The Journal of Nutrition
Nutrient Physiology, Metabolism, and Nutrient-Nutrient Interactions

The Microbiome, Intestinal Function, and Arginine Metabolism of Healthy Indian Women Are Different from Those of American and Jamaican Women[1–3]

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Abstract

Background: Indian women have slower arginine flux during pregnancy compared with American and Jamaican women. Arginine is a semi-essential amino acid that becomes essential during periods of rapid lean tissue deposition. It is synthesized only from citrulline, a nondietary amino acid produced mainly in the gut. The gut is therefore a key site of arginine and citrulline metabolism, and gut microbiota may affect their metabolism.

Objective: The objective of this study was to identify differences in the gut microbiota of nonpregnant American, Indian, and Jamaican women and characterize the relations between the gut microbiota, gut function, and citrulline and arginine metabolism.

Methods: Thirty healthy American, Indian, and Jamaican women (n = 10/group), aged 28.3 ± 0.8 y, were infused intravenously with [guanidino-$^{15}N_2$]arginine, [5,5-$^2H_2$]citrulline, and [$^{15}N_2$]ornithine and given oral [U-$^{13}C_6$]arginine in the fasting and postprandial states. Fecal bacterial communities were characterized by 16S rRNA gene sequencing.

Results: In the fasting state, Indian women had lower citrulline flux than did American and Jamaican women [7.0 ± 0.4 compared with 9.1 ± 0.4 and 8.9 ± 0.2 μmol · kg fat-free mass $(FFM)^{-1} \cdot h^{-1}$, P = 0.01] and greater enteral arginine conversion to ornithine than did American women (1.4 ± 0.11 compared with 1.0 ± 0.08 μmol · kg $FFM^{-1} \cdot h^{-1}$, P = 0.04). They also had lower mannitol excretion than American and Jamaican women (154 ± 37.1 compared with 372 ± 51.8 and 410 ± 39.6 mg/6 h, P < 0.01). Three dominant stool community types characterized by increased abundances of the genera *Prevotella*, *Bacteroides*, and *Bacteroides* with *Clostridium* were identified. Indian women had increased mean relative abundances of *Prevotella* (42%) compared to American and Jamaican women (7% and < 1%, P = 0.03) which were associated with diet, impaired intestinal absorptive capacity, and arginine flux.

Conclusions: These findings suggest that dysregulated intestinal function and a unique gut microbiome may contribute to altered arginine metabolism in Indian women. *J Nutr* 2016;146:706–13.

Keywords: arginine, citrulline, microbiome, intestinal permeability, nonpregnant women

Introduction

Arginine is a semi-essential amino acid that becomes essential during severe illness or physiologic stress and in periods of rapid tissue deposition such as in childhood and during wound healing (1). Because pregnancy is also a period of increased tissue deposition, an adequate arginine supply is critical for a successful pregnancy, because it is necessary for the synthesis not only of new proteins but also for nitric oxide, creatine, and the polyamines involved in cell growth and differentiation (2). In prior studies, we showed that the whole-body arginine flux of Indian women during pregnancy is only approximately half that of American and Jamaican women (3–5). The decreased arginine flux was

[1] Supported by the Bill & Melinda Gates Foundation as well as by federal funds from the USDA Agricultural Research Service under cooperative agreement 58-6250-6001.
[2] Author disclosures: CC Kao, JL Cope, JW Hsu, P Dwarkanath, JM Karnes, RA Luna, EB Hollister, MM Thame, AV Kurpad, and F Jahoor, no conflicts of interest.
[3] Supplemental Table 1 and Supplemental Figure 1 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.
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Manuscript received November 23, 2015. Initial review completed January 7, 2016. Revision accepted February 9, 2016.
First published online March 9, 2016; doi:10.3945/jn.115.227579.

strongly associated with the birthweights of babies born to Indian women, because those women with the slowest fluxes delivered the lightest babies (6). This finding suggests that decreased arginine availability may be a factor contributing to the high incidence of low birth weight in Indian women and led us to the current study to determine whether Indian women on the whole, regardless of pregnancy status, inherently produce arginine at a slower rate than their American and Jamaican counterparts. In an earlier publication of data from the current study, we reported that nonpregnant Indian women have increased arginine catabolism by arginase, an enzyme that hydrolyzes arginine to ornithine and urea, without a compensatory increase in arginine production (7). These findings suggest inherent differences in arginine metabolism in Indian women compared with American and Jamaican women. The underlying cause of these differences in arginine metabolism are not known.

In humans, ~30–40% of dietary arginine undergoes first pass extraction by the splanchnic bed in the postprandial state (7, 8). Most arginine utilization within the splanchnic bed occurs in the small intestinal mucosa, where there is high activity of type II arginase (2). Therefore, the utilization of arginine by arginase in the intestinal mucosa can limit the availability of dietary arginine to extraintestinal tissues (9). Citrulline, the only precursor for de novo arginine synthesis in the kidneys (**Supplemental Figure 1**) (2), is also predominantly synthesized in the enterocyte (10). Because most food sources, other than watermelon, do not contain substantial amounts of citrulline, citrulline synthesis and plasma concentration are potential biomarkers of enterocyte function and mass (11).

Another key element of the gastrointestinal tract that may influence arginine metabolism and its availability is the gut microbiota. There is evidence that gut bacteria utilize dietary-derived amino acids, including arginine (12), for protein synthesis and production of arginine-derived metabolites, including nitric oxide and polyamines (13). Prior studies have demonstrated that diet and biogeography influence gut microbiome composition (14, 15). As such, different gut microbiome composition may exist in Indian compared with American and Jamaican women and have unique effects on arginine metabolism. Although some data of arginine metabolism generated from the present study have already been reported in an earlier publication of this journal (7), the current paper is the first, to our knowledge, to present results concentrating on the relation between arginine and citrulline metabolism and the corresponding gut microbiota of American, Indian, and Jamaican women. The purpose of this part of the larger study was to evaluate intestinal function and its potential link to the gut microbiota in American, Indian, and Jamaican women and to determine whether differences in intestinal function and microbiota could explain the previously described differences in arginine metabolism (8).

Methods

The study was conducted at Baylor College of Medicine in Houston, Texas; St. John's Academy of Health Sciences in Bangalore, India; and the Tropical Metabolism Research Unit, University of the West Indies in Kingston, Jamaica. By advertisement to the general public, 10 women of childbearing age (ages 20–35 y) were recruited at each of the 3 sites. All participants were enrolled after informed consent was obtained. The study protocol was approved by the Institutional Review Board at Baylor College of Medicine, the Institutional Ethical Review Board of St. John's Medical College Hospital, and the Ethics Committee of the University Hospital of the West Indies. All participants underwent screening with a medical history, physical exam, and laboratory testing, and a urine pregnancy test was performed to exclude pregnancy. Nonpregnant women of childbearing age and without any evidence of chronic health problems (such as diabetes mellitus, hypertension, heart disease, or sickle cell disease) were enrolled in the study.

Study procedures

Isotope infusions. All participants underwent isotope infusion studies in both the fasting and the fed states, the order of which was assigned randomly (fasting compared with fed study first). Infusions were performed at the Metabolic Research Unit of the Children's Nutrition Research Center, St. John's Research Institute of St. John's Academy of Health Sciences, and the Tropical Metabolism Research Unit at the University of the West Indies. Sterile solutions of [5,5-$^2H_2$]citrulline, [$^{15}N_2$]ornithine, [guanidino-$^{15}N_2$]arginine, and [U-$^{13}C_6$]arginine (Cambridge Isotope Laboratories) were prepared with use of strict aseptic technique and tested for sterility and lack of pyrogens before infusion. For both the fasting and fed studies, participants were admitted to the research unit following a 10-h fast. For the fasting study, participants remained fasting throughout the infusion. For the fed study, participants immediately started consuming 16 isocaloric and isonitrogenous meals prepared from Ensure Powder (Abbott Nutrition) given every 0.5 h. Isotope infusions were started after 2 h.

An intravenous catheter was placed in the antecubital vein for isotope infusions and in a hand vein in the contralateral hand for blood sampling. The hand was heated to arterialize blood samples. After baseline blood samples were obtained, a primed, continuous, intravenous infusion of [guanidino-$^{15}N_2$]arginine (prime = 4 μmol/kg, infusion = 4 $\mu mol \cdot kg^{-1} \cdot h^{-1}$), [$^2H_2$]citrulline (prime = 1.5 μmol/kg, infusion = 1 $\mu mol \cdot kg^{-1} \cdot h^{-1}$), and [$^{15}N_2$]ornithine (prime = 2 μmol/kg, infusion = 2 $\mu mol \cdot kg^{-1} \cdot h^{-1}$) was started and maintained for 6 h. During the 6-h intravenous infusion, primed, oral doses of [U-$^{13}C_6$]arginine were also administered, with the total dose divided into equal aliquots administered every 30 min (prime = 6 μmol/kg, cumulative oral dose = 4 $\mu mol \cdot kg^{-1} \cdot h^{-1}$).

An oral dose of D_2O (diluted 1:10 with H_2O, 0.1 g/kg) was also given to measure total body water, from which fat-free mass (FFM)[11] was calculated. Blood samples were obtained every 20 min between hours 5 and 6 of the infusion.

Intestinal function test. On the fasting state study day, each participant drank a solution containing 5.0 g of lactulose and 1.0 g of mannitol at the start of the isotope infusion, urine was collected during the 6-h infusion, and an aliquot was stored at −70°C for later analysis.

Stool collection. On the first isotope infusion day, a partial stool sample was collected from each participant in a labeled, sterile container, immediately delivered to study personnel on ice, and stored at −70°C before analysis. Three participants from India did not successfully provide stool samples. All available stool samples from India and Jamaica were shipped on dry ice to Baylor College of Medicine for further processing. A total of 27 samples underwent processing for microbiome characterization.

Sample analyses

Plasma isotopes. The blood samples were drawn into prechilled tubes containing sodium heparin or EDTA. The tubes were centrifuged immediately at 4°C, and the plasma was separated and stored immediately at −70°C for later analysis. The plasma isotope enrichments of citrulline, ornithine, and arginine were measured on their 5-dimethylamino-1-napthalene sulfonamide derivatives by LC-MS/MS as previously described (7, 16).

Mannitol and lactulose. Myo-inositol was added as internal standard to the urine samples, and urinary mannitol concentration was determined by

[11] Abbreviations: FFM, fat-free mass; LMR, lactulose to mannitol ratio; OTU, operational taxonomic unit; PCoA, principal components analysis.

GC-MS with electron ionization mode with use of a modification of a previously described procedure (17). Urinary lactulose concentration was determined by YSI 2300 Stat Plus Analyzer (YSI Incorporated). Total urinary excretion of each sugar was calculated by multiplying the sugar concentration by volume of urine voided during the 6-h collection period.

Stool genomic DNA extraction, sequencing, and postsequencing processing. Stool samples were subjected to a MoBio kit based extraction and sequenced as previously described by the Human Microbiome Project (18). Sequencing of the V3V5 region of the 16S rRNA gene was accomplished on the 454 GSFLX platform (Roche) with use of the forward primer 357F (CCTACGGGAGGCAGCAG) and adapter-tagged reverse primer 534R (CCGTCAATTCMTTTRAGT). Barcodes are provided in **Supplemental Table 1**, and sequences have been deposited with the NCBI Short Read Archive under Bioproject number PRJNA290203.

Postsequencing processing. The Genboree Microbiome Toolset (19), a QIIME-based (QIIME v1.3) (20) platform for 16S rRNA gene sequence analysis, was used to quality filter reads and cluster sequences into operational taxonomic units (OTUs) and calculate Weighted Unifrac distances (21). The quality parameters were the exact barcode and primer match (then removed), longer than 200 bases, no homopolymers over 8 bases, mean quality score above 20, and zero ambiguous bases. Sequences were clustered into OTUs at 97% sequence similarity with CDHIT (22). Chimera Slayer (23) flagged sequences were removed. Taxonomic classifications, at a minimum 50% confidence level, were made with the Ribosomal Database Project (RDP v11.2) (24). All samples were randomly subsampled to an even depth of 2097 sequences/sample.

Tracer kinetic calculations. To standardize the kinetic data, all measurements are expressed per kg of FFM. The total and endogenous fluxes of arginine, citrulline, and ornithine and enteral arginine tracer hydrolyzed to ornithine in the fed and fasting states were calculated as previously described by us (7). Similarly, total body water, from which FFM was determined, was calculated by deuterium dilution as previously described (7).

Intestinal function calculation. Intestinal mucosal absorptive capacity and mucosal integrity were calculated as previously described (25). Briefly, the differential absorption of lactulose and mannitol was used to assess mucosal absorptive capacity and integrity. Mannitol is a small molecule and therefore passes through aqueous pores in enterocyte membranes. Therefore, quantitation of mannitol excretion is an index of intestinal absorptive capacity. Conversely, lactulose, a large molecule, crosses the intestinal epithelium via tight junctions between enterocytes (26), allowing measurement of lactulose excretion to serve as a marker of impaired barrier function. The ratio of excretion of lactulose to mannitol (LMR) eliminates nonmucosal factors, which may affect excretion of these sugars, and therefore serves as an index of intestinal mucosal integrity (27).

Statistical analyses. For kinetic and intestinal function, outcome variables are summarized by group as mean ± SEM. Differences between the 3 groups were assessed by 1-factor ANOVA. If the ANOVA was significant, post-hoc comparisons were performed by use of Tukey's multiple comparison test. Correlations between metabolic and intestinal permeability parameters were determined with use of Pearson's correlation. Tests were considered statistically significant if $P < 0.05$. Data analyses were performed with use of GraphPad Prism software (version 5).

For the microbiota data, all statistical analyses were performed on the subsampled sequence set. Statistical Analysis of Metagenomic Profiles (STAMP v2.0.8) (28) was used to visualize and test for differences in group membership at the genus level by Kruskal-Wallis H-testing (29) with a Benjamini-Hochberg false discovery rate correction (30) of 5%. The level for significance was $q \leq 0.05$. Post-hoc testing was performed by the Tukey-Kramer method with $P \leq 0.05$. We also examined the samples for patterns with respect to β diversity (weighted UniFrac metric) and at the genus level for composition for covariation with metabolic data in the study participants.

Results

A total of 30 participants completed the metabolic portion of the studies. The characteristics of the participants and their habitual dietary intakes have been presented in an earlier report in this Journal (7). Significant differences between the 3 groups of women included the fact that Indian women were significantly shorter, weighed less, and had less FFM than American and Jamaican women. Indian women also had a lower BMI than Jamaican women. In addition, there were more vegetarian Indian participants (5 vegetarians, 50%) than American (1 vegetarian, 10%) or Jamaican (0 vegetarians, 0%) participants ($P = 0.01$). Fat intake per kg FFM was higher in American women than Indian women, and carbohydrate intake per kg FFM was highest in Indian women compared with American and Jamaican women.

Citrulline metabolism. In the fasting state, total and endogenous citrulline flux were significantly lower in Indian women than in American and Jamaican women (**Table 1**). In the fed state, Indian women had significantly lower endogenous and total citrulline flux than American women but not Jamaican women. In both the fasting and fed states, there were no differences in plasma citrulline concentration between the 3 groups (Table 1).

Arginine flux and enteral arginine hydrolysis. Arginine flux and enteral arginine hydrolysis have been presented in an earlier report (7). Whole-body endogenous arginine flux was significantly higher in Jamaican women than in American and Indian women. Enteral arginine conversion to ornithine, an index of intestinal arginase activity, was higher in Indian women than American women in the fasting, but not the fed, state.

Intestinal function and correlations with kinetic parameters. Mannitol excretion, an index of intestinal absorptive capacity, was significantly lower in Indian women compared with both American and Jamaican women (**Table 2**). Lactulose excretion, a marker of impaired intestinal function, was not different between the 3 groups of women. There was a trend, however, toward a higher LMR, an index of intestinal mucosal integrity for which greater values indicate impairment, in Indian women compared with American and Jamaican women ($P = 0.1$).

Significant positive correlations were found between urinary mannitol excretion and citrulline flux ($r = 0.56$, $P < 0.01$) and arginine flux ($r = 0.46$, $P = 0.01$) in the fasting state. There were also significant negative correlations between LMR and citrulline flux ($r = -0.48$, $P = 0.01$), as well as arginine flux ($r = -0.31$, $P = 0.03$).

Microbial community composition: the intestinal microbiome. Altogether, 27 participants completed the microbiome component of the study. Across all participants, the sequenced fecal communities consisted of the genera *Bacteroides* (25% relative abundance), *Prevotella* (14%), *Gemmiger* (11%), *Roseburia* (6%), *Clostridium* sensu stricto (3%), *Ruminococcus* (3%), *Clostridium XI* (3%), *Clostridium IV* (2%), *Parabacteroides* (2%), *Lactobacillus* (2%), and 5% or less of over 70 other groups (**Figure 1**). There were significant differences between the communities based on geographic origin (**Figure 2**). The genus *Bacteroides* was dominant in the American and Jamaican cohorts, comprising a mean of 38% and 14% of the reads generated from each community, respectively, whereas *Bacteroides*

TABLE 1 Whole-body citrulline fluxes and plasma concentrations in American, Indian, and Jamaican women of childbearing age in the fasting and postprandial states[1]

	American	Indian	Jamaican	*P* value
Fasting state				
Total citrulline flux, μmol · kg FFM^{-1} · h^{-1}	10.5 ± 0.41[b]	8.39 ± 0.41[a]	10.5 ± 0.24[b]	<0.001
Endogenous citrulline flux, μmol · kg FFM^{-1} · h^{-1}	9.06 ± 0.41[b]	6.96 ± 0.41[a]	8.91 ± 0.24[b]	<0.001
Citrulline concentration, μmol/L	26.6 ± 1.67	28.2 ± 2.42	22.7 ± 1.25	0.11
Postprandial state				
Total citrulline flux, μmol · kg FFM^{-1} · h^{-1}	12.0 ± 0.53[b]	9.20 ± 0.59[a]	10.9 ± 0.50[a,b]	0.006
Endogenous citrulline flux, μmol · kg FFM^{-1} · h^{-1}	10.5 ± 0.53[b]	7.78 ± 0.60[a]	9.25 ± 0.47[a,b]	0.005
Citrulline concentration, μmol/L	23.0 ± 1.37	25.9 ± 2.20	21.1 ± 1.27	0.15

[1] Values are expressed as means ± SEMs, n = 10/group. Labeled means in a row without a common superscript letter are different, $P < 0.05$ (ANOVA with post-hoc Tukey's multiple comparison test). FFM, fat-free mass.

comprised only 9% of the reads from the Indian cohort on average (P = 0.03). Instead, the genus *Prevotella* was found at greater mean relative abundances in the Indian cohort (42%) than in the American (7%) or Jamaican (<1%) cohorts (P = 0.03). In addition, the Jamaican cohort was characterized by greater relative abundances of 2 different genera from the class *Clostridia*: *Clostridium* sensu stricto (P = 0.02) and *Clostridium* XI (P = 0.03).

The mean relative abundance of *Prevotella* in the American cohort was strongly influenced by a single sample, and more than half of the American samples lacked *Prevotella* completely. In contrast, *Prevotella* was present at very low levels in 6 of the 10 Jamaican samples. One Indian sample had a genus-level profile that closely resembled those from the American cohort [50% *Bacteroides*, 10% *Gemmiger*, absent of *Lactobacillus*, and very low (<1%) in *Prevotella*]. Members of the genus *Bacteroides* were consistently more abundant in the American cohort relative to the Indian and Jamaican cohorts (P = 0.03), although the Jamaican cohort tended to have greater levels than the Indian cohort (P < 0.05). Although the dominant genera detected at each site suggest the influence of local dietary and geographic factors, the 2 American and Indian samples highlighted above suggest that these relations were not purely driven by geographic origin.

Principal components analysis (PCoA) of the weighted Unifrac distance of each sample, as a function of OTU composition, was used to evaluate the relative similarity of participant microbial communities with one another (**Figure 3**). Overlaid with the PCoA are the top 4 genera detected among subjects and differing according to geographic origin. The metabolic data associated with the participants in this study were closely related to variation in community composition, and relations are indicated where detected.

In total, the first 3 PCoA axes explained 75% of the variation in the community composition (Figure 3). PCoA axis 1 captured 41% of the variation in weighted Unifrac distance among specimens and was positively correlated (R^2 = 0.71, P < 0.01) with the relative abundance of *Prevotella* but negatively correlated with the relative abundances of *Ruminococcus* (R^2 = −0.23, P = 0.01), *Faecalibacterium* (R^2 = −0.26, P = 0.01), arginine flux, and urinary mannitol excretion. PCoA axis 2 captured 24% of the variation between communities and showed a strong correlation with *Bacteroides* (R^2 = 0.84, P < 0.01). Major metabolic factors that separate the subjects in PCoA space on axis 1 are: at one end, the high total energy intake, high total carbohydrate intake, low body weight, and low urinary mannitol excretion subjects and at the other end, the low total energy intake, low total carbohydrate intake, high body weight, and high urinary mannitol excretion subjects as shown in Figure 3. Note, the American samples cluster close to the *Bacteroides* overlay in PCoA space, the Indian samples cluster near the *Prevotella* overlay in the PCoA, and the Jamaican samples cluster near the 2 *Clostridia* genera.

Relations between microbiome and metabolic parameters. The correlation of genus-level relative abundances with participant and metabolic variables was performed (Figure 3). The relative abundance of *Prevotella* was negatively correlated with urinary mannitol excretion (R^2 = −0.50), with a very strong effect size (P < 0.01). Increased abundance of this genus tended to occur in tandem with decreased abundances of the genus *Bacteroides* and appears to have driven the majority of the separation observed among subjects along PCoA axis 1. The relative abundance of the genus *Bacteroides* was positively correlated with urinary mannitol excretion (R^2 = 0.25, P = 0.02) and appears to have contributed to the majority of the separation observed along PCoA axis 2.

TABLE 2 Lactulose and mannitol excretion and lactulose:mannitol ratio over 6 h in women of childbearing age from the United States, India, and Jamaica in the fasting state[1]

	American	Indian	Jamaican	*P* value
Urinary mannitol excretion, mg/6 h	372 ± 51.8[b]	154 ± 37.1[a]	410 ± 39.6[b]	<0.001
Mannitol recovery, %	37.2 ± 5.18[b]	15.4 ± 3.71[a]	41.0 ± 3.96[b]	<0.001
Urinary lactulose excretion, mg/6 h	127 ± 13.6	88.7 ± 26.1	136 ± 14.7	0.20
Lactulose recovery, %	2.53 ± 0.27	1.77 ± 0.52	2.71 ± 0.29	0.20
Lactulose mannitol ratio	0.078 ± 0.010	0.54 ± 0.30	0.068 ± 0.005	0.10

[1] Values are expressed as means ± SEMs, n = 10/group. Labeled means in a row without a common superscript letter are different, $P < 0.05$ (ANOVA with post-hoc Tukey's multiple comparison test).

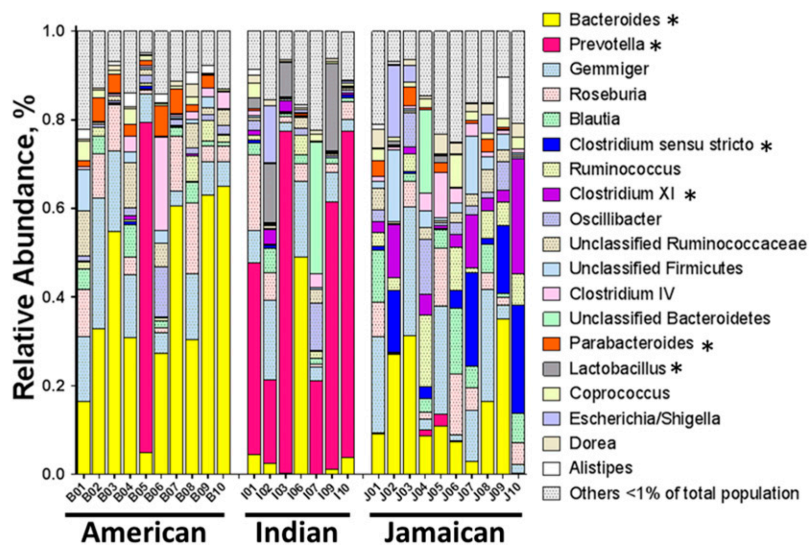

FIGURE 1 Differences in the relative abundance of genera detected in each woman of childbearing age from the United States, India, and Jamaica (n = 27). Note that *Bacteroides*, *Prevotella*, and *Clostridia* differ significantly among cohorts. *Indicates $q \leq 0.05$, with differences as measured by Kruskal-Wallis testing with a Benjamini-Hochberg false discovery rate correction.

Discussion

The purpose of this study was to determine whether American, Indian, and Jamaican women of childbearing age had differences in intestinal function and microbiome composition and whether these differences could contribute to alterations in arginine and citrulline metabolism. The results demonstrate that Indian women have decreased citrulline flux and increased enteral hydrolysis of arginine by arginase in the fasting state. American, Indian, and Jamaican women also had distinct differences in their gut microbiota composition, and these differences correlated with dietary, gut function and metabolic parameters. The decreased citrulline synthesis and increased intestinal arginase activity in combination with increased urinary mannitol excretion indicate altered gut function in Indian women. The implications of these findings are that dysregulated intestinal function and a unique gut microbiome may contribute to altered arginine and energy metabolism in Indian women.

Injury to the intestinal mucosa can disturb the gut barrier function and affect nutrient absorption as well as allow for permeation of substances such as bacteria and toxins (31, 32). The urinary excretion of lactulose and mannitol provides a measure of how much of each sugar is absorbed versus excreted, and therefore a measure of intestinal function is obtained via the lactulose to mannitol ratio (LMR). Increase in the LMR indicates alteration in intestinal permeability, and the change in this ratio may be due to a decrease in mannitol absorption and/or an increase in lactulose absorption. In this study, Indian women had urinary mannitol excretion rates that were approximately half that of American and Jamaican women without substantial change in urinary lactulose excretion. This suggests decreased intestinal absorptive capacity, which may result from

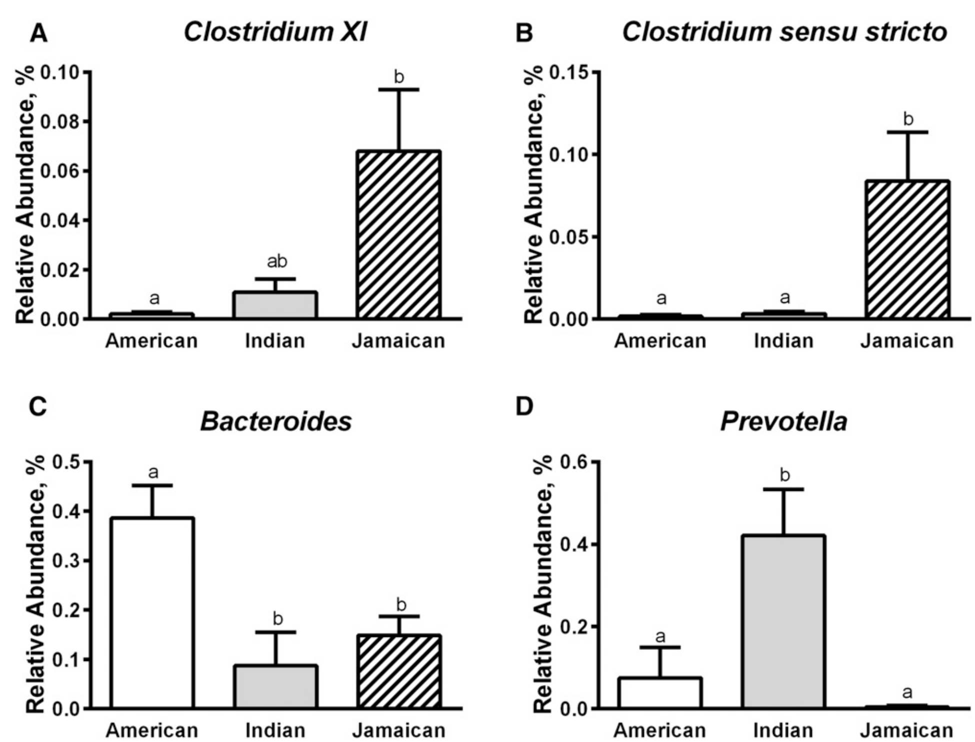

FIGURE 2 Select significant genus level differences as found by Kruskal-Wallis testing with a Benjamini-Hochberg false discovery rate correction in women of childbearing age from the United States (n = 10), India (n = 7), and Jamaica (n = 10). (A) *Clostridium* XI. (B) *Clostridium* sensu stricto. (C) *Bacteroides*. (D) *Prevotella*. Values are means ± SEMs. Labeled means without a common letter differ, $P < 0.05$ by post-hoc testing with the Tukey-Kramer method.

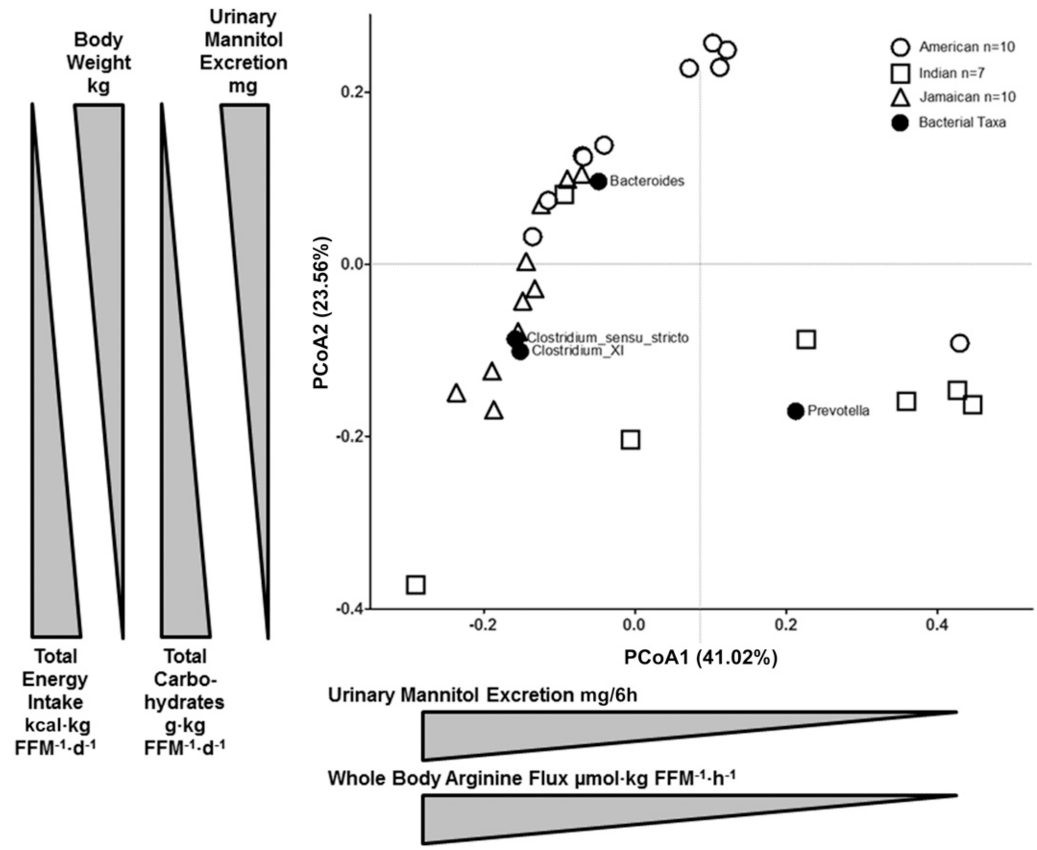

FIGURE 3 Correlations of subject variables with principal component analysis axis loadings in women of childbearing age from the United States (*n* = 10), India (*n* = 7), and Jamaica (*n* = 10). Factors varying along PCoA axis 1 and PCoA axis 2 were identified by correlation of subject variables with axis loadings of weighted UniFrac distance. Centers of variation for 4 bacterial genera are also included. FFM, fat-free mass; PCoA, principal components analysis.

diminished absorptive area without change in the integrity of intestinal tight junctions. There was a trend toward an increased LMR in the Indian women, and half of the Indian women had very high LMR relative to the other women (>0.200), indicating impaired intestinal mucosal integrity. Factors that may contribute to increased intestinal permeability include abnormal gut microbiota, mucosal inflammation, and abnormal epithelial dynamics (31). Malnutrition has been linked to increased permeability of the gut in children (33, 34) and the elderly (35), suggesting that altered intestinal mucosal function may affect absorption of nutrients, including amino acids such as arginine.

Another marker of intestinal function, citrulline flux, was also significantly decreased in Indian women compared with American and Jamaican women. Furthermore, it was positively correlated with urinary mannitol excretion and negatively correlated with LMR. Flux is a dynamic measurement of rate of appearance into a pool—in this case, blood—and may be the sum of multiple points of entry (36). Plasma concentration, on the other hand, reflects the net effect of changes in production and utilization of a substrate. However, it does not provide information about the inflow and outflow rates that lead to increases or decreases in concentration. Although citrulline plasma concentration was not different in Indian women compared with American and Jamaican women, its flux was decreased, indicating a decreased rate of synthesis and implying decreased functional enterocyte mass. In addition, citrulline itself may have an important role in preserving gut barrier function. In a model of intestinal obstruction, pretreatment with a diet containing citrulline preserved gut barrier integrity, modulated the cytokine profile, and increased secretory immunoglobulin A production (37). Citrulline's effect could be modulated by NO, because citrulline is a more potent precursor for NO synthesis than arginine itself (38). Therefore, decreased rate of citrulline production might also lead to a decline in the gut barrier function.

In this study, the rate of conversion of enterally administered arginine tracer to ornithine is representative of the intestinal arginase activity rather than urea cycle activity, because prior studies have demonstrated that there is minimal exchange between extracellular arginine and arginine within the urea cycle (39, 40). Thus, Indian women had increased arginase activity in the fasting, but not in the fed, state.

Distinct differences were also found in the gut microbiota of these women. In all 3 groups of women, the gut microbiota was composed mainly of bacteria from the phyla *Bacteroidetes* and *Firmicutes*, which is consistent with prior reports that *Firmicutes* and *Bacteroidetes*, at different proportions, constitute the majority of the human gut microbiota (41–43). However, at the genus level, the results suggest the presence of 3 dominant states or biotypes in the stool microbiome, which are characterized by increased abundances of *Prevotella*, *Bacteroides*, and *Bacteroides* with *Clostridium*, respectively. These dominant states tend to reflect the geographic origin of the samples, with Indian women having a higher prevalence of *Prevotella*, whereas American and Jamaican women had higher *Bacteroides*.

Gut microbiota may modulate body weight by regulating energy metabolism. For example, studies in mice show that

Firmicutes, as opposed to *Bacteroidetes*, predominate in the microbiota of obese mice (44). This increase in *Firmicutes* is also associated with increased expression of microbial genes involved in carbohydrate metabolism, suggesting that these bacteria facilitate a greater absorption of energy from dietary carbohydrate (45). Malnutrition may also be associated with changes in the gut microbiome. Monira et al. (46) found that children with protein-energy wasting had higher proportions of *Proteobacteria* and lower proportions of *Bacteroidetes* in their fecal microbial communities when compared with healthy children. In this study, correlation of subject phenotypic and metabolic parameters with weighted Unifrac distance demonstrated that body weight increased as the microbiota community shifted from having a *Prevotella*-dominant state to a more *Bacteroides*-dominant state. It is unclear whether this effect was a result of the gut bacteria altering energy absorption or whether it was a consequence of differences in diet.

Correlation of urinary mannitol excretion with weighted Unifrac distances also identified significant relations along PCoA axes 1 and 2. These findings indicate that the *Bacteroides*-dominant subject group had a greater intestinal absorptive capacity than did those subjects whose communities fell within the *Bacteroides* with *Clostridia*- and *Provetella*-dominant groups. The opposite directions of body weight and urinary mannitol excretion along PCoA axis 2 suggest a relation between *Bacteroides* and body weight, which may be mediated through change in the intestinal absorptive function. Finally, endogenous arginine flux increased as the prevalence of *Bacteroides* increased and *Prevotella* decreased. Little is currently known about the effect of gut bacteria on arginine metabolism. In mice, dietary supplementation with arginine induced a shift in the *Firmicutes* to *Bacteroidetes* ratio to favor *Bacteroidetes* in the jejunum and ileum and led to activation of intestinal innate immunity (47). Further investigation is needed to understand the role of *Bacteroidetes* in arginine metabolism.

In summary, significant differences were found in citrulline flux, enteral arginase activity, intestinal absorptive capacity, and gut microbiota composition in Indian women compared with American and Jamaican women. These findings suggest that intestinal permeability and decreased enterocyte mass along with gut microbiota are important factors in the decreased arginine availability in Indian women previously reported by us (7). Interventions to improve intestinal function, as well as modulation of the gut microbiome, such as through diet, may therefore improve arginine bioavailability in Indian women. Further investigation is needed to determine the cause of altered intestinal function in Indian women, as well as to better understand the metabolism of arginine by gut bacteria.

Acknowledgments

The authors thank Grace Tang for her excellent work in the analysis of the samples, Janice Betancourt for her care of the study participants in the Metabolic Research Unit, Karen Prince for figure preparation, and James Versalovic for critical evaluation of the final manuscript. AVK and FJ designed the research; CCK, JWH, PD, JMK, and MMT recruited participants and conducted the experiments; JWH and RAL performed laboratory analyses and calculated and analyzed the data; CCK, JLC, JWH, RAL, EBH, and FJ interpreted the data and wrote the manuscript; CCK and FJ had primary responsibility for the final content. All authors read and approved the final manuscript.

References

1. Morris SM, Jr. Arginine: Beyond protein. Am J Clin Nutr 2006;83:508S–12S.
2. Wu G, Morris SM, Jr. Arginine metabolism: Nitric oxide and beyond. Biochem J 1998;336:1–17.
3. Goodrum LA, Saade GR, Belfort MA, Moise KJ, Jr., Jahoor F. Arginine flux and nitric oxide production during human pregnancy and postpartum. J Soc Gynecol Investig 2003;10:400–5.
4. Thame MM, Fletcher HM, Baker TM, Marini JC, Kao CC, Jahoor F. Arginine flux, but not nitric oxide synthesis, decreases in adolescent girls compared with adult women during pregnancy. J Nutr 2011;141: 71–4.
5. Kurpad AV, Kao C, Dwarkanath P, Muthayya S, Mhaskar A, Thomas A, Vaz M, Jahoor F. In vivo arginine production and nitric oxide synthesis in pregnant Indian women with normal and low body mass indices. Eur J Clin Nutr 2009;63:1091–7.
6. Jahoor F. Arginine production during pregnancy: A study of Jamaican, Indian, and American women. Food Nutr Bull 2013;34:275–6.
7. Kao CC, Hsu JW, Dwarkanath P, Karnes JM, Baker TM, Bohren KM, Badaloo A, Thame MM, Kurpad AV, Jahoor F. Indian women of childbearing age do not metabolically conserve arginine as do American and Jamaican women. J Nutr 2015;145:884–92.
8. Castillo L, Chapman TE, Yu YM, Ajami A, Burke JF, Young VR. Dietary arginine uptake by the splanchnic region in adult humans. Am J Physiol 1993;265:E532–9.
9. Wu G. Intestinal mucosal amino acid catabolism. J Nutr 1998;128: 1249–52.
10. Windmueller HG, Spaeth AE. Source and fate of circulating citrulline. Am J Physiol 1981;241:E473–80.
11. Peters JH, Beishuizen A, Keur MB, Dobrowolski L, Wierdsma NJ, van Bodegraven AA. Assessment of small bowel function in critical illness: Potential role of citrulline metabolism. J Intensive Care Med 2011;26: 105–10.
12. Mardinoglu A, Shoaie S, Bergentall M, Ghaffari P, Zhang C, Larsson E, Backhed F, Nielsen J. The gut microbiota modulates host amino acid and glutathione metabolism in mice. Mol Syst Biol 2015;11:834.
13. Dai Z, Wu Z, Hang S, Zhu W, Wu G. Amino acid metabolism in intestinal bacteria and its potential implications for mammalian reproduction. Mol Hum Reprod 2015;21:389–409.
14. Yatsunenko T, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, Contreras M, Magris M, Hidalgo G, Baldassano RN, Anokhin AP, et al. Human gut microbiome viewed across age and geography. Nature 2012;486:222–7.
15. De Filippo C, Cavalieri D, Di Paola M, Ramazzotti M, Poullet JB, Massart S, Collini S, Pieraccini G, Lionetti P. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. Proc Natl Acad Sci USA 2010;107:14691–6.
16. Kao CC, Wedes SH, Hsu JW, Bohren KM, Comhair SA, Jahoor F, Erzurum SC. Arginine metabolic endotypes in pulmonary arterial hypertension. Pulm Circ 2015;5:124–34.
17. Celli M, D'Eufemia P, Dommarco R, Finocchiaro R, Aprigliano D, Martino F, Cardi E, Giardini O. Rapid gas-chromatographic assay of lactulose and mannitol for estimating intestinal permeability. Clin Chem 1995;41:752–6.
18. Aagaard K, Petrosino J, Keitel W, Watson M, Katancik J, Garcia N, Patel S, Cutting M, Madden T, Hamilton H, et al. The Human Microbiome Project strategy for comprehensive sampling of the human microbiome and why it matters. FASEB J 2013;27:1012–22.
19. Riehle K, Coarfa C, Jackson A, Ma J, Tandon A, Paithankar S, Raghuraman S, Mistretta TA, Saulnier D, Raza S, et al. The Genboree Microbiome Toolset and the analysis of 16S rRNA microbial sequences. BMC Bioinformatics 2012;13(Suppl 13):S11.
20. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena AG, Goodrich JK, Gordon JI, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods 2010;7:335–6.
21. Lozupone C, Knight R. UniFrac: A new phylogenetic method for comparing microbial communities. Appl Environ Microbiol 2005;71: 8228–35.
22. Li W, Godzik A. Cd-hit: A fast program for clustering and comparing large sets of protein or nucleotide sequences. Bioinformatics 2006;22: 1658–9.

23. Haas BJ, Gevers D, Earl AM, Feldgarden M, Ward DV, Giannoukos G, Ciulla D, Tabbaa D, Highlander SK, Sodergren E, et al. Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. Genome Res 2011;21:494–504.
24. Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol 2007;73:5261–7.
25. Iqbal TH, Lewis KO, Gearty JC, Cooper BT. Small intestinal permeability to mannitol and lactulose in the three ethnic groups resident in west Birmingham. Gut 1996;39:199–203.
26. Derikx JP, Luyer MD, Heineman E, Buurman WA. Non-invasive markers of gut wall integrity in health and disease. World J Gastroenterol 2010;16:5272–9.
27. Papadia C, Di Sabatino A, Corazza GR, Forbes A. Diagnosing small bowel malabsorption: A review. Intern Emerg Med 2014;9:3–8.
28. Parks DH, Beiko RG. Identifying biologically relevant differences between metagenomic communities. Bioinformatics 2010;26:715–21.
29. Bluman AG. Elementary statistics: A step by step approach. 6th ed. Boston: McGraw-Hill; 2007.
30. Benjamini Y, Hochberg Y. Controlling the false discovery rate: A practical and powerful approach to multiple testing. J R Stat Soc, B 1995;57:289–300.
31. Salles Teixeira TF, Boroni Moreira AP, Silva Souza NC, Frias R, Gouveia Peluzio Mdo C. Intestinal permeability measurements: General aspects and possible pitfalls. Nutr Hosp 2014;29:269–81.
32. Soeters PB, Luyer MD, Greve JW, Buurman WA. The significance of bowel permeability. Curr Opin Clin Nutr Metab Care 2007;10:632–8.
33. Hossain MI, Nahar B, Hamadani JD, Ahmed T, Roy AK, Brown KH. Intestinal mucosal permeability of severely underweight and nonmalnourished Bangladeshi children and effects of nutritional rehabilitation. J Pediatr Gastroenterol Nutr 2010;51:638–44.
34. Behrens RH, Lunn PG, Northrop CA, Hanlon PW, Neale G. Factors affecting the integrity of the intestinal mucosa of Gambian children. Am J Clin Nutr 1987;45:1433–41.
35. Bolin T, Bare M, Caplan G, Daniells S, Holyday M. Malabsorption may contribute to malnutrition in the elderly. Nutrition 2010;26:852–3.
36. Wolfe RR, Chinkes DL. Isotope tracers in metabolic research: Principles and practice of kinetic analysis. 2nd ed. Hoboken (NJ): Wiley-Liss; 2005.
37. Batista MA, Nicoli JR, Martins Fdos S, Machado JA, Arantes RM, Quirino IE, Correia MI, Cardoso VN. Pretreatment with citrulline improves gut barrier after intestinal obstruction in mice. JPEN J Parenter Enteral Nutr 2012;36:69–76.
38. Cynober L, Moinard C, De Bandt JP. The 2009 ESPEN Sir David Cuthbertson. Citrulline: A new major signaling molecule or just another player in the pharmaconutrition game? Clin Nutr 2010;29:545–51.
39. Castillo L, Chapman TE, Sanchez M, Yu YM, Burke JF, Ajami AM, Vogt J, Young VR. Plasma arginine and citrulline kinetics in adults given adequate and arginine-free diets. Proc Natl Acad Sci USA 1993;90: 7749–53.
40. Yu YM, Young VR, Castillo L, Chapman TE, Tompkins RG, Ryan CM, Burke JF. Plasma arginine and leucine kinetics and urea production rates in burn patients. Metabolism 1995;44:659–66.
41. Arumugam M, Raes J, Pelletier E, Le Paslier D, Yamada T, Mende DR, Fernandes GR, Tap J, Bruls T, Batto JM, et al. Enterotypes of the human gut microbiome. Nature 2011;473:174–80.
42. Ramakrishna BS. Role of the gut microbiota in human nutrition and metabolism. J Gastroenterol Hepatol 2013;28 Suppl 4:9–17.
43. Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, Gill SR, Nelson KE, Relman DA. Diversity of the human intestinal microbial flora. Science 2005;308:1635–8.
44. Ley RE, Backhed F, Turnbaugh P, Lozupone CA, Knight RD, Gordon JI. Obesity alters gut microbial ecology. Proc Natl Acad Sci USA 2005;102: 11070–5.
45. Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature 2006;444:1027–31.
46. Monira S, Nakamura S, Gotoh K, Izutsu K, Watanabe H, Alam NH, Endtz HP, Cravioto A, Ali SI, Nakaya T, et al. Gut microbiota of healthy and malnourished children in Bangladesh. Front Microbiol 2011;2:228.
47. Ren W, Chen S, Yin J, Duan J, Li T, Liu G, Feng Z, Tan B, Yin Y, Wu G. Dietary arginine supplementation of mice alters the microbial population and activates intestinal innate immunity. J Nutr 2014;144:988–95.