

# The Nutrient and Metabolite Profile of 3 Complementary Legume Foods with Potential to Improve Gut Health in Rural Malawian Children

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## Abstract

**Background:** Environmental enteric dysfunction (EED), frequently seen in rural Malawian children, causes chronic inflammation and increases the risk of stunting. Legumes may be beneficial for improving nutrition and reducing the risk of developing EED in weaning children.

**Objective:** The objectives of this study were to determine the nutritional value, verify the food safety, and identify metabolite profiles of 3 legume-based complementary foods: common bean (CB), cowpea (CP), and traditional corn-soy blend (CSB).

**Methods:** Foods were prepared by using local ingredients and analyzed for nutrient composition with the use of Association of Official Analytical Chemists (AOAC) standards (950.46, 991.43, 992.15, 996.06, and 991.36) for macro- and micronutrient proximate analysis. Food safety analysis was conducted in accordance with the Environmental Protection Agency (7471B) and AOAC (2008.02) standards. The metabolite composition of foods was determined with nontargeted ultra-performance LC–tandem mass spectrometry metabolomics.

**Results:** All foods provided similar energy; CB and CP foods contained higher protein and dietary fiber contents than did the CSB food. Iron and zinc were highest in the CSB and CP foods, whereas CB and CP foods contained higher amounts of magnesium, phosphorus, and potassium. A total of 652 distinct metabolites were identified across the 3 foods, and 23, 14, and 36 metabolites were specific to the CSB, CB, and CP foods, respectively. Among the potential dietary biomarkers of intake to distinguish legume foods were pipercolic acid and oleanolic acid for CB; arabinose and serotonin for CSB; and quercetin and  $\alpha$ - and  $\gamma$ -tocopherol acid for CP. No heavy metals were detected, and aflatoxin was measured only in the CSB (5.2 parts per billion).

**Conclusions:** Legumes in the diet provide a rich source of protein, dietary fiber, essential micronutrients, and phytochemicals that may reduce EED. These food metabolite analyses identified potential dietary biomarkers of legume intake for stool, urine, and blood detection that can be used in future studies to assess the relation between the distinct legumes consumed and health outcomes. This trial was registered at [clinicaltrials.gov](http://clinicaltrials.gov) as NCT02472262 and NCT02472301. *Curr Dev Nutr* 2017;1:e001610.

## Introduction

Environmental enteric dysfunction (EED) is generalized subclinical upper small bowel inflammation and is common among young children living in impoverished settings (1–4). EED is associated with increased intestinal permeability, alterations in gut microbial populations, nutrient malabsorption, poor weight gain, stunting, frequent enteric infections, and decreased response to enteric vaccines (1–4). EED predisposes children to clinically manifest



**Keywords:** corn-soybean blend, *Phaseolus vulgaris*, *Vigna unguiculata*, foodomics, metabolomics, malnutrition, enteric disease, environmental enteric dysfunction, complementary foods, infant

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Manuscript received June 30, 2017. Initial review completed August 15, 2017. Revision accepted September 19, 2017. First published September 21, 2017.

Supported by the Feed the Future Food Security Innovation Lab: Collaborative Research on Grain Legumes by the Bureau for Economic Growth, Agriculture, and Trade, the US Agency for International Development under the terms of EDH-A-00-07-00005-00. Additional funding was provided by the Children's Discovery Institute of Washington University and St. Louis Children's Hospital.

Author disclosures: ECB, LZ, IT, NJN, KMM, MJM, and EPR, no conflicts of interest. The opinions expressed herein are those of the authors and do not necessarily reflect the views of the funding agencies.

Supplemental Table 1 is 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://cdn.nutrition.org>.

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Abbreviations used: AOAC, Association of Official Analytical Chemists; CB, common bean; CP, cowpea; CSB, corn-soybean blend; EED, environmental enteric dysfunction; ppb, parts per billion; ppm, parts per million.

forms of malnutrition, including wasting and stunting, and develops during the first 3 y of life, which is a high-risk period marked by the transition from exclusive breastfeeding, to mixed feeding with complementary foods, to an adult diet (1, 2, 4). In sub-Saharan Africa, common complementary foods include maize, cassava, and sorghum, all of which are staples in an unvaried diet that is high in starch and deficient in protein and micronutrients (5). Alternative and culturally acceptable complementary foods that can supply a better balance of nutrients and can provide anti-inflammatory and other gut barrier-protective effects have the potential to reduce EED and its nutritional comorbidities and might also be of benefit to child health and development.

Legumes are a source of essential amino acids, dietary fiber, lipids, micronutrients, and a myriad of phytochemicals, including multiple antioxidants (6, 7). Corn-soybean blends (CSBs) have been used as complementary foods for decades in food-aid and school feeding programs in an effort to deliver higher-quality protein in comparison to a cereal alone (8, 9). Additional evidence supports that other nonsoy legumes, when compared with CSBs, are a rich source of macronutrients, micronutrients, phytochemicals, and prebiotics that can promote gut health (6, 10–14). These include common beans (*Phaseolus vulgaris*), which are digestible and well tolerated in young populations (14) and have been shown to reduce markers of inflammation (15, 16). Furthermore, cowpeas (CPs; *Vigna unguiculata*) are commonly grown in sub-Saharan Africa, where they are considered a staple crop rich in protein, vitamins, and trace minerals including iron and zinc (17, 18). The nutrient profiles of the common bean (CB) and CP, especially their high protein and fiber content, show their potential to improve food and nutrition security in this vulnerable population (13, 19).

Although the CB and CP are readily cultivated in Malawi, they are not commonly utilized as complementary foods. Understanding the nutritional composition and small-molecule profile of foods is crucial in nutrition-based interventions in which this composition will help identify potential dietary biomarkers of intake and disease prevention mechanisms and promote food safety. This methodology, known as food-omics, applies advanced “omics” technologies to identify small compounds in food that can assist in the development of dietary preventive measures against human diseases. The food-omics approach is multifaceted and represents a largely unexploited source in which to identify novel dietary intake biomarkers (20). There have been several studies that reported on the legume metabolome, with a primary focus on improving legume breeding programs (12, 21–24). This includes the Soybean Knowledge Base, an all-inclusive source for soybean translational genomics that provides the integrations of gene, genomics, transcriptomics, proteomics, metabolomics, and phenotype data (22); an investigation on how temperature influences the soybean seed metabolome (23); and an evaluation of diversity among uncooked common beans from 2 centers of domestication (25). In addition, metabolomics has been completed on 17 CP varieties to assess phenolic variations (12).

We conducted a comparative food macro- and micronutrient analysis, a safety assessment, and a metabolomics analysis of 3 legume-based complementary foods—CB, CP, and CSB—collected from Malawi. This analysis will help us assess the future utility of

using legume foods as complementary foods for children. We hypothesized that the CB and CP foods would have higher amounts of protein, dietary fiber, and essential micronutrients and FAs than the CSB. In addition, we used our food metabolomics approach to assist in identifying candidate metabolites for use as future dietary biomarkers of intake that are specific to each legume type.

## Methods

### Identification and preparation of legume foods

Approximately 10 metric tons of CBs and CPs each were purchased from local Malawian markets and wholesalers, generally in 25- or 50-kg sacks. It is estimated that these are aggregates from ~15 farms. CBs and CPs were prepared by dry-roasting the entire lot of hand-sorted beans between 120°C and 130°C for 45–50 min with the use of local Malawian facilities. After dry-roasting, the beans were milled into flour, and the flour was thoroughly mixed. One-kilogram samples of CBs and CPs were taken from this mixture for analysis. The CSB was prepared commercially by extrusion cooking (Rice Milling). The CSB flour for analysis was taken as a composite sample from 20 sacks of CSB that were prepared locally. These milled legume flours were designed to be consumed by adding them in small quantities to the traditional maize porridges, which have been previously and successfully used for legumes in infant and children dietary feeding trials in Malawi and elsewhere (5, 26–28).

### Comparative nutrient analysis

The dietary composition of the foods was measured at Midwest Laboratories. Briefly, 225 g of each legume food was used for proximate analysis (moisture, protein, fat, ash, carbohydrates, and kilocalories), dietary fiber, FA profile, and micronutrient measurements. The methods were based on the Association of Official Analytical Chemists (AOAC) standards as follows: AOAC 950.46 (moisture), AOAC 992.15 (protein), AOAC 996.06 (FA profile), and AOAC 991.36 (fat). For moisture determination, samples were dried in an air oven for 16–18 h at 100–102°C. For protein, samples were digested and distilled to determine total Kjeldahl nitrogen, which was converted into total protein by using a standardized Kjeldahl factor. For fat determination, samples were desiccated and homogenized and fat was extracted by using a solution of petroleum ether, anhydrous sodium sulfate, and defatted cotton. The ash analysis was completed by weighing the sample, heating it to 550°C, and then re-weighing the remaining residue. Carbohydrates and kilocalories were calculated on the basis of the proximate analysis results.

Dietary fiber (soluble and insoluble) was analyzed by using AOAC 991.43. For insoluble dietary fiber, the legume foods were dried and digested with 3 enzymes (protease, amylase, and amyloglycosidase) to break down starch and protein. Ethanol (78% and 95%) was used to precipitate soluble fiber, and insoluble residues were removed with filtration. Residues were weighed to determine insoluble and soluble fiber amounts. Total dietary fiber was the sum of these 2 amounts.

The AOAC 996.06 method was used to analyze the FA profile. Briefly, the methyl ester extracts were injected into a gas

chromatograph that used a flame ionization detector. Thirty-nine FAs were screened for and included butyric (4:0), caproic (6:0), caprylic (8:0), capric (10:0), lauric (12:0), tridecanoic (13:0), myristic (14:0), myristoleic (14:1), myristoleic (14:1n-5), pentadecanoic (15:0), palmitic (16:0), palmitoleic (*trans* 16:1n-9), palmitoleic (*cis* 16:1n-9), heptadecanoic (17:0), 10-heptadecanoic (17:1n-10), stearic (18:0), elaidic (*trans* 18:1n-9), oleic (*cis* 18:1n-9), linoleic (all-*trans* 18:2n-6,9), linoleic (all-*cis* 18:2n-9,12),  $\gamma$ -linolenic (all-*cis* 18:3n-6,9,12), nonadecanoic (19:0),  $\alpha$ -linolenic (all-*cis* 18:3n-9,12,15), arachidic (20:0), 11-eicosenoic (20:1n-11), 11-14-eicosadienoic (20:2n-11,14), homo- $\gamma$ -linolenic (all-*cis* 20:3n-8,11,14), 11-14-17-eicosatrienoic (20:3n-11,14,17), arachidonic (20:4n-5,8,11,14), eicosapentaenoic (20:5n-5,8,11,14,17), heneicosanoic (21:0), behenic (21:0), erucic (*cis* 21:1n-9), docosadienoic (22:2n-13,16), docosapentaenoic (22:5n-4,7,10,13,16), docosahexaenoic (22:6n-4,7,10,13,16,19), tricosanoic (23:0), lignoceric (24:0), and nervonic (24:1n-9) acids. The fat in the legume foods was extracted and saponified, and the FAs were derivatized into FA methyl esters. To quantitate amounts of the identified FAs, the raw chromatographic abundances of each FA in each food sample were compared with those of known standards. Saturated fat, polyunsaturated fat, and monounsaturated fat were totaled and their relative percentages were based on the FA amount measured.

Inductively coupled plasma MS was completed for the following micronutrients: calcium, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc. Briefly, prepared extracts of each of the legume foods were injected into high-energy plasma that forced the elements in the injected sample to emit light wavelengths that were specific to each micronutrient present. The light intensity was detected and correlated to the amounts of micronutrients in the original legume food sample.

### Calculating daily nutrients delivered by 3 legume complementary foods

The daily nutrient intakes for each food were calculated on the basis of serving sizes for weaning children to consume at different ages. The recommendations for the CSB were as follows: 20 g/d (6–8 mo), 30 g/d (9–11 mo), 40 g/d (12–23 mo), and 50 g/d (24–35 mo). The recommendations for CB or CP foods were 21 g/d (6–8 mo), 31.5 g/d (9–11 mo), 42 g/d (12–23 mo), and 52.5 g/d (24–35 mo). The trial registry numbers for clinical trials associated with this research are NCT02472262 and NCT02472301.

### Heavy metal and aflatoxin analyses

Heavy metal and aflatoxin analyses were completed by Midwest Laboratories by using methods described previously (29) and included heavy metal (arsenic, cadmium, lead, and mercury) and aflatoxin contents. The heavy metals analysis was based on the Environmental Protection Agency 7471B method (30). Briefly, samples were dissolved in water, digested with potassium permanganate, and then mixed with water, SSC-hydroxyethyl amine sulfate, and stannous sulfate. The resulting solution was subjected to cold-vapor atomic absorption, where concentration values of heavy metals were based on interpolation to a standard curve of absorbance compared with concentration. Amounts of heavy metals detected in the legume food

were compared with those defined by the USDA as acceptable for human consumption in bean-family foods (31).

Aflatoxin methods were based on AOAC 2008.02. Briefly, legume foods were finely ground and aflatoxins extracted by using a solution of SSC, methanol, and sodium bicarbonate. The resulting extract was centrifuged and filtered to remove contamination, and aflatoxins were separated by using immunoaffinity column isolation. The presence and concentration of aflatoxins in the column-separated filtrate were determined with targeted LC-MS. Amounts of aflatoxins detected in the legume food were compared with those defined by the FDA as acceptable for human consumption (32).

### Comparative nontargeted food metabolomics

The nontargeted food metabolome profile was completed by Metabolon, Inc., as previously published for other whole foods (33). Briefly, each legume food was extracted by using ice-cold 80% methanol for separation and metabolite detection via ultraperformance LC-tandem MS in positive- and negative-ion mode in which samples were quality-controlled within and across assays for individual samples with the use of internal standards. Raw data were extracted and peak-identified by using the Metabolon internal library, and quality-control processed. For each metabolite, peak raw count abundance values were quantified by using AUC analysis, and each metabolite raw abundance was median-scaled by dividing its median raw abundance across the data set into its raw abundance in each legume food. Median-scaled relative abundance  $z$  score was further used to visualize specific metabolites in a given chemical class.  $z$  Scores are expressed as SDs from the mean of the scaled abundance for each metabolite, and data were calculated by using the following formula:  $z = (x - \mu)/\sigma$ , where  $x$  was the relative scaled abundance of the metabolite,  $\mu$  was mean of the scaled relative abundance for the metabolite across the 3 legumes, and  $\sigma$  was the scaled relative abundance SD of the same metabolite across the 3 legume foods. For each food, metabolite profiles were screened for compounds that could serve as biomarkers of legume intake. To be considered a potential biomarker in a given legume food, compounds needed to meet the following criteria: 1) be detected in only one legume food (no others) or 2) have a higher abundance in 1 legume food than in the 2 others, defined as having a  $z$  score of  $\geq 1$  with the other 2 foods having a  $z$  score of  $\leq -1$ .

## Results

### Comparison of targeted nutrients across legume foods

**Table 1** provides details on the macronutrient energy content, which was measured in kilocalories per 100 g, and the micronutrient content, which was measured in parts per million (ppm). All of the legume foods provided similar total energy content (374–391 kcal/100 g). Protein content was higher in CP and CB foods (26 and 23 g/100 g, respectively) than in the CSB (13 g/100 g). Similarly, dietary fiber was higher in CP and CB foods (21 and 28 g/100 g, respectively) than in the CSB (8 g/100 g). Insoluble fiber made up the majority of total dietary fiber. The CSB had twice as much fat (4 g/100 g) as CP and CB foods (2 g/100 g each).

**TABLE 1** Quantified macronutrients, micronutrients, FAs, and food safety profile for each legume food fed to children aged 6–36 mo<sup>1</sup>

Nutrient	Corn-soybean blend	Common bean	Cowpea
<b>Macronutrients</b>			
Energy, kcal/100 g	391	375	374
Carbohydrate, g/100 g	75	66	63
Protein, g/100 g	13	23	26
Fat, g/100 g	4	2	2
<b>Dietary fiber, g/100 g</b>			
Total	8	28	21
Insoluble	8	28	20
Soluble	ND	ND	1
<b>Micronutrients, ppm</b>			
Calcium	2230	2030	1040
Iron	133	117	152
Magnesium	1000	1870	1960
Manganese	10	21	17
Phosphorus	3950	4840	4380
Potassium	6230	15,800	15,900
Sodium	32	12	38
Zinc	39	27	31
<b>FAs,<sup>2</sup> %</b>			
<b>SFAs</b>			
Lauric (12:0)	ND	0.03	0.03
Myristic (14:0)	0.1	0.15	0.2
Pentadecanoic (15:0)	0.02	0.15	0.1
Palmitic (16:0)	12	16	25
Heptadecanoic (17:0)	0.1	0.2	0.1
Stearic (18:0)	3	2	4
Arachidic (20:0)	0.5	0.5	1
Behenic (22:0)	0.3	0.7	3
Tricosanoic (23:0)	ND	0.2	0.3
Lignoceric (24:0)	0.2	1	2
<b>MUFAs, %</b>			
Palmitoleic (16:1 <i>cis</i> )	0.1	0.2	0.1
10-Heptadecanoic (17:1)	0.05	0.07	n.d.
Oleic (18:1 <i>cis</i> )	27	8	8
11-Eicosenoic (20:1)	0.2	0.15	0.4
<b>PUFAs, %</b>			
Linoleic (18:2 <i>cis</i> )	52	29	34
$\alpha$ -Linolenic (18:3)	5	41	21
<b>Heavy metals, ppm</b>			
Arsenic	ND	ND	ND
Cadmium	ND	ND	ND
Lead	ND	ND	ND
Mercury	ND	ND	ND
<b>Aflatoxin, ppb</b>			
Aflatoxin B1	2.50	ND	ND
Aflatoxin B2	ND	ND	ND
Aflatoxin G1	2.67	ND	ND
Aflatoxin G2	ND	ND	ND
Total aflatoxin	5.17	ND	ND

<sup>1</sup> ND, not detected; ppb, parts per billion; ppm, parts per million.

<sup>2</sup> Percentages represent the amount of individual FAs detected in the total amount of FAs identified.

Micronutrient analysis showed that potassium was higher in CP and CB foods (15,900 and 15,800 ppm, respectively) than in the CSB (6230 ppm) food. Phosphorus was also higher in CP and CB foods (4380 and 4840 ppm) than in the CSB (3950 ppm) food. Iron was similar across the 3 groups (CP, CB, and CSB:

152, 117, and 133 ppm, respectively). The CSB food had the highest amounts of calcium (2230 ppm) and zinc (39 ppm) compared with the CP and CB foods. Furthermore, CP and CB foods had more magnesium (CP and CB: 1960 and 1870 ppm, respectively), phosphorus (4380 and 4840 ppm), and potassium (15,900 and 15,800 ppm) than the CSB food (magnesium, phosphorus, and potassium: 1000, 3950, and 6230 ppm, respectively).

An analysis of relative percentages for FA profiles, presented in Table 1, showed that the CP food had the highest amount of saturated fat, at 36%, followed by the CB (21%) and CSB (16%) foods. The SFAs that were highest in the CP food were palmitic acid (25%), stearic acid (4%), behenic acid (3%), and lignoceric acid (2%). The CSB food had the highest amount of MUFAs at 27%, with CB and CP foods both containing 9%. In each legume flour, oleic acid was the largest contributor to MUFA content and represented 8%, 8%, and 27% of total MUFA contents in CP, CB, and CSB foods, respectively. The CB food had the highest amount of PUFAs at 70%, with CSB and CP foods at 57% and 55%, respectively. Among PUFAs, linoleic acid (an n-6 FA) was highest in the CSB food at 52% of the total polyunsaturated fat content (compared with 34% and 29% in CP and CB foods, respectively) and  $\alpha$ -linolenic (an n-3 FA) was highest in the CB food at 41% (compared with 21% and 5% in CP and CSB foods, respectively). Butyric, caproic, caprylic, capric, tridecanoic, myristoleic, elaidic, linoleic,  $\gamma$ -linolenic, nonadecanoic, 11-14-eicosadienoic, homo- $\gamma$ -linolenic, 11-14-17-eicosatrienoic, arachidonic, eicosapentaenoic, heneicosanoic, erucic, docosadienoic, docosapentaenoic, docosahexaenoic, and nervonic acids were not detected in the foods.

### Comparison of daily nutrient intakes provided by legume foods for children

To evaluate how legumes may provide nutrition as complementary foods, Table 2 estimates the essential nutrients for children during weaning across the 3 legume foods, in grams per day, on the basis of their age in months, as well as the recommended levels of intake from the WHO and the National Academy of Medicine (34, 35). The energy needs from complementary foods for children receiving an average amount of breast milk range between 200 and 550 kcal/d (34). By using these energy recommendations, all of the legume foods provided ~40% of daily energy requirements for children aged 6–9 mo (78–118 kcal/d) and ~30–35% for children aged 12–24 mo (156–197 kcal/d).

The CB food provided 45–95% of recommended protein intake (5–12 g/d), 1–5% of recommended fat intake (0.4–1 g/d), and 60–80% of recommended total dietary fiber intake (6–15 g/d). The CP food provided 50–105% of recommended protein intake (5–14 g/d), 1–5% of recommended fat intake (0.4–1.1 g/d), and 45–55% of recommended total dietary fiber intake (4.3–10.8 g/d). The CSB food provided 25–50% of recommended protein intake (3–7 g/d), 3–10% of recommended fat intake (0.9–2.2 g/d), and 20% of recommended total dietary fiber intake (1.7–4.2 g/d).

The total amounts of iron provided by the legume foods were ~20–45% of the recommended level of 11 mg/d (6–9 mo of age) and increased to >70% of the recommended level of 7 mg/d (12–23 mo of age). At 24 mo, the amount of CP food provided reached the recommended levels of iron (8.0 g/d), with CSB and CB foods at

**TABLE 2** Daily nutrients delivered by 3 legumes as a complementary food on the basis of a weaning child's age and serving size<sup>1</sup>

Nutrient	Corn-soybean blend				Common bean				Cowpea				Recommended level of intake <sup>2</sup>			
	6 mo	9 mo	12 mo	24 mo	6 mo	9 mo	12 mo	24 mo	6 mo	9 mo	12 mo	24 mo	6 mo	9 mo	12 mo	24 mo
<b>Macronutrients</b>																
Energy, kcal/d	78	117	156	196	79	118	158	197	79	118	157	196	200 <sup>3</sup>	300 <sup>3</sup>	550 <sup>3</sup>	550 <sup>3</sup>
Carbohydrate, g/d	15	22	30	37	14	21	28	35	13	20	26	33	95	95	130	130
Protein, g/d	3	4	5	7	5	7	10	12	5	8	11	14	11	11	13	13
Fat, g/d	0.9	1.3	1.7	2.2	0.4	0.6	0.8	1.0	0.4	0.6	0.8	1.1	30	30	21	21
Dietary fiber																
Total fiber, g/d	1.7	2.5	3.4	4.2	6.0	9.0	12.0	15.0	4.3	6.5	8.6	10.8	ND	ND	19	19
<b>Micronutrients</b>																
Calcium, mg/d	45	67	89	112	43	64	85	107	22	33	44	55	260	260	700	700
Iron, mg/d	2.7	4.0	5.3	6.7	2.5	3.7	4.9	6.1	3.2	4.8	6.4	8.0	11	11	7	7
Potassium, g/d	0.1	0.2	0.2	0.3	0.3	0.5	0.7	0.8	0.3	0.5	0.7	0.8	0.7	0.7	3.0	3.0
Zinc, mg/d	0.8	1.2	1.5	1.9	0.6	0.9	1.1	1.4	0.7	1.0	1.3	1.6	3	3	3	3
<b>FAs, g/d</b>																
Total SFAs	0.03	0.05	0.06	0.08	0.04	0.07	0.09	0.11	0.08	0.11	0.15	0.19	NA	NA	NA	NA
Total MUFA	0.05	0.08	0.11	0.14	0.02	0.03	0.04	0.05	0.02	0.03	0.04	0.05	NA	NA	NA	NA
Total PUFA	0.11	0.17	0.23	0.28	0.15	0.22	0.30	0.37	0.12	0.17	0.23	0.29	5.1	5.1	7.7	7.7
Linoleic acid	0.10	0.15	0.21	0.26	0.06	0.09	0.12	0.15	0.07	0.11	0.14	0.18	4.6	4.6	7	7
$\alpha$ -Linolenic acid	0.01	0.02	0.02	0.03	0.09	0.13	0.17	0.22	0.04	0.07	0.09	0.11	0.5	0.5	0.7	0.7

<sup>1</sup>NA, not applicable; ND, not detected.<sup>2</sup>Based on the WHO and Institute of Medicine DRIs (34, 35). The recommendations for the corn-soybean blend based on age were as follows: 20 g/d (6–8 mo), 30 g/d (9–11 mo), 40 g/d (12–23 mo), and 50 g/d (24–35 mo).<sup>3</sup>Recommended energy needs of breastfed children from complementary foods (34).

90% (6.7 and 6.1 g/d, respectively). The intake of zinc between the ages of 6 and 9 mo was ~20–40% of the recommended level of 3 mg/d and increased to 40–65% between ages 12 and 24 mo. The CSB food provided the highest amount of zinc at 24 mo (1.9 g/d).

### Food safety analysis

Table 1 shows amounts of heavy metals and aflatoxins detected in the legume foods. Arsenic, cadmium, lead, and mercury were not detected in any of the legume foods. Aflatoxin was only detected in the CSB food at a cumulative concentration of 5.2 parts per billion (ppb).

### Nontargeted food metabolome of legume foods

A total of 652 metabolites were identified collectively across all 3 legume foods and were further organized across 8 classifications, including amino acids, carbohydrates, cofactors and vitamins, energy metabolism, lipids, nucleotides, peptides, and xenobiotics (benzoate metabolism, chemical, drug, and food and plant component). Of the total food metabolome, there were 509 food metabolites identified from CP food, 483 metabolites identified from CB food, and 443 metabolites identified from CSB food (Table 3). Most food metabolites came from amino acid and lipid metabolic pathways (25% and 37%, respectively), and 46–56 metabolites were classified as phytochemicals, indicating they were previously documented as being plant-derived. profile, listing individual metabolites, their associated metabolic pathways, relative abundance, and detection methods, is reported for the 3 foods in Supplemental Table 1.

The Venn diagram shown in Figure 1 illustrates the 396 metabolites out of the 652 total metabolites that were identified in all 3 legume-based foods. This figure also shows the number of metabolites detected between 2 foods or that were present only in a single legume food. This included 23 metabolites identified only in CSB food, 14 metabolites distinctly present in CB food, and 36 uniquely present in CP food. The food metabolites unique to each dietary legume are listed in Table 4, which presents potential biomarkers of intake for each food and are clustered by their respective metabolic pathways. For example, arabinose was only identified from the CSB food. Of the metabolites listed in Table 4, those that had evidence of anti-inflammatory activity included putrescine, ribose, riboflavin (vitamin B-2),  $\alpha$ -tocotrienol, and  $\gamma$ -tocotrienol in the CSB food; serotonin, dimethylglycine, and oleonic acid in the CB food; and hypotaurine, dihydroquercetin (taxifolin), eriodictyol, quercetin 3-galactoside, quercetin 3-glucoside, and quercetin in the CP food (36–42, 44–48).

Figure 2 shows the standard distributions of select metabolites across the amino acid, cofactor and vitamin, FA, and phytochemical pathways. A distinct profile of amino acids across the 3 foods is shown in Figure 2A. CB and CP foods had relative abundance  $z$  scores above the mean abundance across all legumes for all 9 essential amino acids (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine), whereas the CSB relative abundance  $z$  scores were all below the mean. In addition, pipercolic acid and *S*-methylcysteine, common non-protein nitrogen components of *Phaseolus* and *Vigna* species (42), were detected in all 3 foods and had the highest scaled relative abundance in the CB food compared with the CSB and CP foods (Figure 2A).

**TABLE 3** The 652 food metabolites identified from 3 complementary legume foods classified by metabolic pathways

	Number of identified metabolites		
	Corn-soybean blend	Common bean	Cowpea
Metabolic pathways			
Amino acid	114	121	128
Carbohydrate	26	26	26
Cofactors and vitamins	24	19	20
Energy	12	12	13
Lipid	164	179	183
Nucleotide	32	49	47
Peptide	12	16	22
Xenobiotic			
Benzoate metabolism	3	4	4
Chemical	7	7	7
Drug	3	3	3
Food and plant components	46	47	56
Total number of metabolites	443	483	509

Figure 2B shows the distinct profile of FAs identified from the nontargeted metabolomic analysis. Similar to the amino acid metabolite profile, CB and CP foods had relative abundance  $z$  scores above the mean scaled abundance across all legumes for a majority of the FAs. Caprylic acid, capric acid, nonadecanoic acid, and erucic acid were not included in the standard targeted FA analysis, yet were identified by using the nontargeted food metabolome approach. SFAs, including palmitic, stearic, and behenic acids, had the highest relative abundance  $z$  scores in the CP food, which was confirmed to match results from the targeted FA analysis (Table 1). Further targeted metabolomic analyses will be needed to quantify FA amounts in these foods for comparison with the FA amounts reported in Tables 1 and 2.

There were 19 cofactor and vitamin metabolites identified by using metabolomics in the 3 foods. The distinct profile of cofactors and vitamins inherent to each food is highlighted in Figure 2C. When compared with CS and CSB foods, the CP food showed the highest relative abundance  $z$  scores for the B-vitamin metabolites nicotinate (vitamin B-3), trigonelline, and pantothenate (vitamin B-5). The CSB food had the highest relative abundance  $z$  scores for the vitamin B metabolites nicotinamide (vitamin B-3), thiamin (vitamin B-1), and pyridoxine (vitamin B-6) when compared with CP and CB foods. The CSB food also had the highest relative abundance  $z$  scores for ascorbate (vitamin C) and  $\alpha$ -tocopherol (vitamin E) when compared with CP and CB foods. Other vitamin E components, including  $\delta$ -tocopherol and  $\gamma$ -tocopherol or  $\beta$ -tocopherol, showed the highest relative abundance  $z$  scores in the CP food compared with the CS and CSB foods.

Figure 2D shows the distinct profile of all 34 phytochemicals from all 3 foods. Genistein, an isoflavone found primarily in soybeans, was detected in all 3 foods, and the CSB food had the highest relative abundance  $z$  score for genistein compared with the CP and CS foods. Ferulic acid, another abundant phenolic acid in various legumes (12, 49), had the highest relative abundance  $z$  score in the CP food when compared with the CB and CSB foods. Across

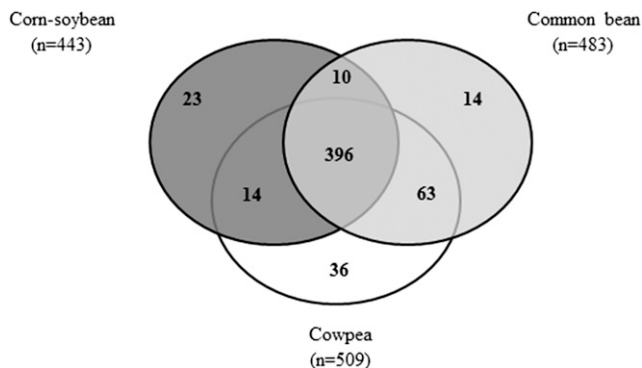
all foods, no additional biomarkers were identified as having a  $z$  score  $\geq 1$  in 1 food while having a  $z$  score of  $-1$  or lower in the other 2 foods.

## Discussion

This study assessed the potential nutritional value of 3 dry-roasted, legume-based complementary foods available to children living in rural Malawi via the integration of targeted nutrient and food safety assessments and global, nontargeted food metabolite profiling. Although a CSB is the traditional complementary food recommended to treat childhood malnutrition in Malawi (50, 51), the nutrient, food safety screening, and metabolite analysis presented herein supports future investigation of alternative legumes, specifically CPs and CBs, as complementary foods.

This targeted nutrient analysis showed that all diets provided similar total energy contents, yet CB and CP foods had higher amounts of dietary fiber and protein (Table 1). The CB and CP foods contributed an estimated 45–80% of the DRI for dietary fiber for these children, compared with 20% in the CSB (Table 2). This finding is similar to a previous report on dietary fiber in legumes (11), which supports that CB and CP foods could serve as high-quality sources of dietary fiber in weaning children. Dietary fiber modulates nutrient absorption and increases fermentation by the beneficial colonic microflora, which can lead to improved gut barrier function and decreased inflammation to reduce the risk of developing EED (10). The high amounts of dietary fiber in CB and CP foods, when compared with the CSB food, support their future potential as additional fiber-rich complementary foods to promote gut health in weaning children.

Table 2 further showed how much the 3 legume foods contributed to recommended daily intakes for key nutrients based on the weaning child's age and needs for complementary foods (34, 35). CB and CP foods provided a higher amount of protein per serving than did the CSB food and provided  $\sim 50$ –75% of the recommended intake in children aged 6–9 mo and 80–100% in children aged 12–24 mo (Table 2). Nontargeted metabolite profiling further showed that the CB and CP foods contained a higher abundance of multiple essential amino acids than the CSB food, including

**FIGURE 1** Venn diagram of the total number of metabolites detected across the 3 legume foods.

**TABLE 4** Potential dietary biomarkers of intake by metabolic pathway for 3 complementary legume foods<sup>1</sup>

Metabolic pathways	Dietary biomarker		
	Corn-soybean blend	Common bean	Cowpea
Amino acid	Putrescine <sup>2</sup> (36), argininosuccinate	3-Methoxytyramine, serotonin <sup>2</sup> (37), tryptophan betaine, dimethylglycine <sup>2</sup> (38)	Ophthalmate, S-methylglutathione, 2-methylbutyrylcarnitine (C5), 3-hydroxy-2-ethylpropionate, 3-hydroxyisobutyrate, 3-methyl-2-oxovalerate, hypotaurine <sup>2</sup> (39), N-acetyltaurine
Carbohydrate	N-acetylglucosamine/N-acetylgalactosamine, arabinose, <sup>3</sup> ribose <sup>2</sup> (40), ribulose/xylulose	—	N-acetyl-glucosamine-1-phosphate
Cofactors and vitamins	Dehydroascorbate, riboflavin (vitamin B-2) <sup>2</sup> (41), $\alpha$ -tocopherol acetate, $\alpha$ -tocotrienol <sup>2,3</sup> (42, 43), $\gamma$ -tocotrienol <sup>2,3</sup> (42, 43)	—	FMN
Energy	Succinylcarnitine	—	—
Lipid	Caproate (6:0), glycerophosphoserine, valerate (5:0)	Diacylglycerol (12:0/18:1, 14:0/16:1, 16:0/14:1), propionylcarnitine (C3), 1-pentadecanoylglycerol (15:0)	Palmitoyl-oleoyl-glycerol (16:0/18:1), adipate, nervonate (24:1n-9), 1-myristoylglycerol (14:0), 1-palmitoyl-2-arachidonoyl-GPE (16:0/20:4), docosadienoate (22:2n-6), N-palmitoyl-sphingosine (d18:1/16:0), stearoyl sphingomyelin (d18:1/18:0)
Nucleotide	—	2'-GMP, 3'-CMP, thymine 3'-UMP	3'-GMP, N2,N2-dimethylguanosine
Peptide	—	Leucylalanine, phenylalanylalanine	Alanylleucine, glycylleucine, lysylleucine, valylglutamine, valylleucine, $\gamma$ -glutamyl- $\alpha$ -lysine, $\gamma$ -glutamylglycine
Xenobiotic	1,1-Kestotetraose, 2-oxindole-3-acetate, chlorogenate, coumaroylquinates 2-5, feruloylputrescine	Oleanolic acid <sup>2,3</sup> (44)	Benzoate, dihydroquercetin (taxifolin) <sup>2</sup> (45), eriocitrin, eriodictyol <sup>2</sup> (46), galacturonate, quercetin 3-galactoside <sup>2,3</sup> (47, 48), quercetin 3-glucoside <sup>2,3</sup> (48), quercetin <sup>2,3</sup> (48), secoisolariciresinol

<sup>1</sup>A total of 24 metabolites were solely detected in the corn-soybean blend, 14 metabolites were unique in the common bean, and 36 metabolites in the cowpea.

<sup>2</sup>Metabolite with evidence of anti-inflammatory activity.

<sup>3</sup>Metabolite that has been previously identified in soybean, common bean, or cowpea and considered a strong potential dietary biomarker for these legume foods.

histidine, isoleucine, leucine, methionine, phenylalanine, threonine, tryptophan, and threonine, which supports its utility in balanced nutrition approaches. High-quality proteins with balanced amino acid profiles, such as those found in CB and CP foods, are important for proper gut barrier and immune system development (52). Given that EED both derives from and contributes to a disturbed microbiome and a dysfunctional gut immune system (53), the high protein content of dry-roasted CB and CP proteins merits further investigation for their use as alternative complementary foods in rural Malawian infants.

Micronutrient analyses confirmed that all of the legume foods naturally provided many essential micronutrients that are important to overall child nutritional status and growth. The CSB and CP foods provided a rich source of both iron and zinc compared with the CB food (Table 1) without being additionally fortified. Iron intake is essential for redox reactions, gene regulation, oxygen delivery to tissues, and cell growth (54, 55), and Malawian children and pregnant and breastfeeding mothers are commonly deficient (56). Efforts have been put forth to increase iron intake in rural Malawian children (57), but elemental iron supplementation increased diarrhea risk and did not reduce EED risk or growth stunting (58). Legume foods, such as CSB and CP foods,

could potentially serve as novel food-based strategies that can be investigated for their effectiveness in increasing total iron intake. Zinc is an essential micronutrient that is also commonly deficient in rural Malawian children (57). Evidence supports a role for zinc to reduce the duration and severity of diarrhea episodes, and it may protect against EED by restoring gut mucosal barrier integrity and by bolstering antibody production against enteric pathogens (59). Consequently, CP and CSB foods may additionally merit attention as a rich source of zinc to improve deficiencies.

Trypsin inhibitors, amylase inhibitors, phytates, and phytoestrogens are traditionally regarded as antinutrients that naturally occur in legumes (60, 61), and concern has been raised that their presence in the diets of young children may reduce micronutrient absorption (62), interfere with protein and carbohydrate digestion, and disrupt estrogen metabolism (63, 64). However, boiling of whole legume seeds, the most common method of preparation for consumption, reduces the activity of these enzymes by ~20-fold to amounts below nutritional significance (65). Decreases in these antinutrient enzymes, as well as in phytoestrogens, were also observed during dry-roasting (66), which was performed locally during food preparation. Moreover, a growing body of research supports that chronic exposure by Malawian children to





Given that these legume foods were harvested and prepared locally, the lack of high amounts of heavy metals and aflatoxins in all of the foods supports their future use as safe complementary foods.

Metabolomic analysis provided an opportunity to quantify, identify, and compare food components and metabolites from the 3 different legume-based foods (Supplemental Table 1, Table 4). Our nontargeted food metabolomics approach successfully identified lipids, amino acids, and other phytochemicals that are established biomarkers or that merit further examination as potential dietary biomarkers of human dietary legume consumption. In all legumes, many of these biomarkers were also associated with intestinal health benefits. Among the 14 potential and identified biomarkers in the CB food was pipercolic acid (Table 4), an established biomarker of legume intake (76). The CB food also contained the potential biomarker oleanolic acid (Table 4), Oleanolic acid, which is the primary MUFA in all legume foods, was isolated in numerous food and medicinal plants and is known to exert anti-inflammatory actions (77). EED is marked by increased oxidative damage to the gut, and by contributing bioactive lipids such as oleanolic acid, CBs may function as a complementary food that could compensate for EED-associated dietary deficiencies and oxidative damage and protect infants against further pathogenesis.

The CP food contained 36 food biomarkers, and among these were quercetin and serotonin (Table 4), which both have established bioactivities in the gut. Quercetin, which was 1 of 14 compounds uniquely detected in the CP food, is a flavonol with natural immunomodulatory and antioxidative properties (78). Quercetin has been shown to control intestinal inflammation via modulation of leukocytes (79) and could therefore be useful in supporting a healthy immune system to lower EED risk. Similarly, serotonin acts on receptors along the intestinal tract to modulate inflammatory responses (37) and thus may be important for maintaining a normal, healthy gut barrier.

Of the 23 biomarkers identified in the CSB, arabinose and  $\alpha$ - and  $\gamma$ -tocopherol (Table 4) have been previously reported in soybean literature (43). Arabinose may be converted into short-chain FAs by bacteria to support healthy enterocyte metabolism, bolster gut mucosal immunity, and beneficially modulate the microbiome (80–82).  $\alpha$ - and  $\gamma$ -Tocopherol possess broad-spectrum antioxidant and immunomodulatory activities (42). As antioxidants, these compounds scavenge free radicals and help prevent cell membrane oxidation (83). Furthermore, as immune-modulators,  $\alpha$ - and  $\gamma$ -tocopherols have been shown to be protective against chronic inflammation, in part via modulation of T helper 2-lymphocyte populations and also by the reduction of the inflammatory mediator PGE2 and the associated enzyme cyclo-oxygenase 2 (42), supporting their use as beneficial immune-modulators in EED protection.

Findings reported herein establish that CP, CB, and CSB legume-based complementary foods are rich in macro- and micro-nutrients, are safe, and have profiles abundant in bioactive small compounds that may have utility as alternative complementary foods for use during weaning. Incorporating a food-omics approach allowed for an in-depth understanding of the variability between metabolites present in the 3 legume foods, especially in

amounts of metabolites that are associated with potential EED prevention in children. This approach also assisted in identifying dietary biomarkers associated with legume foods, which will be useful to assess compliance to different legume food interventions in future dietary clinical trials with these diets. In conclusion, this legume food analysis can guide the future selection of locally available, whole staple foods that are nutritionally adequate and rich in micronutrients and bioactive metabolites that improve health in a population at high risk of EED and stunting.

### Acknowledgments

We thank Theresa N Ngoma, Ulemu K Chimimba, and Nicole S Benzoni for help in developing the initial recipes used to prepare the legume foods, as well as Iman Zarei for internal review of this manuscript before submission. The authors' responsibilities were as follows—ECB, IT, KMM, MJM, and EPR: designed the research; ECB, LZ, and NJN: analyzed the data; ECB and LZ: wrote the first draft of the manuscript; MJM and EPR: had responsibility for the final content; and all authors: read and approved the final manuscript.

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