pearson's correlation of 0.24) correlated with GLP-2 biomarker.

CHAPTER 4- DISCUSSION

4.1 Rice bran feasibility for weaning infants

This study demonstrated that RB was well-tolerated and safe for weaning infants, and its use as a daily food supplement was feasible, with overall high compliance to consumption (90%). In this pilot study, we found RB supplementation supported length of weaning Nicaraguan infants and favorably modulated stool and serum EED biomarkers. After only two months into the intervention, statistical significance was observed at 8 months of age for LAZ ($p<0.01$) in rice bran compared to control. At 12 months of age, infants who received the daily RB supplement were still longer (by length) and showed AAT and GLP-2 biomarkers in stool and serum, respectively to indicate improved intestinal health. Serum metabolite analysis supports the effects of RB consumption with significant increased fold differences amongst some essential amino acids such as methionine, which is a dietary amino acid required for normal growth and development of humans (34). The increase of certain essential amino acids in the RB group is important because WLZ score at birth for those children was significantly lower than infants in the control group (mean of -2.08), and mitigation with low birth weight was seen in linear growth of these infants (35). WAZ was not different between children that consumed RB versus control. Nevertheless, it is possible that the short period of time in which RB consumption and individual factors, such as weight variability at birth and feeding patterns, could have influenced these results (36, 37).

The overall number of diarrheal episodes in the study was low compared to other studies made in the same location, and no difference detected in diarrhea episodes may be due to lower

total incidence in comparison with previous years and the inclusion of rotavirus vaccination program in this region (27, 38).

4.2 Rice bran modulates environmental enteric dysfunctional markers

There is scientific evidence of a direct relationship between EED and growth deficits in children (7, 14, 39). Thus, it is necessary to better understand the relationship among EED biomarkers with nutritional status after RB intake in children (6, 40). In the present study, EED biomarkers measured a series of localized changes in the stool, such as for intestinal absorption and mucosal permeability (AAT), and gut inflammation (CAL, MPO, and NEO). The serum biomarkers (CRP and AGP) are systemic indicators of inflammation and GLP-2 is a biomarker of nutrient absorption and intestinal barrier function that could be involved in reducing intestinal inflammation. (30, 39, 41). Guerrant *et al.* reported that high concentrations of stool AAT and MPO were associated with decreased growth in children (39), and Naylor *et al.* found that high AAT was associated with decreased oral rotavirus vaccine response (42). Adding to the above mentioned, Becker-Dreps, *et al.* in another study carried out in the same city as this trial found that young infants who did not seroconvert to the 1st dose of the rotavirus pentavalent vaccine (RV5) had higher concentrations of NEO, AAT, MPO and CAL as compared to those that did seroconvert, with statistically significant differences observed only for MPO and CAL (43).

In this study population, we did not find significant differences between diet groups for NEO, MPO, and CAL compared to the control group. Some studies reported that exclusively breastfed children have increased levels of stool CAL than mixed fed children (44, 45). The percentage of exclusively breastfed infants was low in this cohort and the mixed breastfed was high (at 12 months control 75% and RB 74%). These factors could contribute to variation in the levels of CAL in both groups.

RB modulation of AAT was consistent with previous findings in neonatal pigs fed RB (46). The significant decrease of AAT of the RB group in this study, may be an indicator of less gut inflammation, which is a food product of gut bacterial metabolism. (46). Umbelliferone sulfate was shown as a known regulator of gut inflammation and microbial overgrown, while also protective against reactive oxygen species. (47, 48).

4.3 GLP-2 correlation with some amino acids metabolites

The higher serum GLP-2 at 12 months in the RB group compared to control reflects reduced inflammation and this marker has been identified as an effective intestinal growth modulator, with trophic effects (49). Furthermore, reparative and cytoprotective properties of RB could have a strong benefit to these children (50). Significant correlation was obtained between the serum biomarker GLP-2 and the amino acid metabolites (5-(galactosylhydroxy)-L-lysine, N2-acetyllysine and Prolylhydroxyproline) important sources of amino acids from RB (22), and the xanthine metabolite (1-methylurate).

4.4 Modulation of the serum metabolome by rice bran consumption

Tryptophan is another amino acid and principal component of the human diet with relevance to the enteric neuro system while also playing a substantial role in the functionality of the gut-brain axis (51-53). Several studies have demonstrated direct relationships between the concentration of tryptophan and its metabolites with various disorders such as irritable bowel syndrome (51), obesity (54), cardiovascular diseases (55), anorexia nervosa (56, 57), and others (58).

In this study, metabolites from the tryptophan metabolic pathway were significantly increased with RB supplementation, including tryptophan, N-acetylkynurenine, serotonin, indolepropionate, and 5-bromotryptophan. Kosek *et al*. reported association between tryptophan

concentration and linear growth in two longitudinal birth cohorts, and increased concentrations of tryptophan was associated with LAZ gain in those infants (59). The association between serum tryptophan concentrations and LAZ merits attention for the RB group, as children showed more growth at 8 and 12 months than infants in the control group.

RB supplementation modulates EED markers and possibly serum metabolites that are associated with improved intestinal health and enhanced nutrient intake. The consumption of heat stabilized RB in weaning infants was associated with increased LAZ scores and changes in serum metabolite levels. RB supplementation warrants further investigation as a practical intervention strategy that could decrease EED prevalence and risk for children from low- and middle-income countries, particularly where rice is grown as a staple food.

4.5 Study limitations

Limitations to this study include the period that those children were followed and consumed RB. Six months of following is considered as a short period of time to assess and evaluate growth indicators; however, this study demonstrated a growth tendency in the group of children that consumed rice bran. According to WHO, there is not a specific schedule to growth assessment but, some countries such as Nicaragua recommends following in the first two years of life (60, 61).

This study includes only metabolites that could be identified in the serum metabolome measured after the six months of RB consumption. A baseline serum metabolomic analysis at six months of age it would have been excellent to compare with the serum metabolites at 12 months of age.

CHAPTER 5- CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Conclusions

The consumption of heat stabilized RB in children in developing countries such as Nicaragua is a feasible strategy to fight against environmental enteric dysfunction and consequently malnutrition on this vulnerable population. In this study, the group of children that consumed RB had a significant enhancement of their length in a short period of time and the presence of significant metabolites with particularly favorable influence on the tryptophan metabolism pathway.

Participants consuming rice bran had decreased inflammatory markers at 12 months compared to control for stool Alpha-1 Antitrypsin ($p=0.02$) and increased levels for serum marker Glucagon-like peptide ($p=0.03$) at 12 months compared to control.

No increase of arsenic serum concentrations were found on this group of study.

5.2 Future directions

This study indicates improved growth outcomes, reduced gut-permeability and inflammation after the addition of dietary RB compared to control. As future analysis, it is important to identify differences in the gut microbiome and metabolome of these Nicaraguan infants consuming RB when compared to age/sex matched controls, and to integrate systems level mechanisms for preventing enteric dysfunction. We could assume that RB promotes favorable gut health via microbiome-mediated metabolism, including production of molecules, which aid nutrient absorption, enhance immunity, and prevent diarrheal episodes during critical windows of immune and microbial co-maturation. Those favorable changes in enteric microbial community dynamics following RB consumption will include native gut probiotics and will

assist in the development of sustainable functional foods that improve maternal and child health outcomes.

REFERENCES

1. WHO. May 2017. Internet: http://www.who.int/mediacentre/factsheets/fs330/en/ (accessed 06/12 2017).
2. Black E R AHL, Bhutta A Z, et al. . Maternal and child undernutrition: global and regional exposures and health consequences. The Lancet 2008:18. doi: 10.1016/S0140-6736(07)61690-0.
3. Bhutta A Z SAR, et al. Global Nutrition Epidemiology and Trends. Ann Nutr Metab 2012;61:9. doi: 10.1159/000345167.
4. Joseph SA, Casapía M, Blouin B, Maheu-Giroux M, Rahme E, Gyorkos TW. Risk Factors Associated with Malnutrition in One-Year-Old Children Living in the Peruvian Amazon. PLOS Neglected Tropical Diseases 2014;8(12):e3369. doi: 10.1371/journal.pntd.0003369.
5. Keusch T G RHI, Denno M D, et al. Implications of acquired environmental enteric dysfunction for growth and stunting in infants and children living in low- and middle-income countries. Food Nutr Bull 2013;34(3):12.
6. Keusch T G DMD, Black E R, et al. Environmental Enteric Dysfunction: Pathogenesis, Diagnosis, and Clinical Consequences. CID 2014;59(4):6.
7. Syed S AA, and Duggan C. Environmental Enteric Dysfunction in Children: A Review. J Pediatr Gastroenterol Nutr 2016;63(1):9.
8. Korpe S P PAW. Environmental Enteropathy: Critical implications of a poorly understood condition. Trends Mol Med 2012;18(6):18.
9. Caulfield E L HLS, and Piwoz G E. Interventions to improve intake of complementary foods by infants 6 to 12 months of age in developing countries: Impact on growth and on the prevalence of malnutrition and potential contribution to child survival. Food and Nutrition Bulletin 1999;20(2):18.
10. Dewey KG, Cohen RJ, Brown KH, Rivera LL. Age of introduction of complementary foods and growth of term, low-birth-weight, breast-fed infants: a randomized intervention study in Honduras. Am J Clin Nutr 1999;69(4):679-86.

11. Smith HE, Ryan KN, Stephenson KB, Westcott C, Thakwalakwa C, Maleta K, Cheng JY, Brenna JT, Shulman RJ, Trehan I, et al. Multiple micronutrient supplementation transiently ameliorates environmental enteropathy in Malawian children aged 12-35 months in a randomized controlled clinical trial. J Nutr 2014;144(12):2059-65. doi: 10.3945/jn.114.201673.
12. Lunn P G N-CCA, Downes R M. Intestinal permeability, mucosal injury, and growth faltering in Gambian infants. The Lancet 1991;338:4.
13. Campbell D I EMaLPG. Growth Faltering in Rural Gambian Infants Is Associated with Impaired Small Intestinal Barrier Function, Leading to Endotoxemia and Systemic Inflammation. J Nutr 2003;133:7.
14. Kosek M HR, Lima A, et al. Fecal Markers of Intestinal Inflammation and Permeability Associated with the Subsequent Acquisition of Linear Growth Deficits in Infants. Am J Trop Med Hyg 2013;88(2):7.
15. Unicef. Internet: https://www.unicef.org/infobycountry/nicaragua_statistics.html 2018).
16. UNICEF W, World Bank Group. Levels and trends in child malnutrition. UNICEF, 2015:6.
17. Lin CC, Chen HH, Chen YK, Chang HC, Lin PY, Pan IH, Chen DY, Chen CM, Lin SY. Rice bran feruloylated oligosaccharides activate dendritic cells via Toll-like receptor 2 and 4 signaling. Molecules (Basel, Switzerland) 2014;19(4):5325-47. doi: 10.3390/molecules19045325.
18. Yang X, Wen K, Tin C, Li G, Wang H, Kocher J, Pelzer K, Ryan E, Yuan L. Dietary rice bran protects against rotavirus diarrhea and promotes Th1-type immune responses to human rotavirus vaccine in gnotobiotic pigs. Clinical and vaccine immunology : CVI 2014;21(10):1396-403. doi: 10.1128/cvi.00210-14.
19. Lei S, Ramesh A, Twitchell E, Wen K, Bui T, Weiss M, Yang X, Kocher J, Li G, Giri-Rachman E, et al. High Protective Efficacy of Probiotics and Rice Bran against Human Norovirus Infection and Diarrhea in Gnotobiotic Pigs. Frontiers in microbiology 2016;7:1699. doi: 10.3389/fmicb.2016.01699.
20. Reddy KV, Maheswaraiah A, Naidu KA. Rice bran oil and n-3 fatty acid-rich garden cress (Lepidium sativum) seed oil attenuate murine model of ulcerative colitis.

International journal of colorectal disease 2014;29(2):267-9. doi: 10.1007/s00384-013-1785-7.

21. Herfel T, Jacobi S, Lin X, van Heugten E, Fellner V, Odle J. Stabilized rice bran improves weaning pig performance via a prebiotic mechanism. Journal of animal science 2013;91(2):907-13. doi: 10.2527/jas.2012-5287.
22. Zarei I, Brown DG, Nealon NJ, Ryan EP. Rice Bran Metabolome Contains Amino Acids, Vitamins & Cofactors, and Phytochemicals with Medicinal and Nutritional Properties. Rice 2017;10(1):24. doi: 10.1186/s12284-017-0157-2.
23. Henderson AJ, Ollila CA, Kumar A, Borresen EC, Raina K, Agarwal R, Ryan EP. Chemopreventive properties of dietary rice bran: current status and future prospects. Advances in nutrition (Bethesda, Md) 2012;3(5):643-53. doi: 10.3945/an.112.002303.
24. MINSA. Norma tecnica de inmunizaciones y manual de procedimientos de inmunizaciones. In: Inmunizaciones PAd, ed. Managua, Nicaragua, 2013:246.
25. Lakkakula NR, Lima M, Walker T. Rice bran stabilization and rice bran oil extraction using ohmic heating. Bioresource Technology 2004;92(2):157-61. doi: http://dx.doi.org/10.1016/j.biortech.2003.08.010.
26. WHO. WHO Anthro and macros. version 3.2.2 ed, 2011.
27. Becker-Dreps S, Bucardo F, Vilchez S, Zambrana LE, Liu L, Weber DJ, Pena R, Barclay L, Vinje J, Hudgens MG, et al. Etiology of childhood diarrhea after rotavirus vaccine introduction: a prospective, population-based study in Nicaragua. The Pediatric infectious disease journal 2014;33(11):1156-63. doi: 10.1097/inf.0000000000000427.
28. Borresen EC GM, Zambrana LE, Perez J, Perez C, Stallones L, Becker-Dreps S, Yuan L, Vilchez S, Ryan EP. . Association between Infant Feeding Practices and Nutritional Status in Healthy Nicaraguan Infants. J Food Nutri Diete 2016;1(3):6. doi: 14000110.
29. George CM, Oldja L, Biswas S, Perin J, Lee GO, Kosek M, Sack RB, Ahmed S, Haque R, Parvin T, et al. Geophagy is associated with environmental enteropathy and stunting in children in rural Bangladesh. Am J Trop Med Hyg 2015;92(6):1117-24. doi: 10.4269/ajtmh.14-0672.
30. Connor EE, Evock-Clover CM, Wall EH, Baldwin RLt, Santin-Duran M, Elsasser TH, Bravo DM. Glucagon-like peptide 2 and its beneficial effects on gut function and health

in production animals. Domestic animal endocrinology 2016;56 Suppl:S56-65. doi: 10.1016/j.domaniend.2015.11.008.

31. Haugen J-E, Tomic O, Kvaal K. A calibration method for handling the temporal drift of solid state gas-sensors. Analytica Chimica Acta 2000;407(1–2):23-39. doi: https://doi.org/10.1016/S0003-2670(99)00784-9.
32. Broccardo CJ, Schauer KL, Kohrt WM, Schwartz RS, Murphy JP, Prenni JE. Multiplexed analysis of steroid hormones in human serum using novel microflow tile technology and LC–MS/MS. Journal of chromatography B, Analytical technologies in the biomedical and life sciences 2013;934:16-21. doi: 10.1016/j.jchromb.2013.06.031.
33. Brown DG, Borresen EC, Brown RJ, Ryan EP. Heat-stabilised rice bran consumption by colorectal cancer survivors modulates stool metabolite profiles and metabolic networks: a randomised controlled trial. British Journal of Nutrition 2017;117(9):1244-56. doi: 10.1017/S0007114517001106.
34. McBreairty LE, Bertolo RF. The dynamics of methionine supply and demand during early development. Applied Physiology, Nutrition & Metabolism 2016;41(6):581-7. doi: 10.1139/apnm-2015-0577.
35. Hack M, Klein NK, Taylor HG. Long-Term Developmental Outcomes of Low Birth Weight Infants. The Future of Children 1995;5(1):176-96. doi: 10.2307/1602514.
36. WHO. Global Database on Child Growth and Malnutrition. In: WHO/NUT/97.4, ed. Geneva: WHO, 1997:74.
37. Guerrant RL, Oria RB, Moore SR, Oria MO, Lima AA. Malnutrition as an enteric infectious disease with long-term effects on child development. Nutrition reviews 2008;66(9):487-505. doi: 10.1111/j.1753-4887.2008.00082.x.
38. Becker-Dreps S, Paniagua M, Dominik R, Cao H, Shah NK, Morgan DR, Moreno G, Espinoza F. Changes in childhood diarrhea incidence in nicaragua following 3 years of universal infant rotavirus immunization. The Pediatric infectious disease journal 2011;30(3):243-7. doi: 10.1097/INF.0b013e3181f87ffe.
39. Guerrant L R LMA, Pinkerton R, et al. Biomarkers of Environmental Enteropathy, Inflammation,Stunting,and Impaired Growth in Children in Northeast Brazil. journalpone 2016;11(9):20.

40. Kosek M, Guerrant RL, Kang G, Bhutta Z, Yori PP, Gratz J, Gottlieb M, Lang D, Lee G, Haque R, et al. Assessment of Environmental Enteropathy in the MAL-ED Cohort Study: Theoretical and Analytic Framework. Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America 2014;59(Suppl 4):S239-S47. doi: 10.1093/cid/ciu457.
41. Campbell RK, Schulze K, Shaikh S, Mehra S, Ali H, Wu L, Raqib R, Baker S, Labrique A, West KP, Jr., et al. Biomarkers of Environmental Enteric Dysfunction Among Children in Rural Bangladesh. Journal of Pediatric Gastroenterology and Nutrition 2017;Publish Ahead of Print.
42. Naylor C, Lu M, Haque R, Mondal D, Buonomo E, Nayak U, Mychaleckyj JC, Kirkpatrick B, Colgate R, Carmolli M, et al. Environmental Enteropathy, Oral Vaccine Failure and Growth Faltering in Infants in Bangladesh. EBioMedicine 2015;2(11):1759-66. doi: 10.1016/j.ebiom.2015.09.036.
43. Becker-Dreps S, Vilchez S, Bucardo F, Twitchell E, Choi WS, Hudgens MG, Perez J, Yuan L. The Association Between Fecal Biomarkers of Environmental Enteropathy and Rotavirus Vaccine Response in Nicaraguan Infants. The Pediatric infectious disease journal 2017;36(4).
44. Asgarshirazi M, Shariat M, Nayeri F, Dalili H, Abdollahi A. Comparison of Fecal Calprotectin in Exclusively Breastfed and Formula or Mixed Fed Infants in the First Six Months of Life. Acta medica Iranica 2017;55(1):53-8.
45. Dorosko SM, Mackenzie T, Connor RI. Fecal calprotectin concentrations are higher in exclusively breastfed infants compared to those who are mixed-fed. Breastfeeding medicine : the official journal of the Academy of Breastfeeding Medicine 2008;3(2):117-9. doi: 10.1089/bfm.2007.0036.
46. Yang X, Twitchell E, Li G, Wen K, Weiss M, Kocher J, Lei S, Ramesh A, Ryan EP, Yuan L. High protective efficacy of rice bran against human rotavirus diarrhea via enhancing probiotic growth, gut barrier function, and innate immunity. Scientific reports 2015;5:15004. doi: 10.1038/srep15004.
47. Duncan SH, Flint HJ, Stewart CS. Inhibitory activity of gut bacteria against Escherichia coli O157 mediated by dietary plant metabolites. FEMS Microbiology Letters 1998;164(2):283-8. doi: 10.1111/j.1574-6968.1998.tb13099.x.

48. Mazimba O. Umbelliferone: Sources, chemistry and bioactivities review. Bulletin of Faculty of Pharmacy, Cairo University 2017. doi: http://dx.doi.org/10.1016/j.bfopcu.2017.05.001.
49. Burrin DG, Petersen Y, Stoll B, Sangild P. Glucagon-like peptide 2: a nutrient-responsive gut growth factor. J Nutr 2001;131(3):709-12.
50. Drucker DJ. Glucagon-like peptide 2. The Journal of clinical endocrinology and metabolism 2001;86(4):1759-64. doi: 10.1210/jcem.86.4.7386.
51. Fitzgerald P, Cassidy Eugene M, Clarke G, Scully P, Barry S, Quigley Eamonn MM, Shanahan F, Cryan J, Dinan Timothy G. Tryptophan catabolism in females with irritable bowel syndrome: relationship to interferon-gamma, severity of symptoms and psychiatric co-morbidity. Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society 2008;20(12):1291-7. doi: 10.1111/j.1365-2982.2008.01195.x.
52. Keszthelyi D, Troost FJ, Jonkers DM, van Donkelaar EL, Dekker J, Buurman WA, Masclee AA. Does acute tryptophan depletion affect peripheral serotonin metabolism in the intestine? Am J Clin Nutr 2012;95(3):603-8. doi: 10.3945/ajcn.111.028589.
53. Kaluzna-Czaplinska J, Gatarek P, Chirumbolo S, Chartrand MS, Bjorklund G. How important is tryptophan in human health? Critical reviews in food science and nutrition 2017:0. doi: 10.1080/10408398.2017.1357534.
54. Strasser B, Berger K, Fuchs D. Effects of a caloric restriction weight loss diet on tryptophan metabolism and inflammatory biomarkers in overweight adults. European journal of nutrition 2015;54(1):101-7. doi: 10.1007/s00394-014-0690-3.
55. Yu E, Ruiz-Canela M, Guasch-Ferre M, Zheng Y, Toledo E, Clish CB, Salas-Salvado J, Liang L, Wang DD, Corella D, et al. Increases in Plasma Tryptophan Are Inversely Associated with Incident Cardiovascular Disease in the Prevencion con Dieta Mediterranea (PREDIMED) Study. J Nutr 2017;147(3):314-22. doi: 10.3945/jn.116.241711.
56. Haleem DJ. Improving therapeutics in anorexia nervosa with tryptophan. Life sciences 2017;178:87-93. doi: 10.1016/j.lfs.2017.04.015.
57. Kaye W. Neurobiology of anorexia and bulimia nervosa. Physiology & behavior 2008;94(1):121-35. doi: 10.1016/j.physbeh.2007.11.037.

58. Widner B, Leblhuber F, Walli J, Tilz GP, Demel U, Fuchs D. Degradation of tryptophan in neurodegenerative disorders. Adv Exp Med Biol 1999;467:133-8.
59. Kosek MN, Mduma E, Kosek PS, Lee GO, Svensen E, Pan WK, Olortegui MP, Bream JH, Patil C, Asayag CR, et al. Plasma Tryptophan and the Kynurenine-Tryptophan Ratio are Associated with the Acquisition of Statural Growth Deficits and Oral Vaccine Underperformance in Populations with Environmental Enteropathy. Am J Trop Med Hyg 2016;95(4):928-37. doi: 10.4269/ajtmh.16-0037.
60. WHO. Training course on child growth assessment. Geneva: WHO, 2008.
61. MINSA. Normativa 097-Manual red comunitaria salud y nutricion en la niñez. In: Nutricion PCdSy, ed. Managua Nicaragua: Ministerio de Salud 2012:124.

LIST OF ABBREVIATIONS

AAT	Alpha-1 antitrypsin
AGP	Alpha 1-acid glycoprotein
Al	Aluminum
As	Arsenic
B	Boron
Ba	Barium
Be	Beryllium
Ca	Calcium
CAL	Calprotectin
Cd	Cadmium
Co	Cobalt
Cr	Chromium
CRP	C-reactive protein
Cu	Copper
DRC	Dynamic Reaction Cell
EED	Environmental Enteric Dysfunction
ELISA	Enzyme-Linked-Immunosorbant-Assay
Fe	Iron
GLP-2	Glucagon-like peptide-2
HESI-II	Heated electro spray ionization
ICP-MS	Inductively coupled plasma mass spectrometry
K	Potassium
LAZ	length-for-age Z score
Li	Li Lithium
LIMS	Metabolon Library Information Management Systems
Mg	Magnesium
Mn	Manganese
Mo	Molybdenum
MPO	Myeloperoxidase
Na	Sodium
NEO	Neopterin
Ni	Nickel
P	Phosphorus
Pb	Lead

RB	Rice Bran
S	Sulfur
Se	Selenium
Sr	Strontium
UPLC-MS/MS	Ultra-performance liquid chromatography tandem mass spectroscopy
USDA-ARS	Unite States Department of Agriculture-Agricultural Research Service
V	Vanadium
W	Tungsten
WAZ	weight-for-age Z score
WHO	World Health Organization
WLZ	weight-for-length Z score
Zn	Zinc