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Original Research Article

A cross-sectional study of associations between the ^{13}C-sucrose breath test, the lactulose rhamnose assay, and growth in children at high risk of environmental enteropathy

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A B S T R A C T

Background: Environmental enteropathy' (EE) is common among children who are highly exposed to enteric pathogens in low-resource settings. We optimized and validated a stable isotope-based breath test of intestinal sucrase activity (^{13}C-SBT) as a noninvasive test of carbohydrate digestion and metabolism.
Objectives: The primary objective of this study was to assess the relationship between the ^{13}C-SBT and the lactulose/rhamnose ratio (LR) and growth in children. Secondary objectives were to assess the relationship between the ^{13}C-SBT and additional biomarkers of EE. We also characterized the relationship between the ^{13}C-SBT and child sex and dietary diversity, as well as household socio-economic status and food security.
Methods: In this cross-sectional study, 12-to-15-mo-old children were recruited in Bangladesh, India, Kenya, and Peru. Children were assessed with a 4-h ^{13}C-SBT and a 90-min LR test. Plasma was collected to determine the citrulline and kynurenine/tryptophan ratio. Length and weight were measured, and other variables were assessed through questionnaires. For a subset of children, anthropometry was re-measured after 3 mo. Linear regression was used to examine associations corresponding to each objective.
Results: Three sites generated ^{13}C-SBT breath curves that enabled pooled analysis. Differences in ^{13}C-SBT breath curves, LR ratios, and other EE biomarkers were observed between sites. No associations were observed for ^{13}C-SBT summary measures and LR or child growth [e.g., the association between LR and cumulative percent dose recovered at 90 min: –0.39; 95% confidence interval (CI): –1.79, 0.70]. Length-for-age and weight-for-age were positively associated with the time to 50% of dose recovered (0.05; 95% CI: 0.01, 0.09, and 0.05; 95% CI: 0.02, 0.07, respectively), and dietary diversity was associated with time at which 50% of the dose recovered by 240 min is recovered and cumulative percent dose recovered at 90 min (–0.10; 95% CI: –0.18, –0.02 and 2.67; 95% CI: 0.47, 4.88, respectively).
Conclusions: In children at risk of EE, there were no associations between the ^{13}C-SBT, LR, or other EE biomarkers encompassing different pathophysiological domains of EE.
This trial was registered at clinicaltrials.gov as NCT04109352.

Keywords: environmental enteropathy, gut function, sucrose breath test, intestinal permeability

Abbreviations: cPDR90, cumulative percent dose recovered at 90 min; EE, environmental enteropathy; KT, the ratio of the plasma concentration of kynurenine to tryptophan multiplied by 1000; LAZ, length-for-age *z*-score; LR, urinary lactulose/rhamnose excretion ratio; SES, socio-economic status; T50, time at which 50% of the dose recovered by 240 min is recovered; VCO2, volume of carbon dioxide produced by the body; WAZ, weight-for-age *z*-score; ρ, pharmacokinetic model-based test statistic of intestinal sucrase activity; %L, percent dose of lactulose recovered in urine during LR test; %R, percent dose of rhamnose recovered in urine during LR test; ^{13}C-SBT, ^{13}C-sucrose breath test.

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Introduction

Environmental enteropathy (EE) is an acquired subclinical entity that is theorized to result from a complex interaction between host innate immunity, nutritional inadequacy, and recurrent oral pathogen exposure, with adverse consequences for child growth and development [1–4]. Duodenal and jejunal biopsies in children and adults at high risk of EE show villous atrophy and a high density of intraepithelial infiltrates [5]. However, apart from morphological identification of these features through the examination of biopsy specimens, EE lacks definitive diagnostic criteria, owing to its complex pathophysiology involving multiple domains of the gut and the unknown temporality of the problem [6,7]. Because biopsy is infeasible in many research settings, the dual sugar assay of intestinal permeability, using lactulose (L) and rhamnose (R) or mannitol sugars, has been repeatedly used to define EE [8]. Based on these dual sugar tests, the prevalence of EE in children aged 15–24 mo ranges between ~40–65% in low and middle country settings [9,10].

Observational evidence from diverse pediatric populations and experimental evidence from animal models provides evidence for the role of EE in growth faltering [11–13]. Growth faltering among children with EE is thought to occur due to reduced nutrient availability or increased requirement due to malabsorption and nutrient shunting for immune response or intestinal repair [14]. However, this mechanism is supported by only limited experimental evidence [11,12]. Although early studies suggested that EE was associated with the malabsorption of carbohydrates, protein, and fat [15,16], a more recent investigation using a state-of-the-art stable isotope-based test to evaluate protein absorption showed no significant difference between young children classified as having EE [a ratio of recovered L to R (LR) $\geq$ 0.068] or not (LR $<$ 0.068) [17].

The present study investigates carbohydrate digestion and metabolism in children at risk of EE by employing the ^{13}C-sucrose breath test (^{13}C-SBT). The ^{13}C-SBT is a promising stable isotope breath test in which the test subject ingests a dose of nonradioactive, ^{13}C-labeled sucrose, which is absorbed and metabolized, appearing on the breath as $^{13}CO_2$ [18]. The ^{13}C-SBT reflects chemotherapy-induced mucositis [19] and congenital sucrase-isomaltase deficiency [20,21] and correlates well with total small intestinal sucrase activity in rats [22]. The cumulative percent of the ^{13}C-sucrose dose recovered in a breath at 90 min (cPDR90) was previously shown to be associated with LR [r = 0.67, 95% confidence interval: 0.42–0.82] in Australian aboriginal children with and without diarrhea [23].

As part of phase 1 of our published protocol [18], the ^{13}C-SBT was optimized to provide higher enrichment with a lower dose and then validated against intestinal sucrase activity when sucrase-isomaltase was inhibited by reducose [24–26]. Zambian adults with biopsy-confirmed EE were reported to have retained the ability to digest and oxidize ^{13}C-labeled sucrose when compared to healthy adults without EE from Scotland [25], presumably because of considerable reserve capacity throughout the small intestine. The present multi-site study, in 4 resource-limited countries, aims to evaluate the field usability of this optimized and validated ^{13}C-SBT in children aged 12–15 mo, at risk of EE and to assess the relationship between the ^{13}C-SBT, LR, and linear growth as a primary objective [18].

Methods

Study sites and design

Three of the 6 initially proposed study sites [18] provided data to the present analysis, which are Dhaka, Bangladesh (the International Centre for Diarrhoeal Disease Research); Bangalore, India (St. John's Research Institute and St. John's National Academy of Health Sciences); and Iquitos, Peru (Asociación Benéfica PRISMA and the University of Virginia). Results from a fourth site, Siaya (Masinde Muliro University of Science and Technology, Kakamega, Kenya), are presented with the qualification that, due to low enrichment of $^{13}CO_2$ in breath samples (likely due to problems during breath collection, sample storage, or sample transport) and differences in test duration (90 min compared with 240 min), ^{13}C-SBT results were not directly comparable to other sites. One site (University of the West Indies, Kingston, Jamaica) was excluded due to SARS-CoV-2 pandemic-induced delays in recruitment and protocol completion, and 1 site (Tropical Disease Research Centre, Ndola, Zambia) was excluded as results have been delayed by laboratory issues. Data was collected throughout 2019 and 2020. Each study site obtained approval from the ethical review board of their respective institution. Written, informed consent was obtained from caregivers on behalf of their children.

Participant details and study protocol

All children were recruited and enrolled through convenience sampling [18]. Two groups of children (lower- and higher-income) were enrolled at each site. We achieved sampling of both low- and high-socio-economic status (SES) children by sampling from lower-income communities (e.g., urban and rural slums) and well-infant clinics in the hospital or community. Children with severe acute malnutrition (weight-for-height z-score ≤ -3 SD), positive HIV status, any chronic medical or surgical conditions contributing to growth faltering, and weight-for-height z-score $>+2$ SD were excluded.

A unified protocol was adhered to by all the sites involved in the coordinated research project registered in clinicaltrials.gov (NCT04109352), details of which have previously been published [18]. Briefly, 1 experimental day was scheduled, during which a combination of standardized tests and procedures were performed. The tests included the ^{13}C-SBT, the LR dual sugar assay of intestinal permeability, along with anthropometric measurements and a fasting (~4 h from the last meal/human milk) plasma sample. The experimental protocol shown in Figure 1 provides the timing of the breath and urine samples collected during the experiment. Briefly, the L (1 g) and R (0.2 g) were weighed on a high-precision electronic analytical balance (OHAUS Adventurer Pro AV264C in Bangladesh; Sartorius QUINTIX224-10IN in India, Radwag AS 220.R2 PLUS in Peru, and HoChoice HC3004 model in Kenya) accurate to 0.0001 g /0.1 mg, for each child. The ^{13}C sucrose dose was prepared by first preparing a stock solution of ~4 mg/100 mL sucrose by accurately weighing 500 mg $^{13}C_{12}$-sucrose into a falcon tube, followed by 12.5 mL sterile water (by weight 0.997 g/mL * 12.5 mL = 12.4625 g), mixed thoroughly to ensure complete dissolution. The exact weight of the final solution was again weighed to calculate the exact concentration of the final stock solution. The use of the research-grade analytical balances (in Bangladesh, an OHAUS Adventurer Pro AV264C inaccurate to 0.0001 g; In India, a Sartorius R200D dual range analytical balance, which reads $\leq$0.00001 g; and in Peru a Radwag AS 220.R2 PLUS laboratory analytical balance accurate to 0.0001 g, and in Kenya an HoChoice HC3004 scale accurate to 0.0001 g) and pipetting ensured minimal error in the actual dose measured for administration. The 1 mL aliquot of the stock solution was deposited into sterile, clearly labeled, sequentially numbered cryovials and frozen at –20°C. To prepare the sucrose dose for each child, a calibrated micropipette was used for pipetting a volume of the stock solution calculated for 4 mg/kg body weight of the child either directly into the LR solution (in Bangladesh,

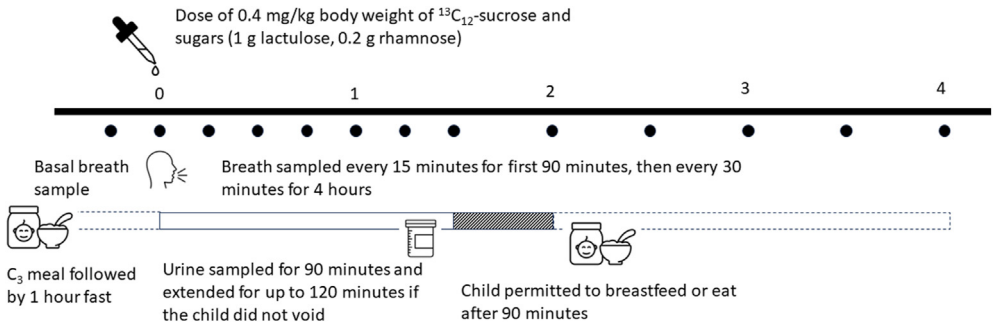

FIGURE 1. Visual representation of protocol for breath and urine collection. The study protocol for breath, urine, and blood collection was adopted with minor modifications by each site.

Peru, and Kenya) or onto a sterile screw cap vial kept on the analytical balance and then into the LR solution (in India). The isotope [0.4 mg/kg body weight of $^{13}C_{12}$-sucrose (99 atom %), and sugars (1 g L, 0.2 g R)] was administered 1 h following a meal comprising of egg, rice, and/or pulses, which was provided at the facility. Children were only allowed to drink water in the first 90 min of the experiment, after which they received another meal or human milk (Figure 1). To establish the reliability of the ^{13}C-SBT, it was repeated 1 wk after baseline in a single site (Peru).

Interviews were conducted with primary caregivers to obtain information about the household's access to improved water and sanitation, 8 selected assets, maternal education, and household income, which was then used to calculate the WAMI (water/sanitation, assets, maternal education, and income) score, a socio-economic index that has previously been validated across diverse low- and middle-income country settings, with a range of 0 (lowest) to 1 (highest) SES [27]. Household food insecurity was assessed using the household food insecurity access scale [28]. Minimum dietary diversity (MDD) was assessed using the standard recommended WHO indicator [29]. Additional diet questions were queried about whether the child had consumed common foods with a higher natural abundance of ^{13}C (maize, sorghum, millet, and sugarcane) on the day prior to the experiment. Questionnaires also assessed child morbidity at baseline using standardized questionnaires. The recumbent length of each child was measured using a SECA 417 infantometer (in India and Bangladesh), a SECA 210 mobile measuring mat (in Kenya), or a locally produced measuring board made to technical standard (in Peru) [30], whereas weight was measured using a SECA 354 weighing scale (In India and Bangladesh), an S014555 electronic scale with an accuracy of 5 g (in Kenya), or a Tanita BD-585 scale with the smallest increment read of 10 g (in Peru). Infants were weighed either nude or with weighed diapers. All measurements were conducted in triplicates. The mean was taken when there was a difference by 0.2 mm (length) in 2 measurements, OR if 2 measurements were the same, that value was taken.

Analytical methods

Breath samples were collected using a mask (covering the nose and mouth of the child) connected with a 1-way valve into a nondiffusible disposable bag, allowing only expired breath to be collected. After each collection, the samples were transferred into 2 10-mL nonsilicon-coated tubes (evacuated tube; Becton Dickinson) and stored at room temperature until analysis. The $^{13}CO_2$ abundance (atom %) in the breath samples was analyzed using an isotope ratio mass spectrometer at St. John's Research Institute (Delta V Advantage, Thermo Fisher Scientific Inc) and at Flinders University (Automated Breath CO_2Analyzer (ABCA), SerCon). The increase in $^{13}CO_2$ enrichment during the fed state of the tracer protocol was expressed as atom percent (atom %) excess over baseline abundance. The precision of the isotope mass ratio spectrometry (IRMS) for carbon dioxide measurement was <0.006%.

Urine void was collected as described earlier [18]. Isotopic standards ($^{13}C_{12}$-L and $^{13}C_6$-R (Sigma-Aldrich) were diluted to a final concentration of 4000, 1000, 500, 50, 5, 0.5 μg/mL. Hydrophilic interaction liquid chromatography was done on an acquity UPLC BEH Amide (130 Å, 1.7 μM, 2.1 mm X100 mm) (Waters) on a vanquish system. Mobile phase A was water with 10 mM ammonium acetate, and phase B in acetonitrile with 10 nM ammonium acetate. The flow rate was 0.25 mL/min. A Thermo Orbitrap ID-X tandem mass spectrometer was used with parallel reaction monitoring parameters were, for MS-1: MSI Res = 60,000, Range –67 to 1000, RF lens = 35, AGC = 25%, and MaxIT-50 and for MS-2 CE = 30 =/–10, Res = 30,000, AGC = standard and MaxIT = Auto. For L, the limit of detection was 0.13 ug/mL, whereas the limit of quantification was 1.32 ug/mL. For R, these were 0.05 ug/mL and 0.5 ug/mL, respectively. There were no samples with values that fell below these limits. The coefficient of variation was the coefficient of variation was 1.16% for L and 1.02% for R (after 20 repetitions).

Plasma was separated from the collected blood samples, stored, and shipped to a central laboratory for analysis [18]. Standards for L-citrulline (Sigma-Aldrich), L-tryptophan [amino acid light version AA mixture (Promega)], and L-kynurenine (Sigma-Aldrich) were serially diluted in water to achieve a 6-point calibration set with ranges of each analyte of 10 μM, 5, 2, 1, 0.5, and 0.1 μM) in water. Quality control solutions were prepared at 2 levels by doping pooled human plasma with reference standards and stored at −80°C. The internal standard was prepared with labeled forms of L-citrulline, L-tryptophan, and L-kynurenine (Cambridge Isotope Laboratories).

Calibrators, quality control, and samples were protein precipitated by vortexing 20 μL of plasma or calibrator with 50 μL of 0.2 molar trifluoroacetic acid containing the internal standards, adding 500 μL of acetonitrile, revortexing, and placing the vial in a −20°C freezer for 10 min. Samples were then centrifuged for 5 min at 5000 × g, and the supernatant was transferred to an autosampler vial for analysis.

Liquid chromatography was performed on a Vanquish UPLC system using an acquity ultra performance liquid chromatography (UPLC) BEH C18 1.7 μM, 2.1 × 100 mm column (Waters). Mobile phase A contained 0.1% (vol/vol) formic acid in water, and mobile phase B contained 0.1%% (vol/vol) formic acid in methanol. A 5-μL sample was introduced, and a gradient was 0–5% B, 0.5–5% B, 8–50% B, 9–98% B, 13–98% B, 13.1–5% B, 15.0–5% B. A Thermo Orbitrap ID-X tandem mass spectrometer was programmed to the transitions previously described [31], with a 600°C ionization temperature and 2000 V ionization voltage in positive ionization mode. The limits of

detection for citrulline, kynurenine, and tryptophan were 0.06 μM, 0.03 μM, and 0.03 μM, respectively, whereas the limits of quantification were 0.61 μM, 0.32 μM, and 0.24 μM, respectively. No samples had values falling below these limits.

Key variable definitions

Increases in atom % excess over baseline abundance (δ) were converted to a percentage dose recovery rate using the formula previously reported [32]. Empirical curves (of the form at^be^{-ct} where a, b, and c are empirical constants) were then fit to the series of breath samples from each participant's breath test. From these curves, the cPDR90 and the time at which 50% of the dose recovered by 240 min were recovered (T_{50}). In addition, ρ, a pharmacokinetic model-based test statistic that disaggregates characteristics of the breath curve likely related to intestinal sucrase activity from those likely resulting from bicarbonate kinetic and carbon dioxide production [26,32], were calculated for each breath test. As previously reported, our a priori primary summary breath test measure was cPDR90. However, estimates of the percentage dose recovery rate may be influenced by misspecification of carbon dioxide production (VCO_2, in mmol/h), which is calculated from estimated body surface area and sex [33]. Therefore, T_{50} was identified, as well as a priori, as a secondary breath test summary measure that is not influenced by the misspecification of VCO_2. The model-based statistic ρ was developed as a posteriori [26,32].

LRs were calculated as the ratio of the percent dose recovered for each probe within the 2 h protocol (90 min collection extended to 120 min, if no void was obtained within 90 min), where the percent L (%L) and R excretion (%R) was estimated as the total urine recovered over time of the urine collection multiplied by the concentration of L or R (in μM/L) and divided by the dosage. Bearing in mind that very young children do not urinate on demand, not all children are provided a urine sample within 120 min. We therefore examined whether the characteristics of children with an LR result, compared with those who did not, varied within each country. We initially proposed to use a high-SES sample to establish cut-off values for %L, %R, and LR to classify children into those with and without EE [18]. However, given the relatively small final number of high-SES children in the sample, we instead used data from a previously described group of high-SES children from the same site in India [34] to establish cut-offs at 2 SDs above (for %L and LR) and below (for %R) the geometric mean of the respective variables. These values were 0.264, 0.705, and 0.093 for %L, %R, and LR, respectively. Because the %L, %R, and LR have a skewed distribution, the median and IQR for these values are presented. The kynurenine–tryptophan ratio (KT) was calculated as the ratio of kynurenine to tryptophan and multiplied by 1000 [31].

To summarize anthropometry and questionnaire data, heights and weights were converted to height-for-age z-scores and weight-for-age z-scores (WAZ) using WHO reference standards [35]; mild, moderate, or severe household food insecurity was calculated using the standard classification [28], and children were classified as meeting recommendations for dietary diversity (MDD) if they consumed 4 or more food groups on the previous day. We also tested whether the presence or absence of any individual food groups (grains, legumes, vitamin A-rich fruits and vegetables, other fruits and vegetables, flesh foods, eggs, and dairy) and human milk in the 24 h prior to the test were associated with the ^{13}C-SBT summary measures.

Statistical methods

Mean ^{13}C-SBT statistics, median LR, %L, and %R, and mean plasma biomarkers of EE, length-for-age z-score (LAZ), WAZ, and questionnaire data were compared between the 3 study sites using analysis of variance and χ^2 tests to identify significant differences between sites.

To assess the reliability of the ^{13}C-SBT, we calculated Spearman correlation coefficients between the ^{13}C-SBT summary measures for children in Peru, for whom the test was repeated on 2 different days on each enrolled child, 1 wk apart.

To assess the relationship between the ^{13}C-SBT and the LR and growth in children (our study's primary objective), we constructed bivariable regression models where the primary, a priori exposure of interest was the child's LR test result (LR), and the primary, a priori outcome was the ^{13}C-SBT test (cPDR90). Because LR statistics are positively skewed, LR (as well as %L and %R) was log normalized in these models. There was little evidence that children with an LR test result were different from those without an LR result. Data were therefore assumed to be missing at random (Supplemental Table 1), and missing data were not imputed. We also evaluated LAZ, WAZ, and MDD as potential confounders. However, as the inclusion of these variables did not substantially alter the direction or significance associations between LR and ^{13}C-SBT test outcome variables, only the bivariate model results are presented. The site was adjusted for a random effect. Residual plots were used to check assumptions of linearity, constant variance, and normally distributed errors.

In addition to this hypothesis-testing analysis, we also conducted secondary analysis of other potential associations between other exposure variables, including EE biomarkers (%L, %R, citrulline, and KT), child anthropometry (LAZ and WAZ), and common risk factors for EE (child sex, MDD, SES, food insecurity, recent diarrheal disease, child age, and sex), and secondary ^{13}C-SBT outcomes (T_{50} and ρ). To conduct these secondary analyses, we constructed additional bivariable regression models to examine the association between each exposure and outcome. The site was included as a random effect. Because these secondary analyses were considered exploratory, their P values were not adjusted for multiple comparisons [36]. For the subset of children with a 3-mo follow-up anthropometry measured, we also constructed bivariable regression models to examine associations between associations between ^{13}C-SBT summary measures at baseline (exposure variables) and change in LAZ/WAZ from the initial visit to the follow-up visit (outcome variable). These models again included the site as a random effect.

All analyses were conducted using R statistical software version 4.4.0 [37].

Results

Characteristics of study population

Our previously reported sample size calculation called for ~100 children per site (N = 600) to detect differences in the ^{13}C-SBT cPDR90 on the order of ~3.3% between children with a high compared with a low LR ratio, as well as differences in the order of ~2.0% between children with stunting compared with children with [18]. Our final sample size was substantially less than what we initially proposed. A total of 87 children from Bangladesh, 31 children from India, 54 children from Peru, and 93 children from Kenya were enrolled in the study. Of these, 56 children from Bangladesh, 31 children from India, and 39 children from Peru completed ≥1 successful breath test. In Kenya, only 32 children completed an analyzable breath test because of low enrichment of breath samples, likely caused by issues during breath collection, breath sample storage, or sample shipping. A total of 87 from Bangladesh, 11 from India, and 33 study children from Peru

were able to complete follow-up after 3 mo, for repeat measurement of height and weight (Supplemental Figure 1). Of the 158 children with an analyzed ^{13}C-SBT breath test, 121 also had a successful LR test, whereas 37 children failed to void within the 90-min test window or the extended 90-to-120-min test window. Because urinary LR excretion prior to 120 min is regarded as a more specific measure of small intestinal permeability than later excretion [38], only LR excretion prior to 120 min was analyzed.

Characteristics of the study children and biomarkers of EE varied by country (Table 1). Children from India were older and more likely to be underweight than children from other sites. Children from Peru were shorter (lower mean LAZ) and had higher mean plasma citrulline values than children from the other sites. Children from Kenya had lower weights for length (lower mean weight-for-length *z*-score) and higher mean LR than the other sites. Only children from Peru had statistically greater height and weight at 3 mo follow-up. Children from Bangladesh had a higher WAMI compared to the other sites. Households in Peru were the least food secure but showed a higher MDD. Children from Bangladesh had higher %L and %R, whereas those from Peru showed a higher LR than children from other sites. Children from Bangladesh and Peru had higher mean KT than children from India, which was mainly driven by the lower mean plasma tryptophan. There were no differences in LR between children from families of higher compared with lower SES (Supplemental Figure 2).

^{13}C-SBT results

^{13}C-SBT results varied by country. Children from Bangladesh tended to have breath curves that peaked later than children from India and Peru (Table 2 and Figure 2). Similarly, these children had a later time to 50% recovery (T_{50}), a lower cPDR90 and ρ, but the difference was not present for other summary measures. This finding could be attributed to the potential systematic misspecification of VCO_2, which is calculated from body surface area calculation (high-SES children from Peru had greater body weight and length). The cPDR90 was relatively higher in high than low-SES children (Supplemental Figure 3).

^{13}C-SBT-retest reliability

Among Peruvian children with 2 breath tests performed (paired n = 39) 1 wk apart, test-retest reliability was low, with a Pearson's correlation of 0.21 (–0.12, 0.49) for cPDR90; –0.15 (–0.12, 0.49) for T_{50} and 0.27 for ρ (–0.05, 0.54). The corresponding coefficients of variation were 37.3%, 11.3%, and 44.6% for cPDR90, T_{50}, and ρ, respectively (Supplemental Figure 4).

Associations between ^{13}C-SBT, LR, anthropometry, other EE biomarkers, and other factors

There were no significant associations between each of cPDR90, T_{50}, ρ, and LR, %L or %R recovery (Table 3, Figure 3 for the overall population, and Supplemental Figure 5 by site). Nor were any

TABLE 1
Demographic, anthropometric, and environmental enteropathy biomarkers of study children by site.

	Bangladesh (BGD)	India (IND)	Kenya (KEN)	Peru (PEL)
N	87	31	93	54
Age - mean (SD)	13.3 (1.1)	14.8 (1.3)	14.1(1.2)	13.6 (1.0)
Male- percentage	52.9%	48.4%	50.5%	51.9%
WAZ - mean (SD)	–0.9 (0.9)	–1.0 (0.9)	–0.2 (1.1)	–0.6 (0.9)
Percent underweight	12.6	16.1%	4.3%	5.6%
LAZ - mean (SD)	–1.1 (0.9)	–1.0 (1.3)	0.8 (1.6)	–1.5 (1.0)
Percent stunted	21.8%	32.3%	5.8%	33.3%
WLZ - mean (SD)	–0.5 (0.9)	–0.7 (0.7)	–1.0 (1.2)	0.2 (1.0)
N with follow-up data	48	12	0	33
ΔWAZ - mean (SD)	–0.2 (0.4)	0.0 (0.3)	—	0.3 (0.6)
ΔLAZ - mean (SD)	–0.1 (0.3)	0.0 (0.6)	—	0.5 (0.9)
Recruited from high-SES community	5 (5.7%)	2 (6.5%)	0	12 (22.2%)
Percentage				
recent diarrheal disease	21.8%	9.7%	37.6%	0.0%
WAMI - mean (SD)[1]	0.7 (0.1)	0.6 (0.1)	0.5 (0.1)	0.6 (0.1)
Food secure - percentage	33.3%	58.1%	6.5%	22.2%
Minimum dietary diversity percentage	48.3%	51.6%	53.8%	70.4%
C4 foods consumed in day prior percentage	10.3%	45.2%	96.8%	92.5%
N with urine (*N* with paired urine and breath)	40 (40)	19 (19)	95 (30)	32 (32)
%L - median (IQR)	0.2 (0.3)	0.1 (0.2)	0.1 (0.1)	0.2 (0.2)
%R - median (IQR)	1.2 (1.9)	1.1 (1.2)	0.2 (0.3)	1.0 (1.2)
LR - median (IQR)	0.1 (0.2)	0.1 (0.1)	0.3 (0.5)	0.2 (0.1)
High LR - percentage	65.0%	72.7%	77.5%	71.9%
N with serum (*N* with paired serum and breath)	87 (56)	24 (24)	0 (0)	54 (38)
Citrulline - mean (SD)	24.8 (8.6)	25.4 (6.3)	—	42.4 (13.2)
Tryptophan (μM) -mean (SD)	35.4 (10.5)	45.5 (8.4)	—	37.1 (7.2)
Kynurenine (μM) - mean (SD)	2.7 (0.9)	2.9 (0.6)	—	2.8 (0.1)
K/T (ratio*1000) - mean (SD)	80.8 (26.7)	64.6 (18.0)	—	77.8 (25.6)

The distribution of study variables in each site is provided as mean and SD (for continuous, normally distributed variables), median and IQR (for non-normally distributed variables), and percentages (for binary variables). Results from the study's Kenyan site are provided for comparison. However, only data for Bangladesh, India, and Peru was used in pooled analyses (Tables 2–5).

Abbreviations: BGD, Dhaka, Bangladesh study site; C4 foods, foods with a high $^{13}C/^{12}C$ isotopic ratio; IND, Bangalore, India study site; IQR, interquartile range; K/T, plasma kynurenine/tryptophan ratio**;** KEN, Siaya, Kenya study site; LAZ, length-for-age *z*-score; LR, lactulose/rhamnose excretion ratio; PEL, Loreto, Peru study site; SD, standard deviation; SES, socio-economic status

[1] WAMI, water/sanitation, assets, maternal education and income index; WAZ, weight-for-age *z*-score; WLZ, weight-for-length *z*-score; ΔLAZ, change in length for-age *z*-score from baseline to 3-mo follow-up visit; ΔWAZ, change in weight-for-age *z*-score from baseline to 3-mo follow-up visit; %L, percent lactulose excretion; %R, percent rhamnose excretion.

TABLE 2
^{13}C sucrose breath test summary statistics of study children by site.

	Bangladesh (BGD)	India (IND)	Peru (PEL)	*P* value for difference	Kenya (KEN)
N	56	31	39		32
δ_0	–22.7 (1.3)	–20.7 (1.6)	–20.0 (2.2)	<0.001	–14.3 (2.4)
cPDR90	18.2 (4.8)	23.6 (6.5)	20.5 (7.6)	0.001	—
T_{peak}	82.2 (19.0)	68.3 (21.4)	67.7 (24.6)	0.001	51.3 (16.9)
T_{50}	106.9 (13.2)	97.7 (16.4)	99.2 (14.0)	0.006	—
ρ	2.9 (1.8)	4.2 (2.2)	3.4 (1.7)	0.012	—

^{13}C-SBT breath curves from the 4 participating sites and breath curve summary statistics: the $^{13}CO_2/^{12}CO_2$ delta value (δ_0), which is the relative difference in parts per thousand between [13C]/[12C] in the sample and the internationally accepted calibration standard ratio R [32]; cumulative percent dose recovered at 90 min (cPDR90), time (in minutes) to breath curve peak (T_{peak}); the time (in minutes) at which 50% of the dose recovered by 240 min was recovered (T_{50}); and ρ, a mechanistic model-based summary statistic developed by our group to support the interpretation of ^{13}C-SBT breath curves by connecting them to the underlying physiological process of interest, i.e., intestinal sucrase activity, was developed a posterior [26,32]. Breath curves from Kenya (KEN) were considered incomparable to other sites due to low enrichment of $^{13}CO_2$ in breath samples (likely due to problems during breath collection, sample storage, or sample transport) and differences in test duration (90 min compared with 240 min). As a result, data from Kenya was excluded from pooled analysis. Only δ_0 and T_{peak} are presented here, as cPDR90, T_{50}, and ρ either cannot be calculated or are incomparable.
Abbreviations: BGD, Dhaka, Bangladesh study site; IND, Bangalore, India study site; KEN, Siaya, Kenya study site; PEL, Loreto, Peru study site; ^{13}C-SBT, ^{13}C sucrose breath test.

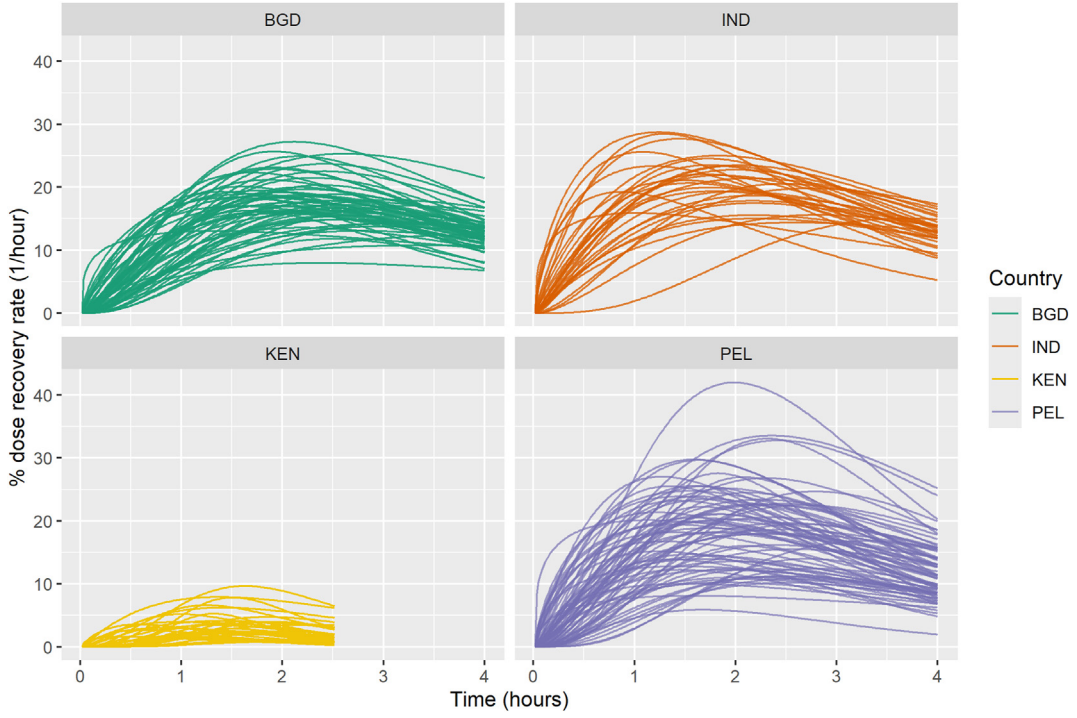

FIGURE 2. ^{13}C-SBT breath curves by country. ^{13}C-SBT breath curves from the 4 participating sites and breath curve summary statistics: the $^{13}CO_2/^{12}CO_2$ delta value (δ_0), which is the relative difference in parts per thousand between [13C]/[12C] in the sample and the internationally accepted calibration standard ratio R [32]; cumulative percent dose recovered at 90 min (cPDR90), time to breath curve peak (T_{peak}); the time at which 50% of the dose recovered by 240 min was recovered (T_{50}); and ρ, a mechanistic model-based summary statistic developed by our group to support the interpretation of ^{13}C-SBT breath curves by connecting them to the underlying physiological process of interest, i.e., intestinal sucrase activity, was developed a posteriori [26,32]. Breath curves from Kenya (KEN) were considered incomparable to other sites due to low enrichment of $^{13}CO_2$ in breath samples (likely due to problems during breath collection, sample storage, or sample transport) and differences in test duration (90 min compared with 360 min). As a result, data from Kenya was excluded from pooled analysis. Only δ_0 and T_{peak} are presented here, as cPDR90, T_{50}, and ρ either cannot be calculated or are incomparable. BGD, Dhaka, Bangladesh study site; IND, Bangalore, India study site; KEN, Siaya, Kenya study site; PEL, Loreto, Peru study site; ^{13}C-SBT, ^{13}C sucrose breath test.

associations observed between the ^{13}C-SBT summary statistics, plasma citrulline, or plasma KT, aside from a nonstatistically significant trend toward faster T_{50} among children with higher plasma citrulline (Table 3). Unexpectedly, a higher LAZ or WAZ, at baseline, was each associated with slower T_{50}, although not with cPDR90 or ρ (Table 4). Children whose caregivers reported that the child had consumed a minimally diverse diet in the day prior to the test had higher cPDR90 and faster T_{50} than children who did not (Table 4). There was no association between ρ and anthropometry, biomarkers of EE, or MDD.

Comparing between EE biomarkers, LR was positively associated with KT (Pearson's correlations of 0.16, 0.37, and 0.06 in Bangladesh, India, and Peru, respectively) and negatively associated with plasma

TABLE 3
Associations between biomarkers of environmental enteropathy and ^{13}C sucrose breath best summary statistics.

	cPDR90 Estimated association (95% CI)	T_{50} Estimated association (95% CI)	ρ Estimated association (95% CI)
Log (%L)	0.59 (–0.83, 1.90) ($P = 0.394$)	–0.02 (–0.07, 0.03) ($P = 0.385$)	0.14 (–0.34, 0.57) ($P = 0.541$)
Log (%R)	1.05 (–0.36, 2.40) ($P = 0.138$)	–0.01 (–0.07, 0.04) ($P = 0.571$)	0.13 (–0.34, 0.58) ($P = 0.579$)
Log (LR)	–0.39 (–1.74, 0.90) ($P = 0.559$)	–0.01 (–0.06, 0.04) ($P = 0.768$)	0.01 (–0.45, 0.44) ($P = 0.958$)
Citrulline (μM)	0.08 (–0.02, 0.19) ($P = 0.135$)	–0.00 (–0.01, 0.00) ($P = 0.083$)	0.01 (–0.02, 0.04) ($P = 0.464$)
Kynurenine (μM)	0.61 (–0.68, 1.90) ($P = 0.356$)	–0.00 (–0.05, 0.04) ($P = 0.873$)	–0.16 (–0.53, 0.21) ($P = 0.399$)
Tryptophan (μM)	0.00 (–0.10, 0.12) ($P = 0.940$)	0.00 (–0.00, 0.01) ($P = 0.618$)	–0.01 (–0.04, 0.03) ($P = 0.513$)
KT (ratio*1000)	0.02 (–0.03, 0.06) ($P = 0.437$)	–0.00 (–0.00, 0.00) ($P = 0.702$)	–0.01 (–0.02, 0.01) ($P = 0.411$)

This table shows estimated associations based on bivariable linear regression models. Dependent (outcome) variables are displayed across the top row. cPDR90, T_{50}, and ρ represent 3 summary measures of the ^{13}C-SBT output. Independent (exposure) variables are displayed down the leftmost column. Only data from Bangladesh, India, and Peru are included in these models. Each model includes a site-specific random intercept ($N = 91$ for %L, %R, and LR models, and $N = 118$ for Citrulline, Kynurenine, Tryptophan, and KT models).
Abbreviations: CI, confidence interval; cPDR90, cumulative percent dose recovered at 90 min; KT, plasma kynurenine/tryptophan ratio; LR, lactulose/rhamnose excretion ratio; T_{50}, time (in minutes) at which 50% of the dose recovered by 240 min is recovered; ρ, pharmacokinetic model-based test statistic of intestinal sucrase activity; %L, percent lactulose excretion; %R, percent rhamnose excretion; ^{13}C-SBT, ^{13}C sucrose breath test.

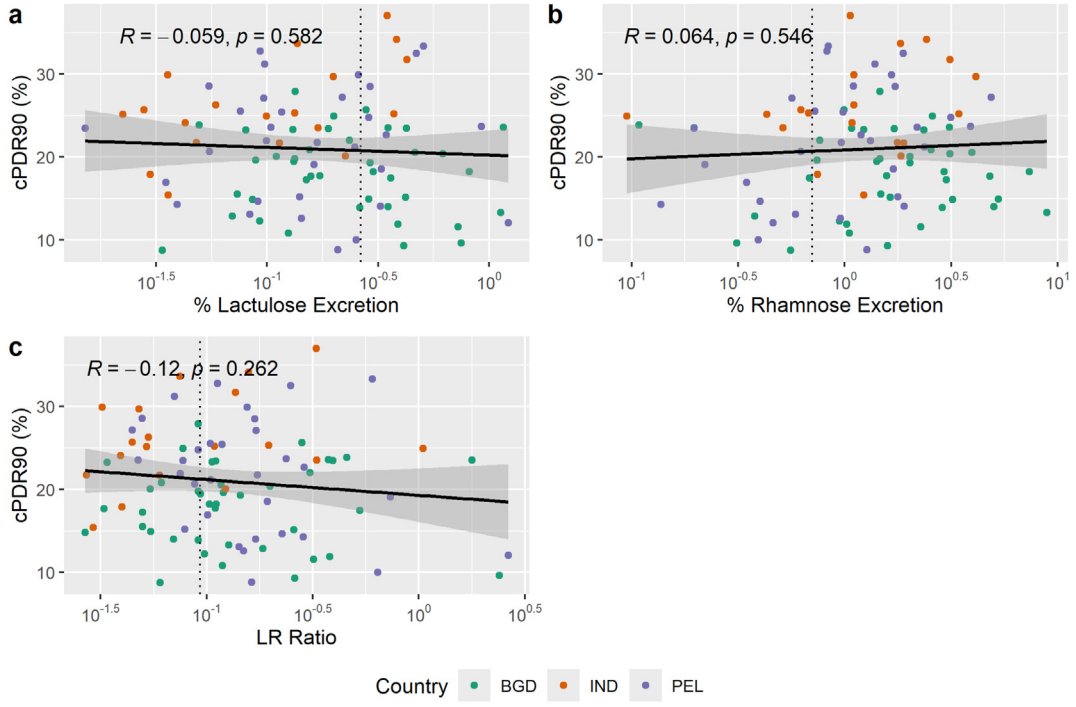

FIGURE 3. ^{13}C-SBT cPDR90 by percentage lactulose excretion, percentage rhamnose excretion, and lactulose rhamnose ratio (LR). Overall estimates of associations between cPDR90 and LR test statistics (percentage lactulose excretion, percentage rhamnose excretion, and the LR). The vertical dotted lines in each plot represent the cut-off for low compared with high values for each of these values. cPDR90, cumulative percent dose recovered at 90 min; R, Pearson's correlation coefficient; ^{13}C-SBT, ^{13}C sucrose breath test.

citrulline concentrations (Pearson's correlations of –0.24, –0.39, and –0.09 in Bangladesh, India, and Peru respectively) although none of these achieved statistical significance.

^{13}C-SBT and child growth

There was no evidence that baseline cPDR90, T_{50}, or ρ was associated with growth (change in LAZ or WAZ) in the 3 mo following the breath test (Table 5).

Discussion

In this study of young children at risk of EE and growth faltering, we identified differences in ^{13}C-SBT breath curves, a measure of sucrose digestion and metabolism, among children across 3 resource-limited settings. However, there was no evidence of an association between the ^{13}C-SBT and urinary LR, the most widely used

TABLE 4
Associations between demographic and anthropometric variables and ^{13}C sucrose breath best summary statistics.

	cPDR90 Estimated association (95% CI)	T_{50} Estimated association (95% CI)	ρ Estimated association (95% CI)
Child age (months)	0.19 (–0.71, 1.25) ($P = 0.696$)	–0.02 (–0.06, 0.01) ($P = 0.265$)	0.14 (–0.13, 0.48) ($P = 0.324$)
Female	0.28 (–1.91, 2.49) ($P = 0.807$)	0.04 (–0.04, 0.12) ($P = 0.316$)	0.15 (–0.51, 0.82) ($P = 0.658$)
LAZ	N/a	0.05 (0.01, 0.09) ($P = 0.024$)	–0.15 (–0.48, 0.18) ($P = 0.373$)
Stunting (reference = not stunted)	N/a	–0.07 (–0.16, 0.02) ($P = 0.157$)	0.11 (–0.62, 0.88) ($P = 0.769$)
WAZ	N/a	0.05 (0.02, 0.07) ($P = 0.032$)	0.04 (–0.32, 0.40) ($P = 0.826$)
Underweight (reference = not underweight)	N/a	0.01 (–0.12, 0.13) ($P = 0.923$)	–0.50 (–1.53, 0.560) ($P = 0.348$)
High-SES community	3.37 (–0.16, 7.03) ($P = 0.067$)	–0.07 (–0.21, 0.06) ($P = 0.322$)	0.39 (–0.68, 1.48) ($P = 0.480$)
WAMI	8.84 (–1.85, 18.20) ($P = 0.075$)	–0.17 (–0.52, 0.23) ($P = 0.399$)	0.60 (–2.71, 3.38) ($P = 0.687$)
Food insecure (reference = food secure)	–0.93 (–3.33, 1.38) ($P = 0.437$)	–0.02 (–0.11, 0.07) ($P = 0.653$)	0.35 (–0.39, 1.03) ($P = 0.329$)
MDD	2.67 (0.47, 4.88) ($P = 0.019$)	–0.10 (–0.18, –0.02) (0.025)	0.13 (–0.55, 0.80) ($P = 0.713$)
C_4 foods consumed in day prior	1.96 (–0.97, 5.03) ($P = 0.212$)	–0.09 (–0.21, 0.02) ($P = 0.122$)	0.36 (–0.50, 1.23) ($P = 0.434$)
Diarrheal disease in month prior	–1.32 (–4.78, 1.93) ($P = 0.477$)	0.01 (–0.11, 0.14) ($P = 0.857$)	–0.09 (–1.13, 0.88) ($P = 0.853$)

This table shows estimated associations based on bivariable linear regression models. Dependent (outcome) variables are displayed across the top row. cPDR90, T_{50}, and ρ represent 3 summary measures of the ^{13}C-SBT output. Independent (exposure) variables are displayed down the leftmost column. Only data from Bangladesh, India, and Peru are included in these models. Each model includes a site-specific random intercept. Associations between anthropometry and cPDR90 are excluded to emphasize the potential for spurious associations between these variables. PDRr may be influenced by the misspecification of carbon dioxide production (VCO2, in mmol/h), which is calculated from the estimated body surface area based on anthropometry.
Abbreviations: C4 foods, foods with a high $^{13}C/^{12}C$ isotopic ratio; CI, confidence interval; cPDR90, cumulative percent dose recovered at 90 min; LAZ, length-for-age *z*-score; MDD, minimum dietary diversity; PDRr, percentage dose recovery rate; SES, socio-economic status; T_{50}, time (in minutes) at which 50% of the dose recovered by 240 min is recovered; VCO2, volume of carbon dioxide produced by the body; WAMI, water/sanitation, assets, maternal education and income index; WAZ, weight-for-age *z*-score, ρ, pharmacokinetic model-based test statistic of intestinal sucrase activity; ^{13}C-SBT, sucrose breath best.

noninvasive test for EE. Children who consumed a minimally diverse diet on the day prior to the test had higher cPDR90 and faster T_{50} than children who did not, primarily through associations with fruit or vegetable consumption and dairy (Supplemental Table 2).

These results should be interpreted in light of other work by our group, which has conclusively demonstrated that the ^{13}C-SBT is reflective of intestinal sucrase activity in in vivo testing with a direct pharmacological inhibitor [25]. Our work here, therefore, suggests that despite prior evidence [23], intestinal sucrose digestion/metabolism may not be appreciably altered in EE, although it may be present in less common or more severe enteropathies [19,22]. This might be the case, for instance, if the expression of factors involved in nutrient absorption is upregulated in EE as an adaptive response to intestinal damage [39]. We also note that although we observed differences both in our primary diagnostic test of EE, the LR ratio, and secondary biomarkers of EE (plasma citrulline and KT) between sites, the interpretation of these tests was inconsistent. For example, children from Peru had a relatively higher LR ratio than children from Bangladesh and India, suggestive of greater small intestinal permeability (i.e., greater risk of EE), but higher plasma citrulline values, suggestive of greater small intestinal enterocyte mass (i.e., reduced risk of EE). In line with a reduced risk of EE, children from Peru also showed a relatively higher cumulative percent dose recovery during the ^{13}C-SBT, faster T_{50}, and higher ρ values, suggestive of greater intestinal sucrase activity. On the contrary, children from India with the lowest LR (but also lower citrulline) showed better intestinal sucrase activity through a higher cPDR90, faster T_{50}, and higher ρ values. Several authors have described limitations related to the LR test as a 'gold standard' for EE [8], and others have reported inconsistencies across EE biomarkers as a methodological challenge [9,40].

We also documented low test-retest reliability for the ^{13}C-SBT based on data from children from Peru. The values we observed were much lower than those of reliability analyses of morphometry (5–13% coefficient of variation), although these were based on comparisons of results collected on the same day [41] although similar to those of another common EE biomarker, fecal myeloperoxidase ($\rho = 0.36$ between 2 samples taken 1 d apart) (personal communication, GOL). Epithelial turnover in humans occurs on the order of ~4–5 d, and this may be accelerated by enteropathogen infection [42]. In animal studies, sucrase activity is also responsive to intervention within ~3 d [43]. It is, therefore, possible that intestinal sucrase activity is not chronically depressed by EE, although still fluctuating on a shorter time scale, possibly in response to recent diet or physical activity. This theory would align with growing evidence that EE may not be a single condition but rather an assemblage of responses by the small intestine to near-constant infectious pathogen exposure [9], resulting in a presentation that may be inconsistent across populations.

Although differences in the ^{13}C-SBT by country were not well explained by LR or by other EE biomarkers or common risk factors for EE, we did observe some evidence of an association between the ^{13}C-SBT and MDD. Children's diets also influence baseline breath $^{13}CO_2$

TABLE 5
Linear regression of associations between ^{13}C sucrose breath test statistics and change in length-for-age *z*-score from baseline to 3-mo follow-up visit and change in weight-for-age *z*-score from baseline to 3-mo follow-up visit. For Peru, India, and Bangladesh only ($N = 89$).

	ΔLAZ Estimated association (95% CI)	ΔWAZ Estimated association (95% CI)
cPDR90	N/a	N/a
T_{50}	0.32 (–0.22, 0.81) ($P = 0.221$)	–0.21 (–0.64, 0.18) ($P = 0.316$)
ρ	–0.03 (–0.09, 0.03) ($P = 0.315$)	0.01 (–0.04, 0.06) ($P = 0.652$)

Dependent (outcome) variables are displayed across the top row. ΔLAZ and ΔWAZ are the changes in length-for-age-*z* and weight-for-age-*z* from the baseline measurement to the 3-mo follow-up. Independent (exposure) variables (summary measures of the ^{13}C-SBT output) are displayed down the leftmost column. Only data from Bangladesh, India, and Peru are included in these models. Each model includes a site-specific random intercept. Associations between anthropometry and cPDR90 are excluded to emphasize the potential for spurious associations between these variables. PDRr may be influenced by the misspecification of carbon dioxide production (VCO2, in mmol/h), which is calculated from estimated body surface area based on anthropometry.
Abbreviations: CI, confidence interval; cPDR90, cumulative percent dose recovered at 90 min; PDRr, percentage dose recovery rate; T_{50}, time (in minutes) at which 50% of the dose recovered by 240 min is recovered; VCO2, volume of carbon dioxide produced by the body; ρ, pharmacokinetic model-based test statistic of intestinal sucrase activity; ^{13}C-SBT, ^{13}C sucrose breath test.

abundance (δ_0) [44], and δ_0 values were associated with cPDR90 and T_{50}, although they are not associated with ρ.

In prior work, our group has compared summary measures of ^{13}C-SBT breath curves and discussed the strengths and limitations of these measures relative to their biological interpretability (ρ) or lack thereof (cPDR90, T_{50}, and time at which the $^{13}CO_2$ breath curve peaks). Our prior mechanistic modeling work suggests that cPDR90 is the most predictive of severe sucrase suppression, although it may be limited for the purposes of studying children at risk of undernutrition due to its sensitivity to misspecification in the estimated carbon dioxide production rate [26].

Limitations of our study include the sample size ($N = 172$), a smaller number of complete breath test results ($N = 126$), paired breath test and LR results ($N = 91$), and fewer study sites (3 compared with 6), compared to what we initially proposed [18]. Our study was, therefore, underpowered to detect smaller differences between LR and the ^{13}C-SBT, such as differences in the order of what has previously been reported for diarrheal and the ^{13}C-SBT [23]. As described in our published protocol [18], this study was designed as a test of "field usability" and validation of the ^{13}C-SBT for studies of infants and young children in resource-limited settings. Other work by our group aims to reduce the duration of the ^{13}C-SBT and the number of breath samples required for reliable estimation [45]. In terms of test usability, although there was a steep learning curve in test administration, the test was very well tolerated by children and caregivers. We also identified challenges in breath sample collection and shipment, challenges in follow-up at some sites, and challenges enrolling high-SES children, limiting the ability to identify a high-income control to better understand reference values for LR. Working through these challenges has enabled the development of protocols to support future studies. In addition, comparable data from 3 low- and middle-income country (LMIC) groups enhances our understanding of test performance and provides greater generalizability of results for this highly novel and ambitious isotope tracer test. Other limitations include the lack of longitudinal data to better understand the relationship between the ^{13}C-SBT as children grow and a lack of detailed dietary data that would support a greater understanding of how dietary intake may affect sucrose metabolism. However, strengths include contextual data for the test from prior adult studies [25] and mechanistic modeling analyses that inform our understanding of the breath curve summary statistics [26,32] and provide strong evidence that the ^{13}C-SBT captures intestinal sucrase activity, as intended.

In conclusion, using a functional test of sucrose digestion and metabolism, we fail to find evidence of impaired intestinal sucrase activity among toddlers with abnormal gut permeability consistent with EE in 3 low- and middle-income country settings despite previously demonstrating that the test responded appropriately to pharmacological inhibition of intestinal sucrase activity. Our results suggest that suboptimal sucrase-isomaltase functioning may not be a major component of intestinal dysfunction in EE. Future work should aim to verify this finding by further characterizing nutrient absorption in EE through field-friendly functional testing.

Author contributions

The authors' responsibilities were as follows– RY, VOO, MNK, PK, DJM, GOL: designed the research; NS, SH, MP-O, SOK, SD, RY: conducted the research; AFB, GOL: analyzed the data; NS, VOO, GOL: wrote the paper; GOL had primary responsibility for final content; and all authors: read and approved the final manuscript.

Conflict of interest

VOO is employed by the funding organization. All other authors report no conflicts of interest.

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Data availability

Data described in the manuscript will be made available upon request to principal investigators of each site (NS: nirupama.s@sjri.res.in, SH: sayeeda@icddrb.org, MP-O: mparedeso@prisma.org.pe, SOK: konyole2000@yahoo.com) according to institutionally required data use agreements.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ajcnut.2024.10.001.

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