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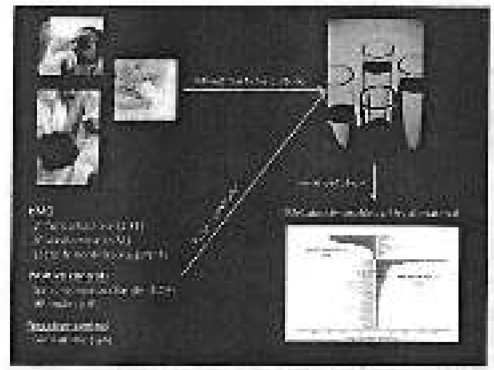
Fecal Metabolomics of Healthy Breast-Fed versus Formula-Fed Infants before and during in Vitro Batch Culture Fermentation

JoMay Chow,[†] Matthew R. Panasevich,[‡] Danny Alexander,[§] Brittany M. Vester Boler,[‡] Mariana C. Rossoni Serao,[‡] Trevor A. Faber,[‡] Laura L. Bauer,[‡] and George C. Fahey*[,‡]

[†]Abbott Nutrition, Columbus, Ohio 43219, United States

[‡]Department of Animal Sciences, University of Illinois, Urbana, Illinois 61801, United States

[§]Metabolon, Inc., Durham, North Carolina 27713, United States



ABSTRACT: Nontargeted metabolomics analyses were used (1) to compare fecal metabolite profiles of healthy breast-fed (BF) and formula-fed (FF) infants before and during in vitro fermentation in batch culture and (2) to evaluate fecal metabolomics in infant diet. Samples from healthy BF (n = 4) or FF (n = 4) infants were individually incubated at 37 °C in anaerobic media containing 1% (wt/vol) galactooligosaccharides, 6′-sialyllactose, 2′-fucosyllactose, lacto-N-neotetraose, inulin, and gum arabic for up to 6 h, and supernatants were analyzed using GC/MS and LC/MS/MS to assess changes in various compounds. Comparison of over 250 metabolites prior to incubation showed that BF samples contained higher relative concentrations ($P \leq 0.05$) of 14 compounds including but not limited to human milk oligosaccharides and other metabolites presumably transferred through breast feeding (linoelaidate, myo-inositol) ($P \leq 0.05$). Conversely, feces from FF infants contained 41 identified metabolites at higher levels ($P \leq 0.05$). Our data are consistent with the notion that carbon-limited cultures catabolize protein and amino acids to obtain energy, whereas the provision of fermentable carbohydrate creates anabolic conditions relying on amino acids for bacterial growth. Results also suggest that fecal metabolomics can be a useful tool for studying interactions among diet, microbes, and host.

KEYWORDS: *infant, feces, metabolomics, metabonomics, oligosaccharides, fermentation*

■ INTRODUCTION

The human gut microbiota play a crucial role in shaping infant health and development. Abnormal patterns of gut microbiota have been clinically linked to late-onset sepsis[1] and necrotizing enterocolitis,[2] two important causes of morbidity and mortality in preterm infants. A growing body of evidence also implicates the intestinal microbiota in the development of colic,[3] allergic disease,[4] and obesity later in life[5] as well as the formation of a key metabolite involved in melamine-induced kidney stone formation.[6] Indeed, the gut microbiota modulate brain development and subsequent adult behavior in animal models.[7]

The composition of the infant gut microbiome is profoundly affected by diet. In general, studies using molecular techniques indicate that a majority of breast- and formula-fed infants harbor significant numbers of bifidobacteria.[8] Feces from exclusively breast-fed infants, however, are dominated by bifidobacteria, whereas those from formula-fed infants also contain other bacterial species, including *E. coli*, *Clostridium difficile*, bacteroides, and lactobacilli.[8,9]

Despite the abundance of studies regarding the effect of diet on infant fecal microbiota and health,[10] relatively little attention has been paid to the influence of diet on the infant fecal metabolome.[11−13] The metabolomics approach, which can sometimes involve the analysis and identification of thousands of small metabolites, offers the potential to gain insight into the complex interactions among gut microbes, diet, and host. In turn, these data may be used to gain a clearer understanding of how diet affects both the gut microbiota and the host and to identify potential areas for future research. Overall, the main objectives of the current study were to use nontargeted metabolomics analysis of fecal samples to (1) compare metabolite profiles of healthy breast-fed to healthy formula-fed infants before and during in vitro fermentation in batch culture and (2) to evaluate the usefulness of metabolomics in assessing the infant diet.

■ MATERIALS AND METHODS

Donors

The infant fecal samples used were collected for use in a previous study.[14] Briefly, eight infants were enrolled for participation in this study between March and June of 2010 from the Champaign-Urbana area, and infants consumed their normal diet of exclusive breast milk (n = 4) or exclusive infant formula (n = 4) for at least 2 months immediately prior to fecal collection. Formula-fed infants were fed one of three lactose-based formulas: Similac Advance (Abbott Laboratories Columbus,

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OH; n = 1), Member's Mark Infant formula (Sam's Club, Bentonville, AR; n = 1), or Enfamil Premium (Mead Johnson, Glenview, IL; n = 2). These formulas contained as much as 4 g/L galactooligosaccharides (GOS), but they did not contain maltodextrins, probiotics, or added oligosaccharides other than GOS. Other inclusion/exclusion criteria included the following: the infant was full term at birth with a gestational age of 38 to 42 wk; the infant was at or above the fifth percentile for weight at birth; the infant had no maternal medical history of diabetes, tuberculosis, or perinatal infection with proven adverse effects on the fetus; were vaginal births; were at least 2 mo of age at study entry but not older than 4 mo of age; had no known cardiac, respiratory, gastrointestinal, or other systemic disease such as urinary tract infection or otitis media; had no history of blood group incompatibility serious enough to result in hematological problems; and were not receiving any medications (except for supplemental vitamins) and have never received antibiotics. The experimental protocol was approved by the University of Illinois Institutional Review Board, and all legally acceptable representatives signed an informed consent prior to initiation of the experiment.

Substrates

Substrates used included galactooligosaccharides (GOS) 95 (GOS; Inalco Pharmaceuticals, Italy), α-(2−6′)-*N*-acetylneuraminyl-lactose sodium salt (6′-sialyllactose) (6′SL; Inalco Pharmaceuticals, Italy); 2′- α-L-fucopyranosyl-D-lactose (2′-fucosyllactose) (2′FL; Inalco Pharmaceuticals, Italy); lacto-*N*-neotetraose (LNnT; Boehringer Mannheim, Germany); Orafti HP inulin (HP; BENEO-Orafti, Belgium); and gum arabic (GA; Fisher Scientific, Pittsburgh, PA).

Fecal Collection

On the day of the in vitro experiments, fecal samples were collected in diapers within 15 min of defecation. Diapers were double-sealed in plastic bags and transported to the laboratory in a cooler containing tepid water. Fecal samples were diluted 1:10 (wt/vol) in anaerobic diluent, homogenized for 15 s in a Waring blender, filtered through four layers of cheesecloth, and sealed in serum bottles under CO_2. Serum bottles containing the inocula were stored at 37 °C until use.

In Vitro Fermentation and Sample Collection

Each substrate (80 mg) was weighed in triplicate for each infant at each sampling time into 16 mL Balch tubes. An aliquot (7.2 mL) of media (Table 1) was aseptically transferred into the Balch tubes, capped with butyl rubber stoppers, and sealed with aluminum caps. Tubes containing HP and GA were stored with media at 4 °C for approximately 12 h to enable hydration of the substrates before initiating fermentation. These tubes were placed in a 37 °C water bath approximately 30 min before inoculation. Due to the limited supply of substrates and unpredictability associated with obtaining fecal samples from infants, tubes containing GOS, 6′SL, 2′FL, and LNnT were hydrated upon obtaining fecal samples. Fecal samples from the individual infants were also incubated in media without added carbohydrate, and these cultures are referred to as "blank" tubes. Supernatant samples were collected at 0, 3, and 6 h of fermentation and were immediately frozen at −80 °C until further analysis.

Metabolomic Profiling

Frozen samples were shipped under dry ice to a commercial laboratory (Metabolon, Durham, NC) for metabolite analysis. Procedures for metabolic profiling have been described previously for the three platforms used in combination for the analysis, including GC/MS[15] and two LC/MS systems,[16] one optimized for positive ionization and one optimized for negative ionization. Peak extraction, data curation, and QC procedures were performed using proprietary software.[17,18] At the time of analysis, samples were thawed and prepared according to the described standard methanol extraction protocol, which is designed to remove proteins, dislodge small molecules bound to protein or physically trapped in the precipitated protein matrix, recover a wide range of chemically diverse metabolites, and split into aliquots for analysis on the three platforms.

Table 1. Composition of Microbiological Medium Used in the in Vitro Experiment

component	concentration in medium
	mL/L
solution A[a]	330.0
solution B[b]	330.0
trace mineral solution[c]	10.0
water-soluble vitamin solution[d]	20.0
folate/biotin solution[e]	5.0
riboflavin solution[f]	5.0
hemin solution[g]	2.5
resazurin[h]	1.0
distilled H_2O	296.1
	g/L
Na_2CO_3	4.0
cysteine HCl-H_2O	0.5
trypticase	0.5
yeast extract	0.5

[a]Composition (g/L): NaCl, 5.4; KH_2PO_4, 2.7; $CaCl_2$-H_2O, 0.16; $MgCl_2$-$6H_2O$, 0.12; $MnCl_2$-$4H_2O$, 0.06; $CoCl_2$-$6H_2O$, 0.06; $(NH_4)_2SO_4$, 5.4. [b]Composition (g/L): K_2HPO_4, 2.7. [c]Composition (mg/L): ethylenediaminetetraacetic acid (disodium salt), 500; $FeSO_4$-$7H_2O$, 200; $ZnSO_4$-$7H_2O$, 10; $MnCl_2$-$4H_2O$, 3; H_3PO_4, 30; $CoCl_2$-$6H_2O$, 20; $CuCl_2$-$2H_2O$, 1; $NiCl_2$-$6H_2O$, 2; Na_2MoO_4-$2H_2O$, 3. [d]Composition (mg/L): thiamin-HCl, 100; *d*-pantothenic acid, 100; niacin, 100; pyridoxine, 100; *p*-aminobenzoic acid, 5; vitamin B_{12}, 0.25. [e]Composition (mg/L): folic acid, 10; *d*-biotin, 2; NH_4HCO_3, 100. [f]Composition: riboflavin, 10 mg/mL in 5 mmol/L of Hepes. [g]Composition: hemin, 500 mg/mL in 10 mmol/L of NaOH. [h]Composition: resazurin, 1 g/L in distilled H_2O.

Data Normalization

Data were collected over multiple platform run days and were adjusted by scaling to the median values for each group-balanced run-day block for each individual compound. This minimizes any interday instrument gain or drift but does not interfere with intraday sample variability. Data were not otherwise adjusted or normalized.

Statistical Analysis

Repeated measures ANOVA was performed to leverage the data from the multiple time points within the study. There were three tests embedded within the repeated measures ANOVA: (1) DIET Main, (2) TIME Main, and (3) Interaction (DIET × MAIN). Essentially, the "DIET" Main effect tested whether the means of the three groups were different from the BLANK when averaged across all time points. The "TIME" Main effect examines whether the means at each time point were different when averaged across the groups. Finally, the "Interaction" asks whether the time profiles are nonparallel between the groups (nonparallel profiles signify a difference during the time-course between the groups). The calculations also took into account the repeated measure inherent in sampling at each time point the cultures from individual infants.

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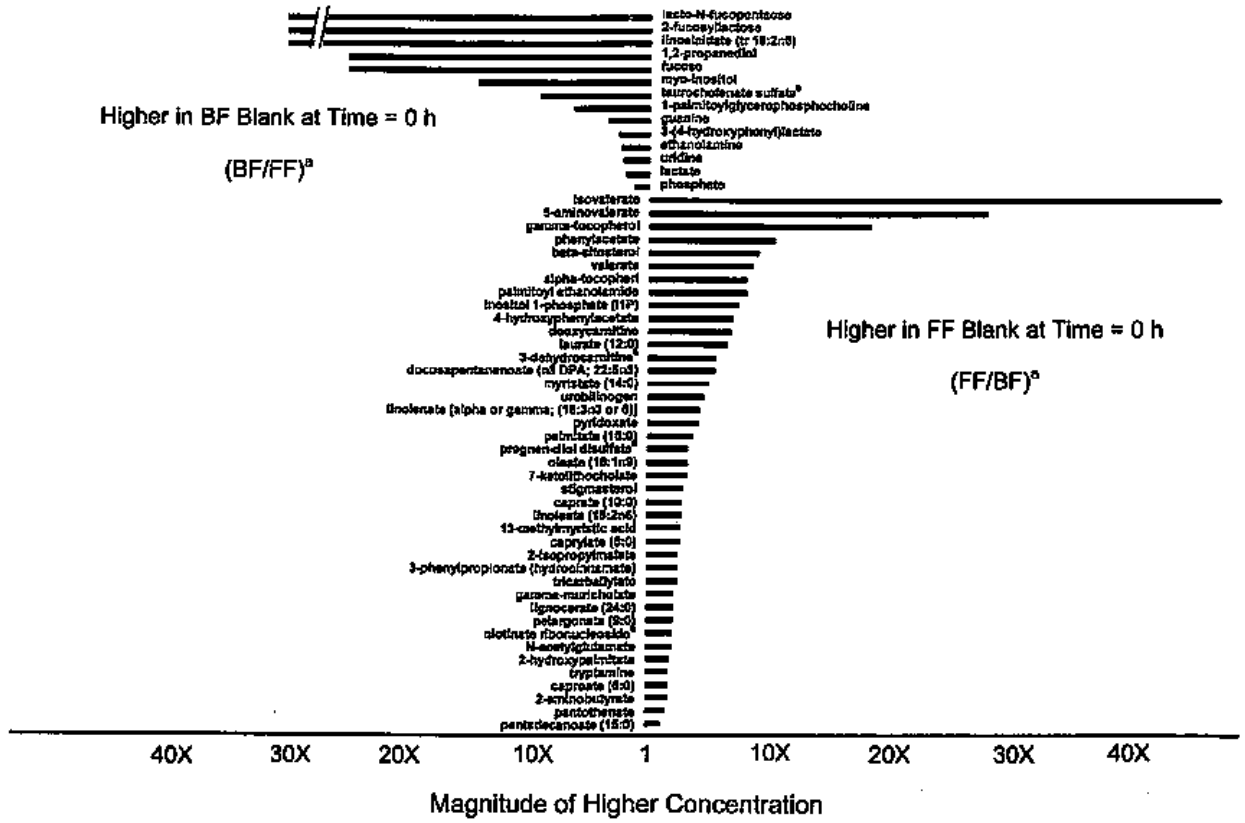

Figure 1. Ratios, as indicated, of breast-fed (BF) and formula-fed (FF) group means for compounds that are significantly different between fecal supernatants at time = 0 in the absence of added carbohydrate substrates (blank). (a) BF/FF = breast-fed to formula-fed and FF/BF = formula-fed to breast-fed. (b) Indicates compound that has not been officially "plexed" (based on a standard), but we are confident in its identity.

Table 2. Metabolic Pathway Classification and Fold-Change Values for Compounds Significantly Higher in Blank BF Samples at Time = 0 h

super-pathway	sub-pathway	biochemical name	BF/FF[a]
amino acid	phenylalanine and tyrosine metabolism	3-(4-hydroxyphenyl)lactate	2.56
carbohydrate	fructose, mannose, galactose, starch, and sucrose metabolism	fucose	25.00
		2-fucosyllactose	>30.00
		lacto-*N*-fucopentaose	>30.00
	glycolysis, gluconeogenesis, pyruvate metabolism	lactate	1.96
	ketone bodies	1,2-propanediol	25.00
energy	oxidative phosphorylation	phosphate	1.25
lipid	long-chain fatty acid	linoelaidate (tr 18:2*n*6)	>30.00
	bile acid metabolism	taurocholenate sulfate[b]	9.09
	glycerolipid metabolism	ethanolamine	2.33
	inositol metabolism	myo-inositol	14.29
	lysolipid	1-palmitoylglycerophosphocholine	6.25
nucleotide	purine metabolism, guanine containing	guanine	3.45
	pyrimidine metabolism, uracil containing	uridine	2.13

[a]BF/FF = breast-fed to formula-fed. [b]Indicates compound that has not been officially "plexed" (based on a standard), but we are confident in its identity.

Missing values (if any) were assumed to be below the level of detection for a particular biochemical with the instrumentation used and were imputed with the observed minimum for that particular biochemical. Welch's Two-Sample *t* tests were used to analyze fold differences between FF and BF groups. For all analyses, missing values were imputed with the observed minimum for that particular compound (imputed values were added after block normalization). The statistical analyses were performed on natural log-transformed data to reduce the effect of any potential outliers in the data. Welch's Two-Sample *t* test comparisons were made between the means of each biochemical using statistical software: Array Studio (Omicsoft, Inc.) or "R" from the Free Software Foundation, Inc. Significance was determined by $P \leq 0.05$.

RESULTS

Comparison of Initial Conditions

Figure 1 displays fold differences of identified metabolites that were present at higher levels ($P \leq 0.05$) in either the BF or in the

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Table 3. Metabolic Pathway Classification and Fold-Change Values for Compounds Significantly Higher in Blank FF Samples at Time = 0 h

super-pathway	sub-pathway	biochemical name	FF/BF[a]
amino acid	glutamate metabolism	*N*-acetylglutamate	2.08
	phenylalanine and tyrosine metabolism	phenylacetate	10.39
		4-hydroxyphenylacetate	6.91
		3-phenylpropionate (hydrocinnamate)	2.52
	tryptophan metabolism	tryptamine	1.85
	urea cycle, arginine and proline metabolism	5-aminovalerate	27.89
	butanoate metabolism	2-aminobutyrate	1.78
carbohydrate	glycolysis, gluconeogenesis, pyruvate metabolism	2-isopropylmalate	2.53
energy	Krebs cycle	tricarballylate	2.48
lipid	essential fatty acid	linoleate (18:2*n*6)	2.87
		linolenate [alpha or gamma; (18:3n3 or 6)]	4.23
		docosapentaenoate (*n*3 DPA; 22:5*n*3)	5.50
	short-chain fatty acid	valerate	8.52
		isovalerate	46.99
	medium-chain fatty acid	caproate (6:0)	1.79
		caprylate (8:0)	2.68
		pelargonate (9:0)	2.19
		caprate (10:0)	2.87
		laurate (12:0)	6.49
	long-chain fatty acid	myristate (14:0)	4.94
		pentadecanoate (15:0)	1.18
		palmitate (16:0)	3.75
		oleate (18:1n9)	3.34
		lignocerate (24:0)	2.20
	fatty acid, monohydroxy	2-hydroxypalmitate	1.89
	fatty acid, branched	13-methylmyristic acid	2.76
	endocannabinoid	palmitoyl ethanolamide	8.00
	carnitine metabolism	deoxycarnitine	6.82
		3-dehydrocarnitine[b]	5.51
	bile acid metabolism	7-ketolithocholate	3.27
		gamma-muricholate	2.26
	inositol metabolism	inositol 1-phosphate (I1P)	7.34
	sterol/steroid	beta-sitosterol	9.02
		stigmasterol	2.97
		pregnen-diol disulfate[b]	3.35
cofactors and vitamins	hemoglobin and porphyrin metabolism	urobilinogen	4.63
	nicotinate and nicotinamide metabolism	nicotinate ribonucleoside[b]	2.16
	pantothenate and CoA metabolism	pantothenate	1.61
	tocopherol metabolism	alpha-tocopherol	8.05
		gamma-tocopherol	18.36
	vitamin B6 metabolism	pyridoxate	4.19

[a]FF/BF = formula-fed to breast-fed. [b]Indicates compound that has not been officially "plexed" (based on a standard), but we are confident in its identity.

FF groups. These differences were calculated by comparing the levels of the various metabolites in the blank tubes at time = 0. Since the contents of the blank tubes were identical except for the fecal samples from the individual infants, any differences in the levels of the various metabolites between the groups presumably represent differences in the relative levels of fecal metabolites between the two diet groups. Samples from BF infants contained significantly greater concentrations of 14 compounds compared to FF infants. As expected, supernatants from BF infants had greater levels of the human milk oligosaccharides (HMO), 2′-fucosyllactose and lacto-*N*-fucopentaose, and the HMO precursor, fucose, than those from FF infants. These two oligosaccharides were not detected in FF samples, but based on detection limits, these values must have been at least 25-fold higher in stool samples from BF infants (Table 2). Furthermore, time = 0 samples from BF infants were higher in a single amino acid super-pathway-related compound, [3-(4-hydroxyphenyl) lactate], a metabolite of tyrosine. Samples from the BF infants also were higher in five lipid super-pathway-related compounds (Table 2) and were particularly elevated in the C18:2, *n*-6 trans fatty acid, linoelaidate (>30-fold) and in the cyclic alcohol structural membrane component, myo-inositol (14.29-fold).

In contrast, supernatants from FF infants contained a total of 41 identified metabolites that were present at significantly higher concentrations compared to those from BF infants (Figure 1). More specifically, samples from FF infants contained seven compounds related to the amino acid super-pathway, a total of two compounds related to carbohydrate and/or energy super-pathways, 26 compounds related to the lipid super-pathway, and seven compounds related to cofactor and/or vitamin

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super-pathways that were present at greater concentrations than those from BF infants (Table 3). The amino acid super-pathway related compounds that were particularly elevated in FF infants included the amino acid catabolites: phenyllactate (10.39-fold), 4-hydroxyphenylacetate (6.91-fold), and 5-aminovalerate (27.89-fold). Likewise, the lipid super-pathway compounds that were noticeably greater in FF infant samples included the following: fatty acids, valerate (8.52-fold) and isovalerate (46.99-fold); the endocannabinoid, palmitoyl ethanolamide (8.00-fold); the sterol beta-sitosterol (9.02-fold); and both α- (8.05-fold) and γ-tocopherol (18.36-fold).

The relative levels of the essential fatty acid, linoleate (18:2, n6), and its omega conversion product, linoelaidate (*trans, trans*-9,12-octadecadienoic acid), in samples from BF and FF infants, are presented in Figure 2. Linoleate was markedly higher in FF

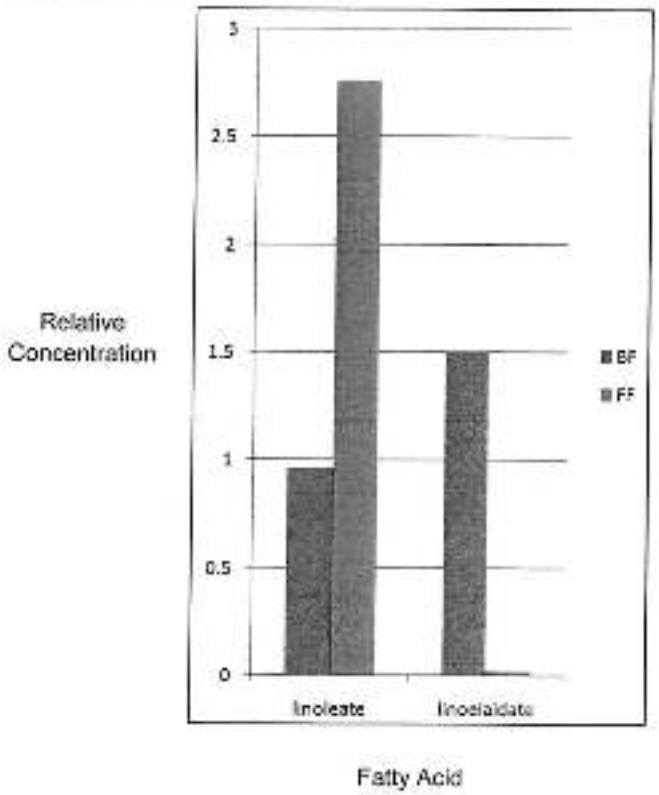

Figure 2. Relative levels of linoleate and its omega conversion product, linoelaidate, in BF versus FF infant samples.

samples, but was also relatively abundant in the BF supernatants. However, the figure also indicates markedly higher levels of *trans, trans* form in the BF infant compared to those from the FF infants, which had almost none.

Infant Variability

Some compounds exhibited high individual infant variation, often being only detected from one infant, or being many fold higher in one individual compared to the population. For instance, one of the BF infants had considerably higher levels of the omega-3 fatty acid, docosahexanoate (DHA), compared to the others, as well as uniquely detectable levels of creatine, acetylcarnitine, and urate (data not shown). Wide variation was also noted in the amount of 2′FL detected in feces from BF and FF infants (data not shown). Other examples of individual infant variation, including differences in several amino acid catabolites and long chain fatty acids, were observed in both the BF and FF groups.

Addition of Exogenous Carbohydrates

A heat map displaying the compounds whose fermentation patterns were altered by the presence of exogenously added carbohydrate in one or both of the diet groups (BF or FF) is shown in Figure 3. Fermentation patterns for GOS, 2′FL, and LNnT were highly similar to each other, with corresponding increases (red) and decreases (green) in various amino acids, carbohydrate, and lipid-related metabolites over the 6 h fermentation. By comparison, the profiles for 6′SL and HP showed similarity to one another but differed substantially from those of GOS, 2′FL, and LNnT. Lastly, the profile for GA was essentially identical to the blank (no carbohydrate added control) and did not resemble those of the other carbohydrate treatments. In addition, Figure 3 indicates that some metabolites that responded differentially to oligo addition between the BF and FF groups (flagged as blue cells in the far right column), which included various metabolites related to amino acid metabolism, the urea cycle, pyruvate metabolism, and specific SCFA (e.g., valerate and isovalerate).

As an example of a differential response to oligos, Figure 4 displays in greater detail changes in the relative concentrations of the fatty acid, valerate, in FF versus BF samples during the course of fermentation. Valerate, a product of proline degradation, accumulated rapidly during the 6 h fermentation in the blank, 6′SL, HP, and GA treatments, but the increase was only observed in samples collected from FF infants. Relative levels of valerate changed very little for BF infants, regardless of carbohydrate substrate. Similar results were observed for isovalerate, a product of valine fermentation (data not shown).

The relative concentrations of selected biogenic amines during the course of fermentation are presented in Figure 5. With a few exceptions, the fold differences between FF and BF (FF/BF) infants changed very little upon addition of carbohydrate. However, the addition of either HP or GA resulted in significantly greater concentrations of putrescine after 6 h of fermentation from FF compared to BF infant inoculum ($P \leq 0.05$). Likewise, supplementation with LNnT led to higher relative concentrations of agmatine in FF compared to BF infants at 6 h ($P \leq 0.05$).

DISCUSSION

Fecal Metabolites: Breast-Fed versus Formula-Fed

A major objective of this study was to compare fecal metabolite profiles of FF and BF infants. To accomplish this objective, we identified compounds present in differing amounts in the time = 0 h blank FF and BF samples, because these compounds would presumably represent those residing in the fecal inocula. Inoculum from FF infants contained elevated levels of many amino acid catabolites such as phenyllactate from the degradation of phenylalanine, 4-hydroxyphenylacetate from the breakdown of tyrosine, and 5-amino valerate from the metabolism of lysine. Samples from this group also contained higher levels of isovalerate and valerate, which are indicative of protein fermentation.

The predominance of protein fermentation, as previously noted by Heavy et al.,[19] may be due to a number of contributing factors. To compensate for differences in the amino acid composition between bovine and human milk, infant formulas have been formulated to contain greater concentrations of protein than human milk,[20] so a greater quantity of protein could potentially escape digestion and absorption in the small intestine. Moreover, samples from FF infants presumably contain a more

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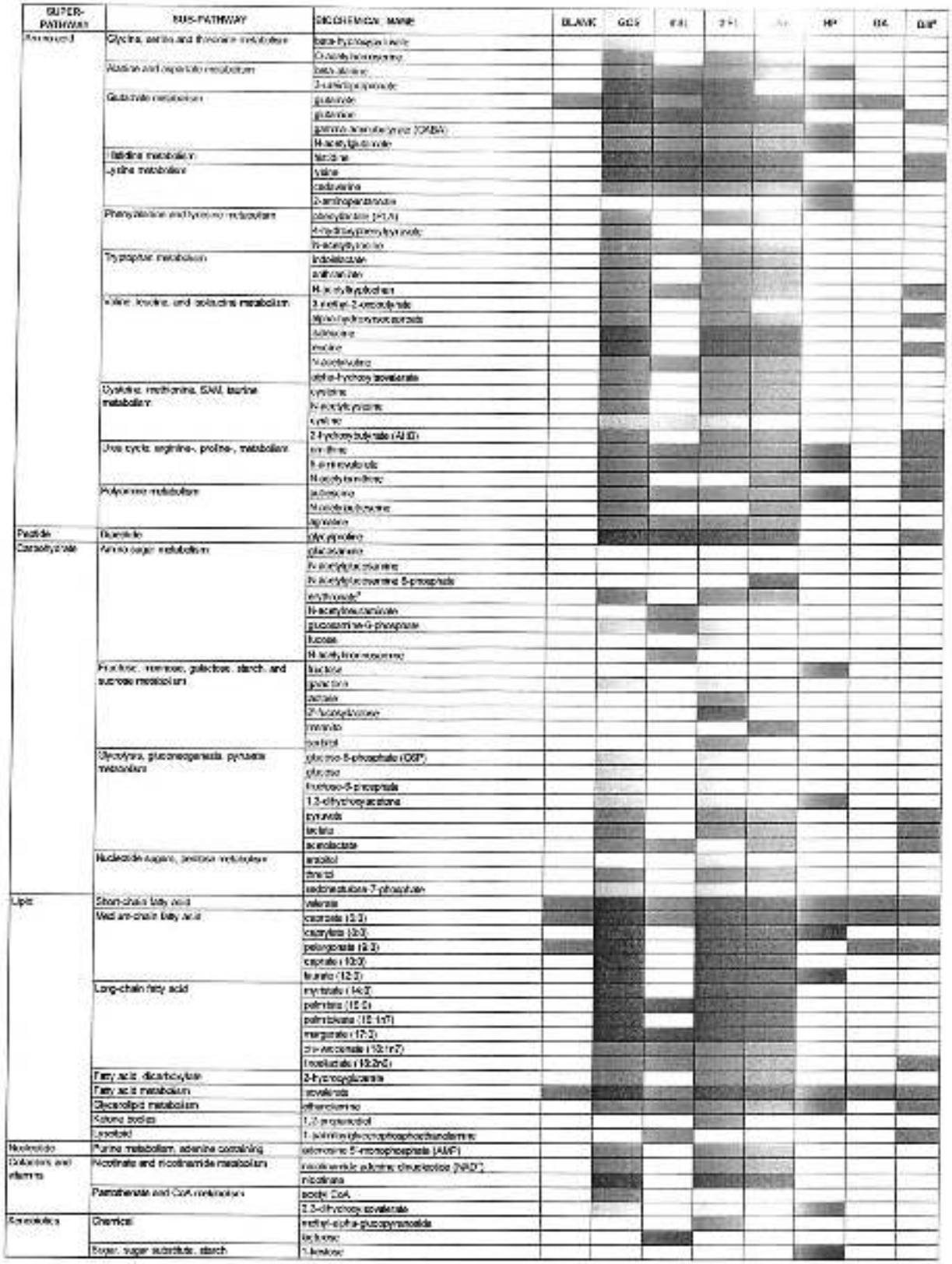

Figure 3. Compounds altered by the addition of exogenously added carbohydrate (relative to the blank) regardless of diet groups. Red cells indicate an increase in the compound; green cells indicate a decrease in the compound; light red cells indicate an initial increase at 3 h with a subsequent decrease at 6 h; blue cells indicate that the compound changed differentially depending on treatment group. (a) Compounds flagged as blue cells responded differentially between the breast-fed and formula-fed groups. (b) Indicates compound that has not been officially "named" (based on a standard), but we are confident in its identity.

diverse fecal microbiome than those from BF infants with a greater proportion of bacteroides and clostridia than BF infants.[8] Because bacteroides and clostridia possess proteolytic and/or amino-acid-fermenting capabilities, samples from FF infants

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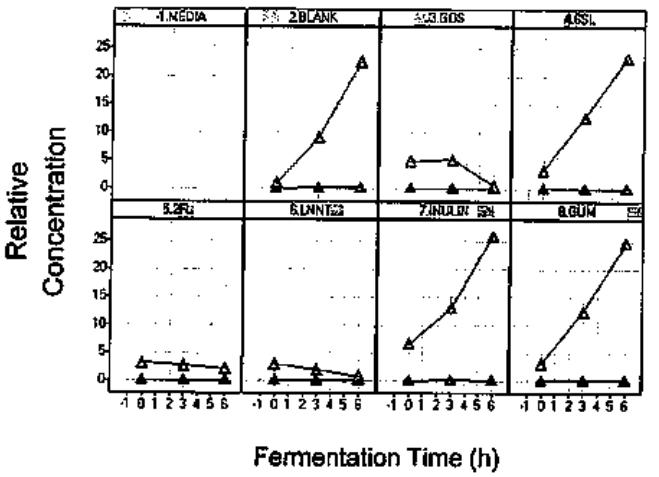

Figure 4. Relative concentrations of valerate over 6 h of fermentation. Each triangle represents the average of four infants in each diet group. Blue triangles = BF, orange triangles = FF.

would be expected to contain higher levels of protein fermentation products.

Very little is known about the fermentation of dietary proteins in the infant gut and its potential effects on human health. However, recently published studies using a formula-fed piglet model suggest that excessive protein intake during the neonatal period could potentially lead to a variety of physiological consequences such as compromised intestinal barrier function,[21] disturbed regulation of intestinal permeability by acetylcholine and vasoactive intestinal peptide,[21] early implantation of ileal microbiota,[22] and altered colonic immune cell development.[23] Indeed, the piglet data[22,23] suggest that neonatal feeding of high protein diets might also result in metabolic consequences later in life by altering sensitivity of the colonic mucosa to pro-inflammatory insults at maturity. Nonetheless, given the limited number of animal studies, the scarcity of mechanistic data, and the qualitative nature of our metabolomics data, it is premature to extrapolate the results to human infants.

Carbohydrate Supplementation

The second objective of the current study was to determine the effect of carbohydrate supplementation on fermentation profiles of the fecal inocula. As expected, the 0 h blank cultures (no carbohydrate added), as well as those treated with GA, mainly produced various fatty acids such as caproate, isovalerate, and valerate as a result of protein fermentation. By contrast, the addition of fermentable carbohydrates, such as GOS, 2′FL, and LNnT, to the cultures reduced the levels of various amino acids, decreased the accumulation of amino acid-related metabolites, and increased the levels of metabolites related to energy generation. These observations are consistent with the idea that cultures lacking a source of fermentable carbohydrate will ferment amino acids to obtain energy, whereas the provision of carbon would obviate the need for metabolism of amino acids for energy and create anabolic conditions that would require utilization of amino acids for growth.

A more detailed examination of these data revealed that cultures from FF infants responded differently to carbon limitation than those of BF infants. Although both BF and FF samples generated amino acid super-pathway-related compounds in response to carbohydrate supplementation, only those from FF infants also fermented protein under carbon-limited conditions (e.g., 0 h blank or GA). Continuous degradation of amino nitrogen by samples from FF infants during carbon-limited conditions was likely due to the presence of bacteria that are able to exclusively utilize amino acids and/or peptides such as clostridia, *Shigella*, enterococci, bacteroides, *Escherichia coli*, or staphylococci.[24]

Interestingly, fecal microbiota from all four FF infants quickly degraded 2′FL in vitro within the first 3 h of incubation, but those from BF infants varied considerably in their ability to ferment this oligosaccharide (data not shown). The rapid disappearance of 2′FL in cultures from FF infants is consistent with previous work showing that the introduction of foods other than breast milk to infants improves the ability of fecal bacteria to ferment complex carbohydrate[25] including HMO.[26] More specifically, the rapid degradation of 2′FL might possibly be attributed to the presence of a significant population of bacteroides in FF infants, some strains of which have been documented to readily utilize HMO as a sole source of carbohydrate.[27] In contrast, the variation in the ability of microbiota from BF infants to catabolize 2′FL might have been caused by either the presence or absence of bifidobacteria species that are capable of utilizing HMO[28] or differences in the levels of bifidobacteria species that are capable of utilizing HMO in the gut. Although we did not determine whether the mothers of the infants who participated in the study were secretors or nonsecretors,[29] it is also possible that variability in the fucosylated oligosaccharide content of breast milk could influence the ability of the infant colonic microbiota to at least degrade this particular HMO. Additional experiments are needed to verify the difference in the ability of fecal microbiota from BF and FF to degrade various HMO and to determine whether

COMPOUND	Fold of Change (FF / BF)[a] Formula-Fed vs. Breast-Fed																				
	Blank			GOS			6'SL			2'FL			LNnT			HP			GA		
	0h	3h	6h	0h	3h	6h	0h	3h	6h	0h	3h	6h	0h	3h	6h	0h	3h	6h	0h	3h	6h
gamma-amino butyrate (GABA)	0.77	0.47	0.32	0.54	0.63	1.15	0.38	0.58	1.02	0.30	0.60	0.67	0.69	0.89	0.61	0.58	0.51	1.19	1.53	0.51	0.86
cadaverine	2.87	3.11	0.39	1.71	1.80	6.04	2.70	2.73	0.94	0.94	3.65	0.96	3.09	1.88	0.81	6.19	1.62	1.15	1.99	0.13	2.32
tyramine	0.71	1.14	0.88	0.73	0.68	1.23	0.51	1.10	0.47	0.62	2.43	1.02	0.91	1.74	1.31	0.46	1.29	0.94	0.78	0.98	1.50
tryptamine	1.85	1.27	1.22	1.23	1.23	1.22	1.28	1.50	1.30	1.32	1.37	1.23	1.82	1.24	0.98	1.11	1.22	1.64	1.10	1.35	1.29
putrescine	2.99	1.84	0.44	2.00	0.19	3.63	0.92	8.39	1.50	0.34	7.98	2.94	1.42	3.10	2.57	0.85	1.28	12.90	1.28	1.45	16.89
agmatine	4.15	0.38	0.09	0.96	0.38	0.40	0.71	0.43	0.09	0.80	2.02	1.10	0.93	0.68	0.62	1.37	0.78	1.56	1.42	0.14	0.19
spermidine	2.00	1.26	0.89	1.36	1.09	0.83	1.43	1.32	1.03	1.34	0.70	0.68	1.42	0.78	0.87	1.18	1.32	0.92	1.52	1.20	1.38

[a]Shaded cells indicate $P \leq 0.05$ (red indicates that the mean values are significantly higher for that comparison; green values significantly lower). Blue-bolded text indicates $0.05 < p \leq 0.10$. Noncolored text and cells indicate that mean values are not significantly different for that comparison.

Figure 5. Fold changes (FF/BF) of biogenic amines in FF vs BF infants during 6 h fermentation.

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secretor status influences the ability of the microbiota to degrade fucosylated oligosaccharides.

Biogenic Amines

Emerging research has linked the intestinal microbiome to both brain development and behavior[7] via the brain–gut–enteric microbiota axis.[30] This relationship is mediated in part through signaling molecules generated by the gut bacteria[31] such as the biogenic amine/neurotransmitter gamma-amino butyric acid.[32] On the basis of previous work showing elevated levels of the biogenic amine, tyramine, in feces from healthy infants fed either cow's milk or cow milk formula compared to those who were breast fed,[33] we originally hypothesized that (a) stool samples from the FF infants would contain higher relative levels of the various amines than those from BF infants and (b) the addition of fermentable carbohydrate would suppress accumulation of amines. However, our limited data suggest that this was not necessarily the case, at least with the biogenic amines that were identified in the current study. To illustrate, at time = 0 h, out of seven biogenic amines identified, only a single amine, tryptamine, was higher in FF than in BF infants (Table 3). Furthermore, provision of fermentable carbohydrate actually resulted in increases rather than decreases in several biogenic amines (e.g., GABA, cadaverine, and agmatine) regardless of diet (Figure 3). Lastly, the excess production of amines by samples from FF infants depended on the specific amine analyzed and on the type of carbohydrate that was added to the cultures (Figure 5). Although we did not anticipate that supplementation with fermentable carbohydrate would increase generation of amines, our data are consistent with the observation that the ability to produce amines is widespread among human intestinal bacteria.[34]

Other Compounds of Dietary Origin

Direct comparison of the time = 0 h blank samples also showed that BF and FF infants contained elevated levels of several metabolites that presumably originated from breast milk and infant formula, respectively. To illustrate, samples from FF infants contained at least 8-fold more α- and γ-tocopherol than those of BF infants. Because tocopherols are considered an essential nutrient and cannot be synthesized endogenously, the presence of elevated levels of tocopherols in the stool samples of FF suggest two possible scenarios: (a) tocopherols are being provided in the diet at levels in excess of infant need or (b) tocopherols provided in infant formula are not completely absorbed by in the small intestine. If (a) were true, one might consider lowering the amount of tocopherols that are incorporated into infant formula. On the other hand, if (b) were true, further research would be needed to identify methods for improving tocopherol absorption by infants.

■ CONCLUSIONS

In this study, we used metabolomics analyses to compare the fermentation profiles generated by fecal inocula from BF and FF infants. Comparison of the samples at time = 0 h revealed signs of carbon limitation and predominant protein fermentation in samples from FF infants versus the presence of HMO and less carbon restriction in the BF group. Furthermore, the comparison revealed differences in relative levels of some compounds that were most likely acquired through either breast milk (e.g., linoelaidate) or infant formula (e.g., tocopherols, soy-based compounds). Supplementation with fermentable carbohydrates led to the accumulation of compounds indicative of energy generation. Cultures from BF infants that were not supplemented with carbohydrate (blank) did not accumulate compounds indicative of protein fermentation, but those from FF infants continued to accumulate compounds involved in protein catabolism. Although our results suggest that supplementation with fermentable carbohydrates had little effect on the accumulation of biogenic amines, further investigations are needed to first determine the biological significance of amino acid catabolites, including biogenic amines, on infant gut health and, more broadly, on overall infant health. In light of these findings, fecal metabolomics appears to be a useful tool for assessing the quality of infant diets regardless of whether the source of nourishment comes from breast feeding or formula feeding.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: gcfahey@illinois.edu. Fax: 217-333-7861. Tel.: 217-333-2361.

Notes

The authors declare the following competing financial interest(s): J.M.C. is an employee of Abbott Nutrition. M.R.P. is a University of Illinois graduate student and co-op student employee of Abbott Nutrition.

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■ ABBREVIATIONS USED

GOS, galactooligosaccharide; GA, gum arabic; 2′FL, 2′-fucosyllactose; 6′SL, 6′-sialyllactose; LNn, lacto-*N*-neotetraose; HP, inulin; SCFA, short-chain fatty acids; BF, breast-fed; FF, formula-fed; HMO, human milk oligosaccharides

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