




Daily Rice Bran Consumption for 6 Months Influences Serum Glucagon-Like Peptide 2 and Metabolite Profiles without Differences in Trace Elements and Heavy Metals in Weaning Nicaraguan Infants at 12 Months of Age

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ABSTRACT

Background: Environmental enteric dysfunction (EED) is associated with chronic gut inflammation affecting nutrient absorption and development of children, primarily in low- and middle-income countries. Several studies have shown that rice bran (RB) supplementation provides nutrients and modulates gut inflammation, which may reduce risk for undernutrition.

Objective: The aim was to evaluate the effect of daily RB dietary supplementation for 6 mo on serum biomarkers in weaning infants and associated changes in serum and stool metabolites.

Methods: A 6-mo randomized-controlled dietary intervention was conducted in a cohort of weaning 6-mo-old infants in León, Nicaragua. Anthropometric indices were obtained at 6, 8, and 12 mo. Serum and stool ionomics and metabolomics were completed at the end of the 6-mo intervention using inductively coupled plasma MS and ultra-high performance LC–tandem MS. The α 1-acid glycoprotein, C-reactive protein, and glucagon-like peptide 2 (GLP-2) serum EED biomarkers were measured by ELISA.

Results: Twenty-four infants in the control group and 23 in the RB group successfully completed the 6-mo dietary intervention with 90% dietary compliance. RB participants had higher concentrations of GLP-2 as compared with control participants at 12 mo [median (IQR): 743.53 (380.54) pg/mL vs. 592.50 (223.59) pg/mL; $P = 0.04$]. Metabolite profiles showed significant fold differences of 39 serum metabolites and 44 stool metabolites from infants consuming RB compared with control, and with significant metabolic pathway enrichment scores of 4.7 for the tryptophan metabolic pathway, 5.7 for polyamine metabolism, and 5.7 for the fatty acid/acylcholine metabolic pathway in the RB group. No differences were detected in serum and stool trace elements or heavy metals following daily RB intake for 6 mo.

Conclusions: RB consumption influences a suite of metabolites associated with growth promotion and development, while also supporting nutrient absorption as measured by changes in serum GLP-2 in Nicaraguan infants. This clinical trial was registered at <https://clinicaltrials.gov> as NCT02615886. *Curr Dev Nutr* 2021;5:nzab101.

Keywords: undernutrition, rice bran, environmental enteric dysfunction biomarkers, metabolome, trace elements, glucagon-like peptide 2, heavy metals

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Abbreviations used: AGP, α 1-acid glycoprotein; CRP, C-reactive protein; EED, environmental enteric dysfunction; GLP-2, glucagon-like peptide 2; ICP-MS, inductively coupled plasma MS; LAZ, length-for-age z-score; LMIC, low-and middle-income country; LOD, limit of detection; OD, optical density; PES, pathway enrichment score; RB, rice bran; UPLC-MS, ultra-high performance LC–tandem MS; WAZ, weight-for-age z-score.

Introduction

The high prevalence of undernutrition in low- and middle-income countries (LMICs) has negative consequences on the growth and development of children during the first 5 y of life (1–3), and stunting and wasting conditions are associated with an increased risk of death (2, 4, 5). Risk factors for undernutrition include, but are not limited to, low birth weight, inadequate breastfeeding, improper complementary feeding, and recurrent infections (4, 6). Diarrheal diseases are also one of the primary causes of undernutrition in children under 5 (1, 4, 6).

Environmental enteric dysfunction (EED) is an acquired condition of the small intestine that most affects children in LMICs (7–9). Although there is little evidence for a specific cause for EED, observational studies suggest that chronic exposure to enteric pathogens from the environment early in life is a major contributor (10–12). EED in children reflects altered gastrointestinal function such as mucosal inflammation, intestinal malabsorption, and increased intestinal permeability, which lead to protein loss (8, 9). It has been challenging to conclusively identify the causes and implications of EED when there is no accepted case definition, and there are no validated, noninvasive diagnostic tests or sets of diagnostic criteria for EED (13). The current gold standard to diagnose EED is upper gastrointestinal endoscopy with biopsy, and this method is quite limited for LMICs due to costs and concerns about safety, which limit its utility for routine diagnosis (14, 15).

In the last few years, a variety of promising EED markers have been studied as indicative of structural, functional, and metabolic changes (16, 17). α 1-Acid glycoprotein (AGP) and C-reactive protein (CRP) are markers of systemic inflammation and glucagon-like peptide 2 (GLP-2) is found in the intestinal damage/repair domain and is related to the nutrient absorption function by the intestine. No single biomarker for EED has been universally accepted.

Dietary interventions that provide isolated nutrients (e.g., vitamin A, zinc, iron) or improve the nutrient density of complementary feeds have not been successful in substantially improving growth outcomes in children with EED (18–20). The negative impacts of EED on children involve failure in a number of gut mucosal immune mechanisms that leads to impaired growth and development (21–23). This is a significant issue for Nicaraguans, whereby the prevalence of chronic malnutrition, which includes stunting in children under the age of 5, is 17.3% (24, 25).

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 (26–28). It is a globally accessible food ingredient with a distinct stoichiometry of phytochemicals and prebiotics that induce nonspecific gut mucosal immune responses (26, 27, 29, 30). A recent study showed macronutrients and secondary metabolites present in RB to be beneficial to human health due to their antioxidant, anti-inflammatory, antimicrobial, and cancer-prevention properties (31). Several mechanisms of gut mucosal immune induction have been identified to support how increased dietary RB intake reduces host susceptibility to enteric infections (32). We previously published changes in growth outcomes and differences in fecal EED markers and microbiota as a result of an RB intervention in Nicaraguan weaning infants. Importantly, RB supplementation resulted in a higher length-for-age z-score (LAZ) and lower fecal α 1-anti-trypsin as compared with control-group children at 12 mo of age (33). This current study evaluated changes to serum

EED biomarkers at 12 mo, targeted serum and stool trace elements, micronutrients and heavy metals, and nontargeted assessment of serum and stool metabolites in weaning infants from León, Nicaragua, where there remains a high burden of diarrheal disease and malnutrition (34).

Methods

Study design

A 6-mo randomized-controlled dietary intervention was conducted in weaning infants residing in León, Nicaragua.

This was an unblinded study in which both the research team, as well as the parents and/or guardians were told if they were part of the intervention group or control. The randomization assignments to intervention and control groups were completed by the study coordinator and ensured balance between groups for sex and health post locations. All infants were recruited from public health rosters provided by the local Health Ministry from the Perla Maria and Sutiava health sectors.

Recruitment of infants began at 4 mo of age, and with eligibility confirmed for enrollment at 6 mo of age. Infants could not have had a diarrheal episode between 4 and 6 mo of age or have taken antibiotics in the prior month to avoid changes to the gut microbiome composition that could confound the effect of RB. Additionally, all eligible participants had no history of malnutrition and received all 3 doses of the rotavirus vaccine per regular administration through the Immunization Program in Nicaragua (35). Initiation of the intervention occurred at 6 mo of age in accordance with the Nicaraguan Ministry of Health (MINSA) recommendation for exclusive breastfeeding during the first 6 mo of life.

The Institutional Review Boards at Colorado State University, Universidad Nacional Autónoma de Nicaragua–León, the University of North Carolina at Chapel Hill, and Virginia Polytechnic Institute and State University approved this study (protocol nos. 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 and they allowed access to the complete infant health record. Infants at 6 mo of age who met the eligibility criteria were randomly assigned within the 2 health sectors (Perla Maria Norori and Sutiava) by sex to either the RB dietary intervention or control group, which was not provided with RB. The RB and control groups shared local feeding practices apart from the provided RB. The intervention occurred between March and October 2015 (NCT02615886). The study team used standard reporting forms to monitor which types of food the child consumed during the study period and this information was registered via the questionnaire twice a month. RB food products are not normally consumed in the Nicaraguan population and families did not have any knowledge about RB as a food ingredient for humans before our study. Brown rice was the only other similar food product known to this population, yet brown rice is rarely consumed due to lack of accessibility and high prices as well as limited availability in local grocery stores.

RB packaging for consumption

The USDA–Agricultural Research Service Dale Bumpers National Rice Research Center provided RB that was polished from the US 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°C for 5 min to inactivate the lipase/lipoxygenase enzymes that cause rancidity

(36). 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 1-g increments, separated into water-proof sachets, and heat-sealed to ensure the RB would not be contaminated.

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

Study intervention

As reported previously (33), the study team (doctor, nurse, and study coordinator) provided a 2-wk 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 mo of age, participants in the RB group consumed 1 g RB/d (1 sachet). Between the ages of 7 and 9 mo, participants consumed 2 g RB/d (2 sachets). At 10 mo of age, participants consumed 3 g RB/d (3 sachets). The amount increased to 4 g RB/d (4 sachets) and 5 g RB/d (5 sachets) at 11 and 12 mo 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 (6 mo of age), infants' parents or guardians were instructed and monitored daily for 1 wk by study personnel so that guardians knew how to administer and record the amount of RB consumed. Compliance with the RB intervention 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 to calculate the amount of RB consumed by children.

Participants in the control group did not receive any RB during the 6-mo study duration and there were no reports of brown rice consumption during that time frame. The study doctor, nurse, and study coordinator visited each participant together every 2 wk during the 6-mo intervention to assess information for any event related to RB consumption that occurred in the preceding 2 wk. Parents or a responsible guardian would contact the study coordinator or nurse at any time by phone call in the case that the infant presented diarrhea or had any possible adverse event. This helped with the prompt collection of the diarrheal sample. The parents or responsible guardian in both study groups could also reach the study team for questions or concerns at any time. Additional household visits occurred when participants were 6, 8, and 12 mo old for anthropometric measures (weight and length) and stool sample collection; a blood sample was obtained from each participant at 12 mo of age. 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, weight-for-age z-score (WAZ), and weight-for-length z-score (WLZ) scores following the WHO child growth standards using the WHO Anthro software (version 3.2.2) (37). Outcomes related to anthropometric measures were reported previously.

Blood was collected via venipuncture in a yellow-top tube (HumaTube Serum Gel-C/A 73030; Human Diagnostics Worldwide, Germany) at the 12-mo visit only. Blood was centrifuged at 4 °C and 750 × g for 10 min to separate for serum and stored in a –80 °C freezer until analysis. Stool was collected from a study-provided disposable

diaper. Stool was placed on ice following collection and frozen at –80 °C until analysis. All biospecimen samples were stored at –80 °C at National Autonomous University of Nicaragua–Leon (UNAN-Leon) and then shipped to Colorado State University on dry ice, where they were relocated into a –80 °C freezer prior to analysis.

A study questionnaire was completed by the participant's caregiver (e.g., mother, father, or grandparent) at baseline to collect the mother's educational level, drinking water source, household flooring type, and animals present in the household. Additional surveys were administered twice monthly to assess for duration of breastfeeding, types and timing of introductions to complementary foods, and any antibiotic use. The breastfeeding questions included whether or not the child was receiving breast milk and/or had been receiving any formula (38). The complementary feeding history included a list of 11 common Nicaraguan foods that are introduced to infants during weaning. Infants' parents or guardians recorded how often the infant consumed each of the 11 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. Analysis of breastfeeding and formula feeding patterns, complementary feeding practices, as well as associations with nutritional status for both groups at 6 mo old (i.e., baseline) were reported previously (38).

Serum analysis for EED markers

Serum collected at 12 mo of age was analyzed for systemic inflammation markers AGP and CRP, and the intestinal damage/repair domain GLP-2 (16, 39). 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). Samples were diluted to 3000-fold for determination of CRP concentrations (ThermoFisher Scientific). GLP-2 concentrations were determined directly without any dilution step (EMD Millipore Corporation). Final concentrations were determined from averages of replicate assays and duplicate optical density (OD) readings and interpolated using Graphpad Prism 6.0 (GraphPad Prism Software) according to standards measured on each 96-well plate.

To generate the standard curve for each set of samples assayed, an average duplicate OD reading was obtained for each standard, control, and sample that was used to calculate the concentration. Samples below the limit of detection (LOD) were considered nondetected and replaced with zero. No arbitrary values were assigned below the LOD as this may introduce an artificial lack of variance. We did not encounter any samples with concentrations above the upper LOD during the EED marker analyses.

Serum trace elements and heavy metals

Serum was analyzed for elemental concentrations via inductively coupled plasma MS (ICP-MS) at the Proteomics and Metabolomics Facility at Colorado State University. For sample preparation, 150 μL serum was added to a 13- × 100-mm culture tube and mixed with 643 μL of 70% nitric acid (BDH Aristar® Plus) followed by 30 μL internal standard solution (10 ppm 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 h at 120 °C. After samples cooled, 100 μL hydrogen peroxide (30% Ultrex® II Ultrapure reagent; J.T. Baker) was added to

each sample and was heated again in a sand bath for 1 h at 120 °C and then allowed to cool to room temperature. Solution was transferred to a 15-mL centrifuge tube and diluted to 15 mL using pure water. Samples had an internal standard concentration of 20 ppb in 3% nitric acid.

Elemental concentrations were measured using an Elan DRC (dynamic reaction cell) II mass spectrometer (PerkinElmer) connected to a Seaspray™ MEINHARD nebulizer and a quartz cyclonic spray chamber. Samples were introduced using an ASX-520 autosampler (CETAC Technologies). Lithium (Li), beryllium (Be), boron (B), sodium (Na), phosphorus (P), sulfur (S), magnesium (Mg), potassium (K), calcium (Ca), tungsten (W), iron (Fe), and lead (Pb) were measured in standard mode. Cadmium (Cd), selenium (Se), and arsenic (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/s)—0.86 and 8.25, respectively. A daily performance check was also run, which ensured that the instrument was operating properly and obtained a $\text{CeO}^+:\text{Ce}^+$ of 0.026 and a $\text{Ba}^{++}:\text{Ba}$ 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 1 mL of each digested individual sample) was run every 10th sample.

Data were processed whereby each element was subjected to internal standard corrections and subsequently drift corrected (40). Corrections were chosen based on minimizing the CV for the quality-control samples. After drift correction, samples were corrected for the dilution factor. LODs and limits of quantification were calculated 3 times or 10 times the SD of the blank divided by the slope of the calibration curve, respectively (41). Final concentrations are given in parts per billion ($\mu\text{g/L}$). Measured calculations below the limits of quantification were assigned to the limits of quantification value for that element.

Stool trace elements and heavy metals

Stool samples were sent to Metabolon, Inc. (Durham, NC, USA), for ionomics. Stool was homogenized in 1% nitric acid with zirconium beads at a rate of 20 mg/1 mL. One hundred microliters of stool homogenate was transferred to a 48-well plate and digested with nitric acid, hydrochloric acid, and hydrogen peroxide. The digest was dried and reconstituted in 1 mL 0.5% nitric acid. The final unit for the concentration was $\mu\text{g/kg}$ (dry stool). Metal ions were measured using ICP-MS. The samples were diluted in deionized- H_2O and introduced into the ICP-MS instrument via an ESI Prep-Fast autosampler including dilution with aqueous nitric acid. The Thermo ICAP-RQ instrument was operated in positive ionization and used a multipoint external calibration curve that preceded the sample into the instrument.

Serum and stool metabolomics

Serum and stool were sent to Metabolon, Inc., for nontargeted metabolite profiling via ultra-high performance LC–tandem MS (UPLC-MS). All samples were accessioned into the Metabolon Library Information Management Systems (LIMS) and stored at -80 °C until metabolome analysis. They were prepared using the automated MicroLab Star® system (Hamilton Company, Switzerland). Eight to 10 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 and dissociate small molecules bound to protein or trapped in the precipitated protein matrix. The resulting extract was divided into 5 fractions: 2 for analysis by 2 separate reverse-phase UPLC-MS methods with positive ion mode electrospray ionization, 1 for analysis by reverse-phase UPLC-MS methods with negative ion mode electrospray ionization, 1 for hydrophilic interaction LC UPLC-MS with negative ion mode electrospray ionization, and 1 sample for backup. All samples were placed briefly on a concentration evaporator (TurboVap® Zymark) to remove organic solvent.

UPLC-MS methods utilized a Waters ACQUITY UP-LC and a ThermoScientific 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 were extracted, peak-identified, and processed for quality control using Metabolon's hardware and software (systems built on a web-service platform utilizing Microsoft's .NET technologies, which run on high-performance application servers and fiber-channel storage arrays in clusters to provide active failover and load-balancing), rescaled to set the median equal to 1.

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™ plug-in for Cytoscape, version 2.8.3) (42). Pathway enrichment scores (PESs) were calculated using the equation $\frac{k/m}{n/N}$, where “k” represents the number of significant metabolites in a pathway ($P \leq 0.05$), “m” the total number of identified metabolites in that pathway, “n” the total number of significant metabolites in the dataset, and “N” the total number of identified metabolites in the complete dataset (43, 44). Metabolic pathways with a PES $<$ or $>$ 1 indicated that the pathway contained ≥ 1 metabolites with a statistically significant fold-difference (between RB group and control at 12 mo) compared with all other pathways within the matrix (45). Each metabolite is symbolized as a circle node, whose size corresponds to the z-score using the relative abundance mean value. This node is extending from a central submetabolic pathway node and the hexagon node represents the super metabolic pathway.

Statistical analysis

Statistical analyses for anthropometric measures (length and weight), serum EED biomarkers, and serum/stool elemental concentrations were completed using SAS version 9.4 (SAS Institute). Normality was evaluated by visual inspection. For anthropometric variables, 2-sample *t* tests were used to compare means for the 2 treatment groups (RB and control) separately at birth and 6 mo (prior to start of treatment).

For EED biomarkers and serum/stool trace element and heavy metals concentrations, the nonparametric Wilcoxon rank-sum test was used to test for differences between the treatment groups.

Serum and stool metabolite data normalization by each compound was corrected in run-day blocks by registering the medians to equal 1 (1.00) and normalizing each data point proportionately. Following log transformation and imputation of missing values, if any, with the minimum observed value for each compound, a Welch's 2-sample *t* test was used on serum metabolites to identify biochemicals that differed

significantly between experimental groups at 12 mo of age. For stool metabolites, a 2-factor ANOVA with repeated measures identified biochemicals exhibiting significant interaction and main effects for experimental parameters of cohort, time point, and treatment. A P value of <0.05 was used for statistical significance and estimated false discovery rate (q -value) was calculated for nontargeted analysis of metabolites to take into account the multiple comparisons that are typical of metabolomic-based studies. Metabolomics statistical analyses were performed in ArrayStudio using median scaled-log transformed data.

Results

A total of 62 healthy, 4-mo-old infants were recruited and randomly assigned to 1 of 2 intervention groups, but they did not receive RB until 6 mo of age. Participants were followed when they were 4 to 6 mo old to ensure they continued to meet inclusion criteria before starting the intervention. A total of 12 infants were withdrawn during this time period due to antibiotic use ($n = 6$), diarrhea episode ($n = 4$), and hospitalization ($n = 2$). Twenty-four infants in the control group and 23 infants in the RB group successfully completed the 6-mo dietary intervention. A total of 3 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 and was reported as an unanticipated problem to our institutional review boards. The CONSORT (Consolidated Standards of Reporting Trials) flow of participants is shown in [Figure 1](#).

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 6 mo of age. [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 mo 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-mo intervention (90%). No adverse events were reported during this period.

Diarrheal episodes among infants from 6 to 12 mo of age

Overall, 9 episodes of diarrhea were reported among the infants between months 6 and 8. In the RB group there were 5 episodes (21.7%) and there were 4 episodes (16.7%) in the control group. Diarrhea episodes were associated with a maximum of 6 stools per 24-h 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 who experienced a diarrheal episode. The most commonly detected organisms in the diarrheal samples were rotavirus (33.3%; 2 out of 5 episodes in RB and 1 out of 4 episodes in controls), enteropathogenic *Escherichia coli* (22.2%; 0 out of 5 RB

participants and 2 out of 4 controls) and adenovirus (11.1%; 1 out of 5 RB participants and 0 out of 4 controls).

Biomarkers of EED

[Table 2](#) illustrates serum mean concentrations for each EED biomarker. For serum biomarkers, the RB participants had significantly increased GLP-2 concentrations at 12 mo compared with controls ($P = 0.03$); CRP and AGP did not show significant differences between the RB and the control group. Stool EED biomarkers were reported previously ([33](#)).

Serum and stool concentrations of trace elements and heavy metals at 12 mo of age

[Tables 3](#) and [4](#) illustrate serum and stool concentrations of trace elements, micronutrients, and heavy metals at 12 mo of age compared between control and RB groups. No significant differences were detected for serum analytes between study groups. Trends for higher micronutrients such as calcium, iron, manganese, potassium, sodium, and sulfur in serum of infants consuming RB were noted, whereas heavy metals such as arsenic, barium, and lead were trending lower.

The stool profile showed significant differences for only manganese and chromium ([Table 4](#)), with increased excretion in the RB group. The RB group stool samples trended towards greater excretion of aluminum, barium, lead, nickel, strontium, and vanadium when compared with the control group.

RB consumption influences serum metabolome in Nicaraguan infants

Serum metabolite analysis of infants at 12 mo of age resulted in the detection of 1081 biochemicals, of which 772 compounds were of known structural identity and 309 compounds of unknown structural identity. [Tables 5](#) and [6](#) show 39 metabolites with significant fold-differences between children consuming RB compared with controls. Significant fold-differences occurred for 15 amino acids, 3 peptides, 13 lipids, 4 nucleotides, and 4 plant/food components in infants consuming RB compared with controls. Amino acid metabolites of significant nutritional importance and that increased with RB intake were lysine (1.91-fold, N-acetyllysine), tryptophan (1.18-fold, tryptophan; 1.81-fold, serotonin), proline (1.31-fold, prolylhydroxyproline), and methionine (1.27-fold) metabolic pathways. Within the broad chemical class of lipids, we observed differences among phosphatidylcholine (1.30-fold, 1-palmitoyl-2-palmitoleoyl-GPC; 1.14-fold, 1-palmitoyl-2-oleoyl-GPC), sphingolipid (0.64-fold, N-behenoyl-sphingadienine), and secondary bile acids (0.40-fold, glycodeoxycholate sulfate). Significantly increased metabolites from fatty acid metabolism associated with RB intake included di-homo-linolenoyl-choline (1.64-fold) and oleoylcholine (1.50-fold). Many of those significant serum metabolites between RB and control group listed in [Tables 5](#) and [6](#) were reported to be components of the RB food metabolome ([31](#)).

[Figure 2](#) visualizes the spectrum of serum metabolites and metabolic pathways affected by RB consumption using a metabolite pathway network analysis. [Figure 2A](#) focuses on amino acids, with a significant pathway enrichment score of 4.7 for the tryptophan metabolic pathway and 5.7 for polyamine metabolism. Lipids (fatty acid metabolism/acetylcholine) represent another significant metabolic pathway affected by RB consumption, with a pathway enrichment score of 5.7 ([Figure 2B](#)).

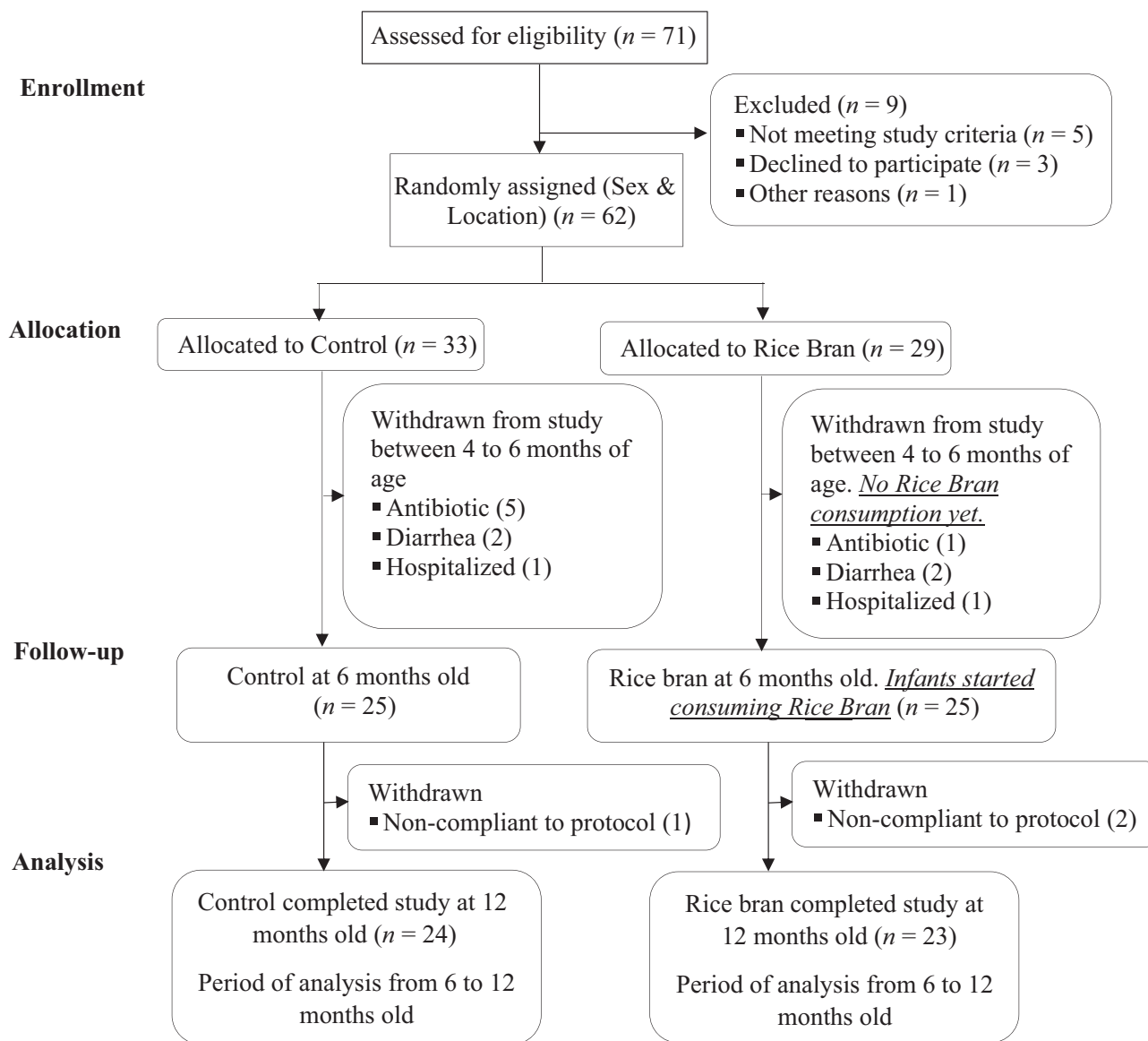


FIGURE 1 Study recruitment and participation based on CONSORT statement guidelines. CONSORT, Consolidated Standards of Reporting Trials.

RB consumption influences the stool metabolome in Nicaraguan infants

Daily supplementation with RB for 6 mo in Nicaraguan infants was examined at 12 mo of age for changes in the stool metabolome compared with controls. The stool biochemical profile contained 1449 compounds, of which 1017 biochemicals were of known structural identity and 432 biochemicals had not been structurally identified. [Tables 7](#) and [8](#) show 44 metabolites with significant differences between the 2 groups. A significant fold-difference in stool metabolites was reported for 4 amino acids, 1 peptide, 1 carbohydrate, 19 lipids, 3 nucleotides, 3 cofactors and vitamins, and 13 xenobiotics from infants consuming RB compared with the control group. Certain stool metabolites increased with RB intake, such as tyrosine (2.71-fold, dopamine 3-*O*-sulfate), disaccharides (2.06-fold, sucrose), and fatty acid–dicarboxylate

metabolic pathways (2.18-fold, 2-hydroxyadipate; 1.80-fold, pimelate). These metabolites were significantly different at 12 mo of age. We also observed significant decreases in stool lysophospholipid metabolism pathway and secondary bile acid metabolism and identified significant increases within the fatty acid metabolism pathway for palmitoylcholine (2.31-fold), oleoylcholine (3.54-fold), linoleoylcholine (1.88-fold), and stearoylcholine (1.48-fold).

RB compounds identified in infant serum and stool

A list of serum or fecal metabolites that differed between RB and control groups and that were also reported in the RB food metabolome is shown in [Tables 5–8](#). The RB metabolite profile that was fed to infants had 448 identified compounds, of which 13 were significantly detected in serum of RB infants when compared with controls at 12 mo of age. There were

TABLE 1 Baseline participant characteristics of Nicaraguan infants¹

Variable	Rice bran (n = 23)	Control (n = 24)	P
Sex, n (%)			
Male	12 (52.0)	14 (58.0)	0.67
Female	11 (48.0)	10 (42.0)	
Water source, n (%)			
Indoor municipal	23 (100)	24 (100)	
Untreated ground water	0 (0)	0 (0)	
Delivery type, n (%)			
Vaginal	16 (69.6)	11 (45.8)	0.09
Cesarean	7 (30.4)	13 (54.2)	
Sanitation system, n (%)			
None	1 (4.3)	0 (0)	0.11
Community latrine	0 (0)	0 (0)	
Latrine	9 (39.1)	4 (16.7)	
Indoor toilet	13 (56.5)	20 (83.3)	
Mother's education, n (%)			
None	0 (0)	1 (4.2)	0.59
Some primary	7 (30.4)	3 (12.5)	
Completed primary	2 (8.7)	3 (12.5)	
Some secondary	5 (21.7)	8 (33.3)	
Completed secondary	5 (21.7)	4 (16.7)	
University	4 (17.4)	5 (20.8)	
Breastfeeding status, n (%)			
6 mo	19 (82.6)	23 (95.8)	0.14
Household animals, ² n (%)			
Poultry	9 (37.5)	3 (12.5)	0.36
Livestock	2 (8.7)	2 (8.3)	
Domesticated pets	16 (69.6)	17 (70.8)	
None	5 (21.7)	7 (29.2)	
Anthropometry ³			
Weight at birth, kg	2.94 ± 0.38	3.17 ± 0.39	0.05
Weight at 6 mo, kg	7.93 ± 0.89	8.09 ± 1.10	0.58
Length at birth, cm	49.55 ± 3.03	50.67 ± 1.93	0.15
Length at 6 mo, cm	66.26 ± 2.90	66.38 ± 2.10	0.87

¹P values for sex, delivery type, breastfeeding status, and household animals were calculated by chi-square test. Anthropometric

P values were calculated by 2-sample t test.

²More than 1 category per house.

³Values are means ± SDs.

14 potentially RB-derived compounds in stool that were significantly different from the control group. We observed that 5-methyluridine, a metabolite found in RB, showed a significant fold-change in both stool and serum after intake at 12 mo (increased 1.18-fold in serum and decreased 0.37-fold in stool). [Table 5](#) highlights amino acids and peptides. [Table 6](#) shows lipids, nucleotides and xenobiotics found in the RB food that showed significant differences in serum after nutritional intervention. Amino acid compounds that increased with RB intake and that were likely derived from RB intake were tryptophan (1.18-fold,

tryptophan; 1.81-fold, serotonin) and methionine (1.27-fold, methionine). We also found significant fold-differences in 1 amino acid, 1 carbohydrate, 7 lipids, 1 nucleotide, 2 cofactors and vitamins, and 1 xenobiotic derived from RB in stool. The amino acid metabolite skatol decreased by 0.77-fold in the tryptophan metabolic pathway and sucrose significantly increased by 2.07-fold in the disaccharides metabolic pathway. Within the class of lipids, decreased fold-change was observed for 1-oleoyl-GPC (18:1; 0.31-fold), 1-linoleoyl-GPC (18:2; 0.41-fold), 1-palmitoyl-GPE (16:0; 0.38-fold), and 1-linoleoyl-GPE (18:2; 0.49-fold). In

TABLE 2 EED biomarkers in serum at 12 mo of age¹

EED biomarker (serum, 12 mo)	Control group (n = 24)	Rice bran group (n = 23)	P ²
C-reactive protein, mg/L	2.32 (3.52)	2.06 (2.06)	0.96
α1-Acid glycoprotein, mg/mL	0.73 (0.84)	1.16 (0.96)	0.08
Glucagon-like peptide 2, pg/mL	593 (224)	744 (381)	0.04

¹Values are medians (IQR). EED, environmental enteric dysfunction.

²Nonparametric Wilcoxon rank-sum test was used to test for differences between the treatment groups.

TABLE 3 Concentrations of trace elements and heavy metals in serum at 12 mo of age

Trace element	Control (n = 24)		Rice bran (n = 23)		P ¹
	Median	IQR	Median	IQR	
Micronutrient, ppb					
Calcium (Ca)	84,200.00	18,300.00	89,100.00	20,000.00	0.30
Cobalt (Co)	1.54	0.51	1.50	0.90	0.86
Copper (Cu)	1190.00	445.00	1180.00	287.00	0.63
Iron (Fe)	1390.00	1190.00	1530.00	1050.00	0.74
Lithium (Li)	18.20	5.38	18.80	6.76	0.80
Magnesium (Mg)	18,800.00	4710.00	19,000.00	4780.00	0.83
Manganese (Mn)	10.60	15.10	13.00	14.10	0.38
Molybdenum (Mo)	4.18	0.87	4.04	1.37	0.44
Phosphorus (P)	100,000.00	26,300.00	107,000.00	17,700.00	0.39
Potassium (K)	166,000.00	38,500.00	172,000.00	23,600.00	0.93
Selenium (Se)	71.10	19.30	71.00	34.00	0.32
Sodium (Na)	2,620,000.00	254,000.00	2,720,000.00	286,000.00	0.32
Strontium (Sr)	38.60	10.70	43.90	13.20	0.31
Sulfur (S)	907,000.00	201,000.00	971,000.00	185,000.00	0.34
Zinc (Zn)	846.00	201.00	845.00	285.00	0.76
Heavy metal, ppb					
Arsenic (As)	15.10	2.41	14.70	1.76	0.42
Barium (Ba)	64.50	8.11	61.10	15.00	0.46
Cadmium (Cd)	3.82	1.57	3.88	3.05	0.89
Lead (Pb)	5.95	1.41	5.76	1.19	0.53
Nickel (Ni)	5.16	1.93	5.18	1.00	0.64
Other, ppb					
Aluminum (Al)	11,300.00	1220.00	11,000.00	2750.00	0.48
Tungsten (W)	0.54	0.00	0.54	0.24	0.55
Vanadium (V)	82.70	35.70	75.00	47.10	0.61

¹Nonparametric Wilcoxon rank-sum test was used.

addition, we also found a significant increase in quinate (2.92-fold) by the food component/plant metabolic pathway and pyridoxine (4.65-fold) by the vitamin B-6 metabolic pathway.

Discussion

This study demonstrated that RB supplementation favorably modulated the GLP-2 serum EED biomarker and impacted a variety of chemical classes of serum and stool metabolites without major changes to targeted trace element concentrations. In our study, serum metabolites from the tryptophan metabolic pathway were significantly increased with RB supplementation, including tryptophan (1.18-fold), N-acetylkynurenine (2.8-fold), serotonin (1.81-fold), and 5-bromotryptophan (1.2-fold). Tryptophan is an essential amino acid and principal component of the human diet with relevance to the enteric neurological system while also playing a substantial role in the functionality of the gut-brain axis (46–48). Several studies have demonstrated direct relations between the concentration of tryptophan and its metabolites with various disorders such as irritable bowel syndrome (46), obesity (49), cardiovascular diseases (50), anorexia nervosa (51, 52), and others (53). Furthermore, serotonin is thought to mediate the vomiting reflex in children with rotavirus and norovirus (54). Kosek et al. (55) reported associations between tryptophan concentration and linear growth in 2 longitudinal birth cohorts, and increased concentrations of tryptophan were associated with LAZ gain in those infants. The association between serum tryptophan concentrations and LAZ in this

cohort merits continued attention for RB supplementation to the diet, particularly as infants showed more growth at 8 and 12 mo than infants in the control group (33). N-acetylkynurenine, a derivative of N-acetyl tryptophan, and observed in this RB intervention group, has been associated with regulating inflammation by inhibiting macrophage activation. Recent research has examined the anti-inflammatory properties of N-acetylkynurenine and its role in autoimmune disease treatment and tissue remodeling (56).

Serotonin, which is synthesized from tryptophan and is a metabolite associated with metabolic alteration in infants with EED, also increased 1.81-fold. Whether elevated serum serotonin metabolite reflects increased gut permeability is unclear; nevertheless, tryptophan increases nutrient absorption important for growth and gut function (57, 58). This supports the positive growth outcomes observed in infants consuming RB, where, after only 2 mo of the intervention, there was a statistically significant increase at 8 mo of age for LAZ ($P < 0.01$). Likewise, at 12 mo of age, infants who received the daily RB supplement were still longer (by length) and showed a significantly higher serum GLP-2 biomarker, indicating improved intestinal health (anthropometric outcomes shown in a previous published article) (33). Notably, we also found an important decrease in the metabolite indolepropionate (0.69-fold) resulting from tryptophan metabolism. Indolepropionate is a well-established antioxidant (59) and biomarker of *Clostridium sporogenes* (60), although studies have shown that serum concentrations of indolepropionate are positively correlated with dietary fiber intake and negatively correlated with CRP concentrations (61).

TABLE 4 Concentrations of trace elements and heavy metals in stool at 12 mo of age

Trace element	Control (n = 23)		Rice bran (n = 23)		P ¹
	Median	IQR	Median	IQR	
Micronutrient, ppb					
Calcium (Ca)	25,700,000.00	43,200,000.00	33,500,000.00	35,600,000.00	0.54
Cadmium (Cd)	95.00	79.00	122.00	112.00	0.52
Cobalt (Co)	285.00	235.00	321.00	218.00	1.00
Copper (Cu)	30,900.00	23,700.00	29,100.00	31,500.00	0.74
Chromium (Cr)	961.00	820.00	1890.00	1530.00	0.03
Iron (Fe)	274,000.00	259,000.00	220,000.00	311,000.00	0.74
Magnesium (Mg)	5,300,000.00	2,520,000.00	6,590,000.00	3,580,000.00	0.06
Manganese (Mn)	46,000.00	38,000.00	70,600.00	63,200.00	0.04
Molybdenum (Mo)	781.00	955.00	704.00	771.00	0.76
Potassium (K)	10,900,000.00	4,730,000.00	11,000,000.00	5,130,000.00	0.71
Selenium (Se)	487.00	478.00	552.00	504.00	0.96
Sodium (Na)	2,330,000.00	3,840,000.00	1,670,000.00	1,720,000.00	0.17
Strontium (Sr)	45,100.00	26,800.00	48,300.00	34,200.00	0.48
Zinc (Zn)	352,000.00	440,000.00	346,000.00	298,000.00	0.74
Heavy metal, ppb					
Arsenic (As)	123.00	74.40	148.00	98.60	0.59
Barium (Ba)	26,700.00	10,200.00	31,900.00	19,900.00	0.28
Lead (Pb)	332.00	322.00	451.00	747.00	0.29
Nickel (Ni)	1800.00	1540.00	2380.00	1790.00	0.12
Other, ppb					
Aluminum (Al)	415,000.00	535,000.00	418,000.00	644,000.00	0.94
Antimony (Sb)	48.30	20.60	54.10	29.90	0.46
Silver (Ag)	36.30	50.90	36.50	84.20	0.96
Thallium (Tl)	20.10	16.90	18.20	19.40	0.99
Vanadium (V)	4940.00	4320.00	4300.00	4320.00	0.54

¹Nonparametric Wilcoxon rank-sum test was used.

Serum metabolite analysis supports the effects of RB consumption with significant increased fold-differences among essential amino acids, such as methionine, a dietary amino acid required for normal growth and development of humans (62). Methionine is the most limiting amino acid in the diet, and it is often found at low concentrations in cereal grains (63). Despite this, methionine increased 1.27-fold after RB intake. In addition, methionine has been associated with linear growth in infants from Malawi (64). The increase in certain essential amino acids in the RB group is important because LAZ was significantly greater in the RB group as compared with controls at 8 mo. WAZ was not significantly different between infants that consumed RB compared with controls. 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 (65, 66).

There is evidence of a direct relation between EED and growth deficits in children (9, 23, 67). Thus, it is necessary to better understand the relation among EED biomarkers with nutritional status after RB intake in children (8, 68). Serum biomarkers CRP and AGP are systemic indicators of inflammation and increases have been associated with yearly decline in infants' LAZ scores (16). In addition, GLP-2 is an intestinotrophic hormone, secreted by enteroendocrine L cells of the intestinal epithelium. It has been shown to increase epithelial proliferation, inhibit apoptosis, enhance barrier function, and increase digestion, nutrient absorption, and blood flow (69–72). In this study population, the higher serum GLP-2 at 12 mo in the RB group compared with the control group reflects reduced inflammation

correlated with RB consumption. Such results are of exceptional significance as GLP-2 is a critical mediator of lipid absorption, and for its ability to reduce inflammation and repair intestinal damage by increasing nutrient absorption (73–75). Reparative and cytoprotective properties of RB could also provide further powerful health benefits to these infants (76).

Interestingly, we noticed decreases in several serum lipid metabolites in the RB group compared with the control group at 12 mo of age. These findings demonstrate that lipid metabolites present in RB may have been metabolized or bioavailable to developing organ systems. To support this hypothesis, we noticed that RB intake increased 3-hydroxyisobutyrate metabolite (1.52-fold), which is a ketone body and can be used for energy production, as carbon sources for lipogenesis by the amino acid metabolic pathway (77), and is involved in energy metabolism to develop brain and lung cells (78). We also observed that glutarate (pentanedioate) lipid metabolite increased significantly (2.00-fold), which is likely due to its production in tryptophan and lysine metabolism as it relates to RB consumption (79). These findings support the growing evidence that RB supplementation not only improves nutrient intake and metabolite profiles important to infant growth and development but also enhances intestinal health and reduces EED.

The stool metabolites such as tyrosine (2.71-fold, dopamine 3-O-sulfate), disaccharides (2.06-fold, sucrose), and fatty acid–dicarboxylate (2.18-fold, 2-hydroxyadipate; 1.80-fold, pimelate) metabolic pathways were also noteworthy for relevance to reduced gut inflammation. Improvements in nutritional status and immune function may relate

TABLE 5 Serum amino acid and peptide metabolites significantly modulated by RB supplementation compared with control at 12 mo of age¹

Chemical class	Metabolic pathway	Metabolite	HMDB ²	Fold-difference ³	P	q Value	
Amino acids	Glycine, serine, and threonine metabolism	Sarcosine	HMDB00271	1.46↑	0.000	0.30	
		N2-acetyllysine**	HMDB00446	1.91↑	0.011	0.78	
		5-(galactosylhydroxy)-L-lysine	—	1.17↑	0.015	0.78	
	Tryptophan metabolism	Tryptophan**	HMDB00929	1.18↑	0.028	0.78	
		N-acetylkynurenine (2)	—	2.80↑	0.000	0.30	
		Serotonin**	HMDB00259	1.81↑	0.021	0.78	
	Leucine, isoleucine, and valine metabolism	Indolepropionate	Indolepropionate	HMDB02302	0.69↓	0.034	0.78
			5-bromotryptophan	—	1.20↑	0.039	0.78
		3-hydroxyisobutyrate	3-hydroxyisobutyrate	HMDB00336	1.52↑	0.008	0.74
			Methionine**	HMDB00696	1.27↑	0.008	0.74
		Urea cycle; arginine and proline metabolism	N-acetylariginine**	HMDB04620	1.41↑	0.002	0.30
			N-acetylcitrulline	HMDB00856	2.10↑	0.001	0.30
	Polyamine metabolism	Polyhydroxyproline	Polyhydroxyproline	HMDB06695	1.31↑	0.001	0.30
N1, N12-diacetylspermine			HMDB02172	0.66↓	0.036	0.78	
4-acetamidobutanoate**		HMDB03681	1.23↑	0.046	0.78		
γ-Glutamyl amino acid	γ-Glutamylmethionine**	γ-Glutamylmethionine**	HMDB29155	1.44↑	0.032	0.78	
		γ-Glutamylthreonine**	HMDB29159	1.47↑	0.012	0.78	
Polypeptide	Polypeptide	HWESASX*	—	2.14↑	0.046	0.78	

¹The table displays metabolites with statistically significant differences between RB and control groups. *Indicates compounds with level 2 annotation that have not been officially confirmed based on a standard but have relative confidence for identity. **Indicates dual identification from RB food metabolome. Upward arrows indicate increased fold-difference in RB compared to control. Downward arrows indicate decreased fold-difference in RB compared to control. RB, rice bran; SAM, S-adenosyl methionine.

²HMDB refers to the Human Metabolome Database. Access numbers are provided for each metabolite identified in the database.

³Fold-difference between study groups was calculated by dividing the scaled relative abundance in children randomly assigned to RB vs. control.

TABLE 6 Serum lipid, nucleotide, and xenobiotic metabolites significantly modulated by RB supplementation compared with control at 12 mo of age¹

Chemical class	Metabolic pathway	Metabolite	HMDB ²	Fold-difference ³	P ⁴	q Value ⁵	
Lipids	Medium-chain fatty acid	Heptanoate (7:0)**	HMDB00666	0.73↓	0.033	0.78	
	Fatty acid, dicarboxylate	Glutarate (pentanedioate)	HMDB00661	2.00↑	0.031	0.78	
	Fatty acid metabolism (acyl choline)	Undecanedioate**	HMDB00888	0.77↓	0.002	0.30	
		Oleoylcholine	—	1.50↑	0.017	0.78	
	Endocannabinoid	Dihomo-linolenoyl-choline	—	1.64↑	0.010	0.78	
		N-oleoylserine	—	1.21↑	0.039	0.78	
	Phosphatidylcholine (PC)	1-palmitoyl-2-palmitoleoyl-GPC	HMDB07969	1.30↑	0.046	0.78	
		1-palmitoyl-2-oleoyl-GPC	HMDB07972	1.14↑	0.022	0.78	
	Diacylglycerol	Linoleoyl-linolenoyl-glycerol [2]	HMDB07250	0.53↓	0.008	0.74	
		Sphingolipid metabolism	N-behenoyl-sphingadienine	—	0.64↓	0.029	0.78
		Androgenic steroids	Epiandrosterone sulfate	—	0.55↓	0.027	0.78
	Nucleotides	Secondary bile acid metabolism	Androstenediol (3 α , 17 α) monosulfate (3)	—	0.59↓	0.037	0.78
		Purine metabolism, adenine containing	Glycodeoxycholate sulfate	—	0.40↓	0.018	0.78
			Adenine**	HMDB00034	1.56↑	0.017	0.78
		Pyrimidine metabolism, uracil containing	N1-methyladenosine	HMDB03331	1.21↑	0.020	0.78
Pseudouridine**			HMDB00767	1.18↑	0.024	0.78	
Xenobiotics		Xanthine metabolism	5-methyluridine (ribothymidine)**	HMDB00884	1.18↑	0.028	0.78
	Caffeine		HMDB01847	1.85↑	0.032	0.78	
		1-methylurate	HMDB03099	1.69↑	0.023	0.78	
	Umbelliferone sulfate	—	0.00↓	0.015	0.78		
	Eugenol sulfate	—	0.21↓	0.017	0.78		

¹The table displays metabolites with statistically significant differences between RB and control groups.

**Indicates dual identification from RB food metabolome. Upward arrows indicate increased fold-difference in RB compared to control. Downward arrows indicate decreased fold-difference in RB compared to control.

RB, rice bran.

²HMDB refers to the Human Metabolome Database. Access numbers are provided for each metabolite identified in the database.

³Fold-difference between study groups was calculated by dividing the scaled relative abundance in children randomly assigned to RB vs. control.

⁴Serum P values calculated by Welch's 2-sample t test.

⁵q-Value threshold accounts for multiple comparisons and false-discovery rate.

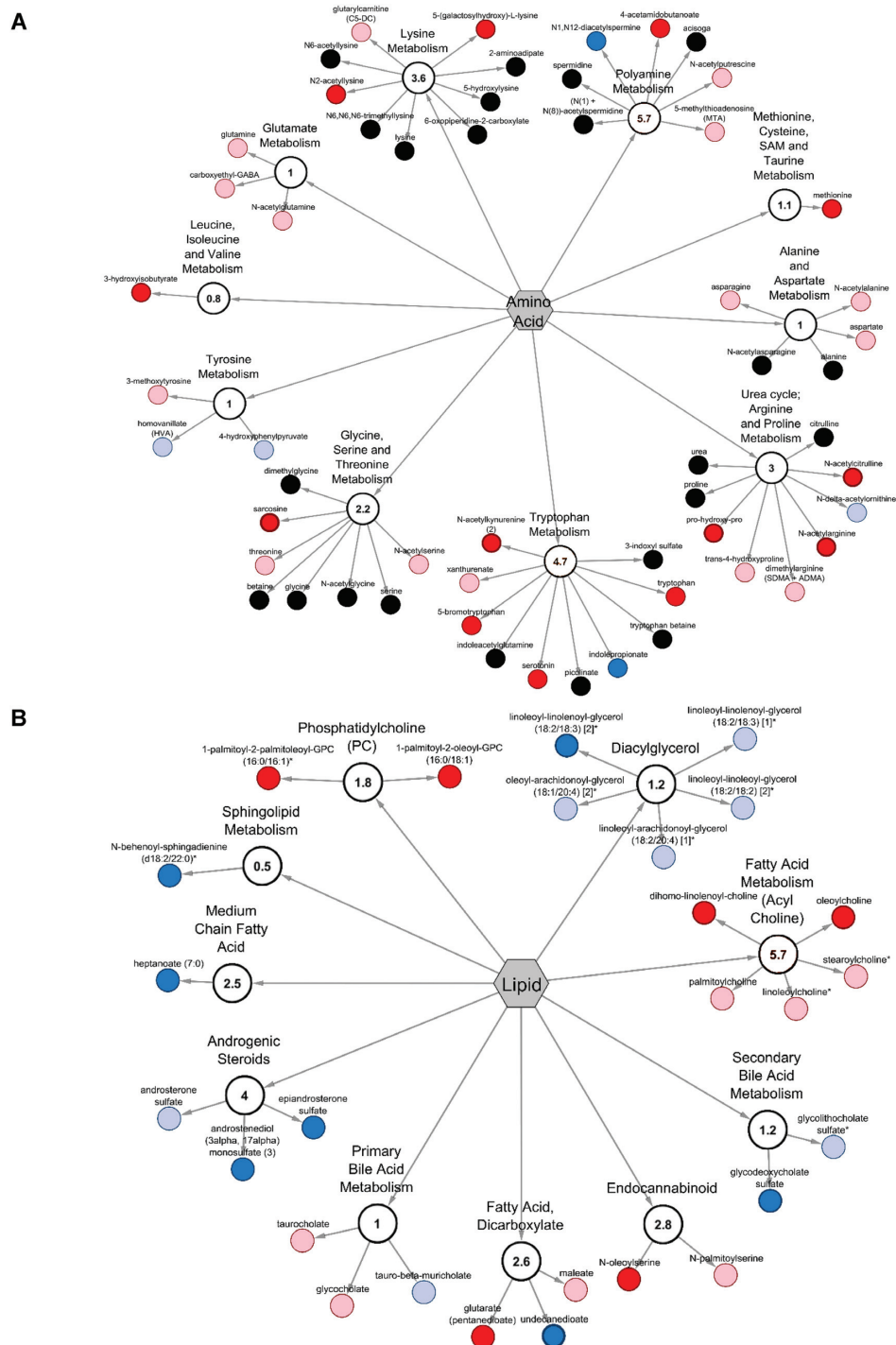


FIGURE 2 Cytoscape network analysis of serum lipid and amino acid metabolites in RB-fed infants at 12 mo compared with the control group at 12 mo. (A) Pathway-specific network visualization for serum lipid metabolites. (B) Pathway-specific network visualization for serum amino acid metabolites. Scores $<$ or $>$ 1 represent PESs that indicate that the pathway contains ≥ 1 metabolites with statistically significant fold-differences between the RB group and control at 12 mo compared with all other pathways within the matrix. Red nodes indicate significant increased difference ($P < 0.05$) between the RB and the control groups, with a metabolite ratio of ≥ 1 . Light red nodes indicate narrowly missed statistical increase cutoffs for significance ($0.05 < P < 0.10$) between the RB and control groups. Blue nodes indicate significant decrease differences ($P < 0.05$) between the RB and the control groups, with a metabolite ratio of < 1 . Light-blue nodes indicate narrowly missed statistical decrease cutoffs for significance ($0.05 < P < 0.10$) between the RB and control groups, and black nodes indicate nonsignificant differences between the RB and control groups. Each metabolite is represented as a node extending from a central submetabolic pathway node, also identified as the PES. The central hexagon is the super metabolic pathway. PES, pathway enrichment score; RB, rice bran. SAM, S-adenosyl methionine. *Indicates dual identification from RB food metabolome.

TABLE 7 Stool amino acid, peptide, carbohydrate, and lipid metabolites significantly modulated by RB supplementation compared with control at 12 mo of age¹

Chemical class	Metabolic pathway	Metabolite	HMDB ²	Fold-difference ³	P ⁴	q Value ⁵	
Amino acids	Tyrosine metabolism	Dopamine 3-O-sulfate	HMDB06275	2.71↑	0.045	0.99	
		Vanillic alcohol sulfate	—	1.93↑	0.038	0.99	
		Skatol	—	0.65↓	0.007	0.63	
Peptides	Tryptophan metabolism	Methionine, cysteine, SAM, and taurine	HMDB00466	0.68↓	0.032	0.88	
		Acetylated peptides	HMDB02005	2.48↑	0.013	0.67	
Carbohydrates Lipids	Disaccharides and oligosaccharides	4-hydroxyphenylacetyl-glycine	—	2.06↑	0.047	0.99	
		Sucrose**	HMDB00258	2.06↑	0.013	0.67	
		2-hydroxyadipate**	HMDB00321	2.18↑	0.045	0.99	
	Fatty acid, dicarboxylate	Fatty acid metabolism (acyl choline)	3-methyladipate**	HMDB00555	0.47↓	0.030	0.87
			Pimelate (heptanedioate)**	HMDB00857	1.80↑	0.023	0.82
			Palmitoylcholine	—	2.31↑	0.001	0.25
	Lysophospholipid	Fatty acid metabolism (acyl choline)	Oleoylcholine	—	3.54↑	0.040	0.99
			Linoleoylcholine*	—	1.88↑	0.013	0.67
			Stearoylcholine*	—	1.48↑	0.024	0.82
			1-oleoyl-GPC (18:1)**	HMDB02815	0.31↓	0.047	0.99
			1-linoleoyl-GPC (18:2)**	HMDB10386	0.41↓	0.027	0.86
			1-palmitoyl-GPE (16:0)**	HMDB11503	0.38↓	0.003	0.43
Glycolipid metabolism	Fatty acid metabolism (acyl choline)	1-linoleoyl-GPE (18:2)*,**	HMDB11507	0.49↓	0.005	0.61	
		1-linoleoyl-2-linolenoyl-digalactosylglycerol (18:2/18:3)*	—	0.52↓	0.001	0.25	
		Linolenoyl-linolenoyl-glycerol (18:3/18:3) [1]*	HMDB07278	0.58↓	0.022	0.82	
Diacylglycerol	Secondary bile acid metabolism	Deoxycholate	HMDB00626	0.31↓	0.005	0.61	
		Glycodeoxycholate	HMDB00631	0.47↓	0.000	0.006	
Carbohydrates	Disaccharides and oligosaccharides	Taurolithocholate	HMDB00722	0.52↓	0.031	0.87	
		Glycoursodeoxycholate	HMDB00708	0.37↓	0.025	0.82	
		Dehydrolithocholate	HMDB00502	0.25↓	0.011	0.67	
Lipids	Fatty acid, dicarboxylate	6-oxolithocholate	—	0.18↓	0.011	0.67	

¹Metabolites with statistically significant differences between RB and control groups are shown. *Indicates compounds with level 2 annotation that have not been officially confirmed based on a standard but have relative confidence for identity. **Indicates dual identification from RB food metabolome. Upward arrows indicate increased fold-difference in RB compared to control. Downward arrows indicate decreased fold-difference in RB compared to control. RB, rice bran.

²HMDB refers to the Human Metabolome Database. Access numbers are provided for each metabolite identified in the database.

³Fold-difference between study groups was calculated by dividing the scaled relative abundance of RB vs. control.

⁴Stool P values calculated by ANOVA.

⁵q-Value threshold accounts for false-discovery rate.

TABLE 8 Stool nucleotide, cofactor, and vitamin and xenobiotic metabolites significantly modulated by RB supplementation compared with control at 12 mo of age¹

Chemical class	Metabolic pathway	Metabolite	HMDB ²	Fold-difference ³	P ⁴	q Value ⁵	
Nucleotides	Purine metabolism, (hypo)xanthine/inosine containing	Xanthosine**	HMDB00299	0.35↓	0.011	0.67	
		N6-dimethylallyladenine	—	0.44↓	0.005	0.61	
	Pyrimidine metabolism, adenine containing	5-methyluridine (ribothymidine)**	HMDB00884	0.37↓	0.029	0.87	
		Nicotinate and nicotinamide metabolism	Nicotinamide**	HMDB01406	1.82↑	0.004	0.57
Cofactors and vitamins	Hemoglobin and porphyrin metabolism	L-urobilin	HMDB04159	0.41↓	0.047	0.99	
		Pyridoxine (vitamin B-6)**	HMDB02075	4.65↑	0.001	0.25	
	Vitamin B-6 metabolism	Caffeic acid sulfate	HMDB41708	2.18↑	0.040	0.99	
		Xanthine metabolism	Retinal	HMDB01358	0.71↓	0.009	0.67
	Food component/plant	Coumaroylquininate (4)	Coumaroylquininate (4)	—	1.40↑	0.031	0.87
			Feruloylglycine (1)	—	2.32↑	0.041	0.99
		Quinate**	Quinate**	HMDB03072	2.92↑	0.045	0.99
			1,2-dilinenoyl-digalactosylglycerol (18:3/18:3)	—	0.49↓	0.008	0.63
		Drug	1-palmitoyl-2-linolenoyl-digalactosylglycerol (16:0/18:3)	—	0.40↓	0.021	0.81
			2-hydroxyacetaminophen sulfate*	—	3.16↑	0.009	0.67
Chemical	Drug	2-methoxyacetaminophen sulfate*	—	2.57↑	0.048	0.99	
		Clotrimazole	HMDB01922	0.52↓	0.020	0.81	
	Chemical	Loratadine	HMDB05000	1.21↑	0.011	0.67	
		2-acetamidophenol sulfate	—	2.62↑	0.000	0.12	
		4-thiouracil	—	0.52↓	0.021	0.81	

¹Metabolites with statistically significant differences between RB and control groups are shown. *Indicates compounds with level 2 annotation that have not been officially confirmed based on a standard but have relative confidence for identity. **Indicates dual identification from RB food metabolome. Upward arrows indicate increased fold-difference in RB compared to control. Downward arrows indicate decreased fold-difference in RB compared to control. RB, rice bran.

²HMDB refers to the Human Metabolome Database. Access numbers are provided for each metabolite identified in the database.

³Fold-difference between study groups was calculated by dividing the scaled relative abundance of RB vs. control.

⁴Stool P values calculated by ANOVA.

⁵q-Value threshold accounts for false-discovery rate.

to increased vitamins (4.65-fold, pyridoxine). Important decreases in stool lipid metabolites were observed that indicated lipid absorption in reference to the RB compositional lipid profile, as well as other metabolites such as amino acids, peptides, nucleotides, and xenobiotics (Tables 7 and 8). We also noticed significant differences in stool lysophospholipid metabolites [1-oleoyl-GPC (18:1), 1-linoleoyl-GPC (18:2), 1-palmitoyl-GPE (16:0), and 1-linoleoyl-GPE (18:2)] and secondary bile acid metabolites (deoxycholate, glycodeoxycholate, tauroolithocholate, glycoursoxydeoxycholate, dehydrolithocholate and 6-oxolithocholate), which have been established as sensitive indicators of gastrointestinal function and microbiome studies (80). Other stool metabolites with significant fold-change that increased with RB intake were sucrose (2.06-fold), 2-hydroxyadipate (2.18-fold), and pimelate (1.80-fold). Sucrose is a disaccharide of 6-O-sinapoyl sucrose metabolism occurring in human gut microbiota and a significant fold-increase is correlated with the growth of beneficial bacteria (*Bifidobacterium*) and supports gut development and protection in infants (81). Skatole is a critical precursor for serotonin and melatonin by the tryptophan metabolic pathway (82). Skatole biosynthesis may have physiological importance herein and based on reported roles in infant fecal microbiota metabolism and neurodevelopment (83).

Nicotinamide changes have been shown for different foods and showed significant fold-differences related to RB intake and possibly also related to tryptophan metabolism. The modified microbiome metabolism and GLP-2 serum biomarker expression could have been increased via shunted tryptophan metabolism toward anti-inflammatory pathways (84). This is related to the observed increase in quinate (2.92-fold), which has shown evidence in controlling oxidative and inflammatory stress conditions by modulating other metabolic pathways (85). Stool pyridoxine had the highest 4.65-fold difference in the stool metabolome, and merits attention for bioaccessibility of pyridoxine. Pyridoxine availability to the host depends on many factors such as temperature, pH of the gastrointestinal tract, and dietary fiber for bonding to polysaccharide and polypeptides (86). Therefore, we suggest that RB supplementation not only increased pyridoxine and nutrient uptake but also enhanced other metabolic pathways for reducing EED risk and especially in the function of the nervous system (87). Pyridoxine, or vitamin B-6, has a role in cognitive development, immune function, and hemoglobin formation (88). There is also other evidence that vitamin B-6 may have an antioxidant function and concentrations have been correlated with amino acid metabolism, nutrition, growth, and infant development (88).

Finally, we observed that 5-methyluridine, which is an endogenous methylated nucleoside found in human fluids and has also been explored as a therapeutic target for hypomethylating agents (89), was detected with significant differences in serum and stool samples. According to this finding, 5-methyluridine, which is found in RB, increased (1.18-fold) in serum after intake and significant differences were also found in stool. In addition, increases in serum 5-methyluridine were positively associated with height-for-age z-score in young infants from rural Malawi (90), and other studies have suggested the importance of this metabolite in cancer prevention (91). It is pertinent to note that high q values in the serum and stool metabolite outcomes were a concern but metabolites with significant P values were encouraging for dual detection of RB metabolites that revealed changes between intervention and

control. Future research is warranted to deepen our understanding of the importance of RB in infant nutrition during these critical stages of growth and development.

Study limitations

This study had limitations such as the small cohort size and short study duration of the diet intervention. The sample size was comparable to other studies with metabolome outcomes (33, 92, 93). Furthermore, we only analyzed 3 established serum EED marker. The evaluation of other EED-related biomarkers was previously conducted with stool. Gut epithelial integrity and permeability can be measured in urine using lactulose to mannitol ratios (94), although urine was not collected in our study. Also, no allergies to the rice bran were expected (95). EED and systemic inflammation biomarkers and metabolome analysis were measured and evaluated for differences between groups at the end of the study (12 mo of age). Future phase 2 studies with RB in diverse populations using an intent-to-treat study design are warranted.

Conclusions

The consumption of heat-stabilized RB in weaning infants was associated with positive changes to GLP-2, serum, and stool metabolites. RB supplementation and changes in serum GLP-2 has implications as a candidate biomarker of nutrient absorption with other serum metabolites associated with improved intestinal health. RB supplementation warrants further investigation as a practical intervention strategy to reduce EED prevalence and risk for children from LMICs, particularly where rice is grown as a staple crop.

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