Non-invasive Methods for Assessing Nutritional Regulation of Neonatal Gut Gene Expression and Host-Microbe Interactions

Sharon M. Donovan, PhD, RD

Department of Food Science & Human Nutrition
University of Illinois, Urbana, IL, 61801, USA

Presentation Outline

- **Introduction**
  - **Benefits of breastfeeding**
  - **Factors affecting development of the gut microbiome**
- **Non-invasive Detection of Intestinal Epithelial Gene Expression**
  - Experimental Approach
  - Impact of infant diet on infant gut epithelial gene expression
- **Host-Microbe Interactions in the Neonate**
- **Conclusions**

Human Infants are Vulnerable to Nutritional Insults

"Infancy is a uniquely vulnerable period of rapid growth and development and, as such, feeding changes have the potential to impart benefit or harm in the short term, into early childhood, and even later into adulthood"

IOM Committee on the "Evaluation of the Addition of Ingredients New to Infant Formula", 2004.

Pediatric Nutrition

- Proper nutrition is critical for health, growth, and development

- Human milk is the ideal nutrition for infants because it provides all necessary nutrients for normal growth and development and reduces risk of many diseases **(American Academy of Pediatrics, 2012)**

- Pediatric nutrition is ***not*** just about providing nutrients
  - Feeding involves social and tactile interactions
  - Human milk contains bioactive components that serve non-nutritional roles, including stimulating development of the gut microbiota

Pediatrics 2012; 121: e827-e41.

TABLE 2 Dose-Response Benefits of Breastfeeding[a]

Condition	% Lower Risk[b]	Breastfeeding	Comments
Otitis media[13]	23	Any	—
Otitis media[13]	50	≥3 or 6 mo	Exclusive BF
Recurrent otitis media[15]	77	Exclusive BF ≥6 mo[d]	Compared with BF 4 to <6 mo[d]
Upper respiratory tract infection[17]	63	>6 mo	Exclusive BF
Lower respiratory tract infection[13]	72	≥4 mo	Exclusive BF
Lower respiratory tract infection[15]	77	Exclusive BF ≥6 mo[d]	Compared with BF 4 to <6 mo[d]
Asthma[13]	40	≥3 mo	Atopic family history
Asthma[13]	26	≥3 mo	No atopic family history
RSV bronchiolitis[16]	74	>4 mo	—
NEC[19]	77	NICU stay	Preterm infants Exclusive HM
Atopic dermatitis[27]	27	>3 mo	Exclusive BFnegative family history
Atopic dermatitis[27]	42	>3 mo	Exclusive BFpositive family history
Gastroenteritis[13,14]	64	Any	—
Inflammatory bowel disease[32]	31	Any	—
Obesity[13]	24	Any	—
Celiac disease[31]	52	>2 mo	Gluten exposure when BF
Type 1 diabetes[13,42]	30	>3 mo	Exclusive BF
Type 2 diabetes[13,43]	40	Any	—
Leukemia (ALL)[13,46]	20	>6 mo	—
Leukemia (AML)[13,45]	15	>6 mo	—
SIDS[13]	36	Any >1 mo	—

Infections

Protective Effect of BF:

- Dosage effect
- Interacts with genetic risk and

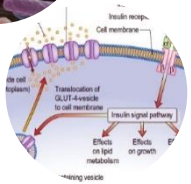
Human Milk as a Developmental Modulator

Growth

Cognitive Development

Gut Microbiome & Immune Development

Metabolic programming



Interaction Between GI, Microbiome and Immune Development

Early Nutrition
(Breast vs Formula feeding)

- Establishment of the Microbiome
- Intestinal Development
- Immune Development

Factors Impacting Establishment of the Intestinal Microbiota

Host Genetics

Term vs. Preterm Delivery

- **Preterm**: Slower colonization and less diversity

Route of Delivery

- **C-section**: less *Bifido* and *Bacteroides*; more *E. coli* & *C. difficile*

Perinatal Antibiotics

- Reduced overall diversity and numbers

Other

- Siblings, pets in the home, smoking, daycare, etc

Type of Nutrition

- Milk oligosaccharides (HMO)
- Bacteria in milk
- Bacteria on maternal skin

- Type of formula
- Prebiotics/Probiotics

Adlerberth & Wold. Establishment of the gut microbiota in Western infants. *Acta Paediatr* 2009; 98: 229-38.

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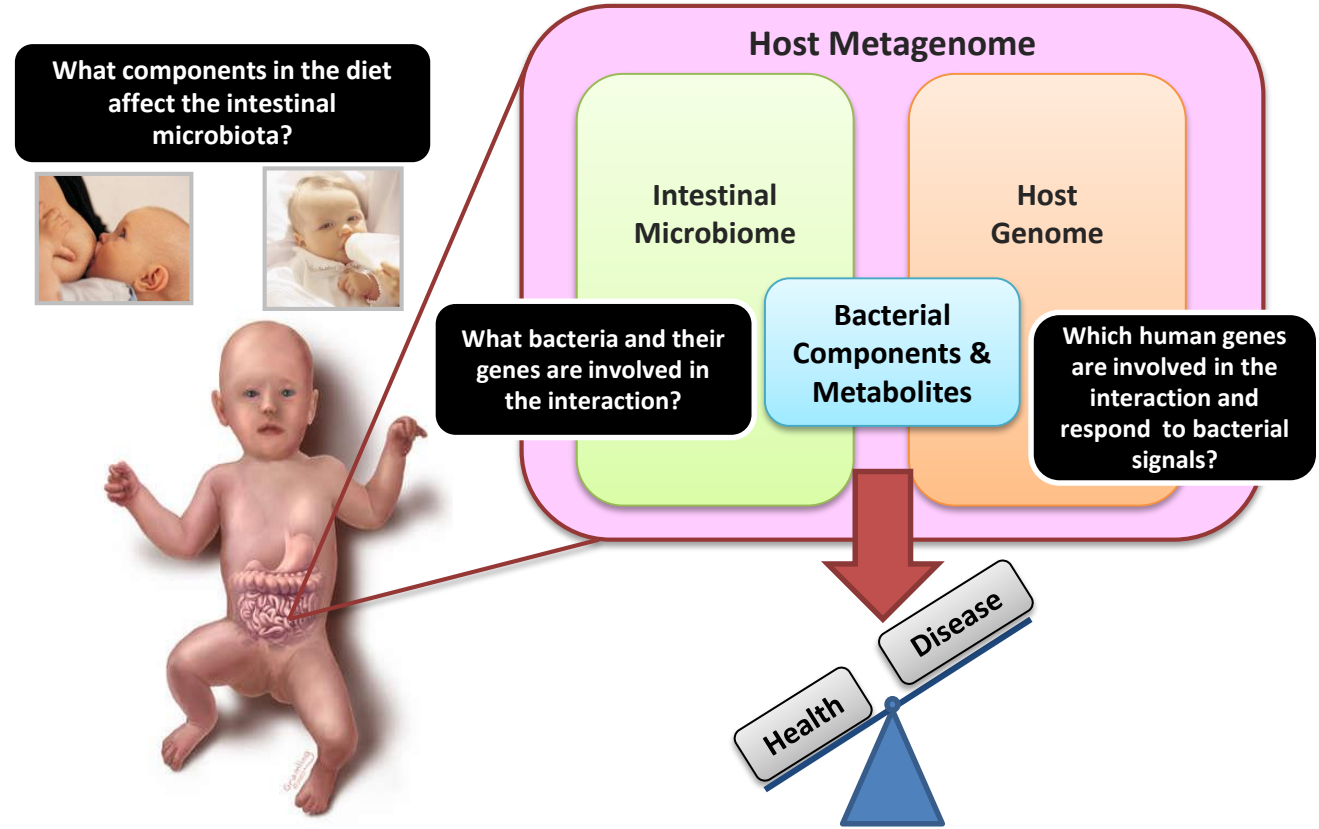
Looking into the "Black Box": Host-Microbe Interactions in the Neonate



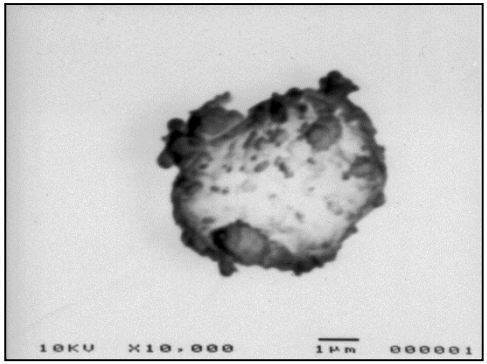
Adapted from: Hattori & Taylor. The human intestinal microbiome: A new frontier of human biology. *DNA Res* 2009

Development of a Non-Invasive Approach

- Defining how early nutrition regulates gut development in human infants has been limited by the lack of ***non-invasive approaches suitable for use in healthy human infants***.

- Exfoliated intestinal cells may provide a means investigate the impact nutrition on intestinal development and function **(Davidson et al., 1995)**

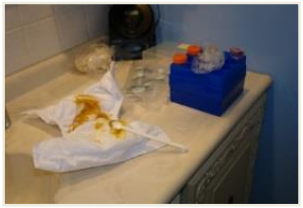
- Approximately 1/6 to 1/3 of epithelial cells are shed daily (>10^{10} cells/day) **(Potten et al., 1979)**

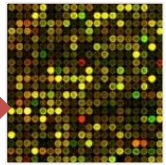


Electron micrograph of sloughed epithelial cell from stool

Overall Experimental Approach

Chapkin RS, Zhao C, Ivanov I, Davidson LA, Goldsby JS, Lupton JR, Mathai RA, Monaco MH, Rai D, Russell WM, Donovan SM, Dougherty ER. Noninvasive stool-based detection of infant gastrointestinal development using gene expression profiles from exfoliated epithelial cells. *Am J Physiol* 2010; 298:G582-9.





Intestinal Gene Expression

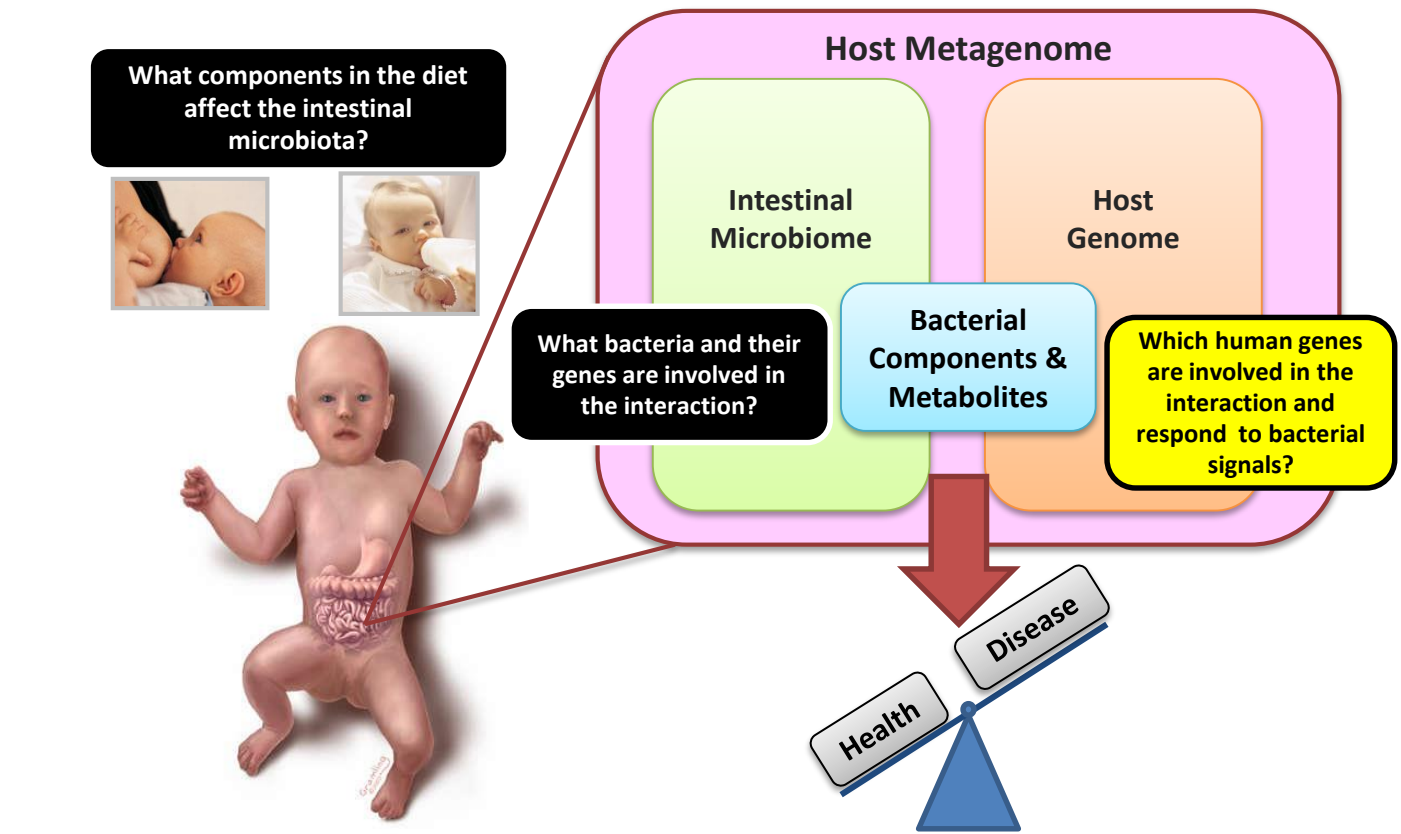
Wang M, Li M, Wu S, Lebrilla CB, Chapkin RS Ivanov I, Donovan SM. Fecal microbiota composition of breast-fed infants is correlated with human milk oligosaccharides consumed. *JPGN* 2015; 60:825-33.

Human Milk Oligosaccharides

Microbiome (16S rRNA) & Metagenome (DNA)

Schwartz S, Friedberg I, Ivanov I, Davidson LA, Goldsby JS, Dahl DB, Herman D, Wang M, Donovan SM, Chapkin RS. A metagenomic study of diet-dependent interaction between gut microbiota and host in infants reveals differences in developmental and immune responses. *Genome Biology* 2012; 13:R32.

Host-Microbe Interactions in the Neonate



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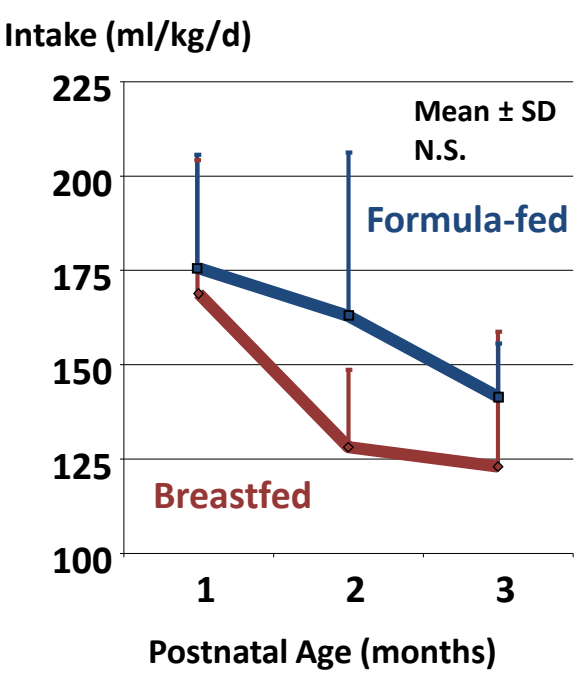
Experimental Subjects

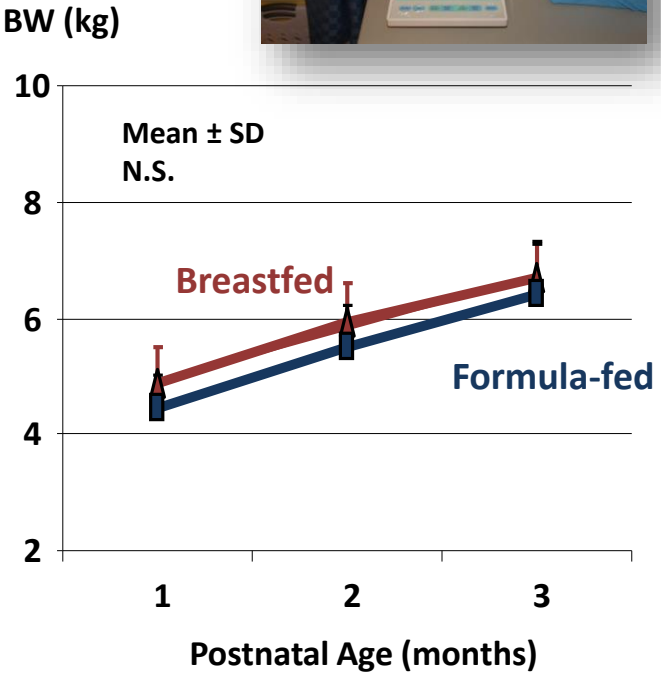
- Vaginally-delivered, term infants of second parity mothers that were medically certified as healthy
- Exclusively breast-fed or fed Enfamil Lipil formula (Mead Johnson, Evansville, IN) until 3 months of age
- Exclusion criteria: formula intolerance, combined breast milk/formula, non-study formula, juice or solid foods

	Breastfed (BF)	Formula-fed (FF)
N =	16	10
Maternal Age (years)	29.5 ± 4.2	29.8 ± 4.9
Infant Birth Weight (kg)	3.78 ± 0.56	3.51 ± 6.2
Infant Birth Length (cm)	52.5 ± 5.5	51.0 ± 2.8

Chapkin RS et al. Noninvasive stool-based detection of infant gastrointestinal development using gene expression profiles from exfoliated epithelial cells. *Am J Physiol* 2010; 298:G582-9.

Milk Intake & Infant Growth

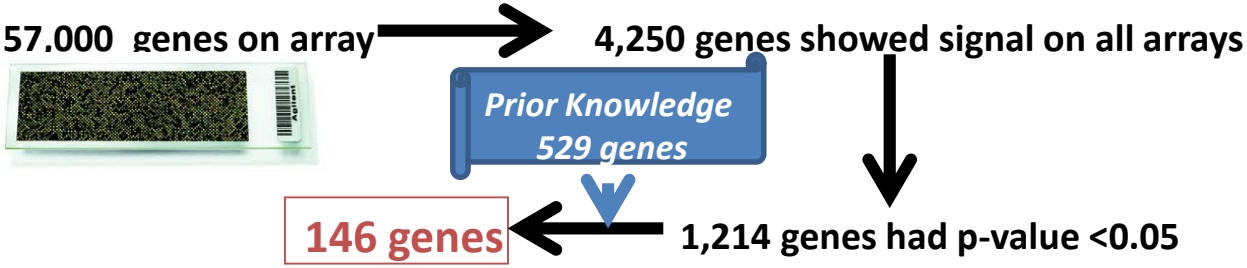




- No significant difference in intake or weight gain

Stool Sample Processing

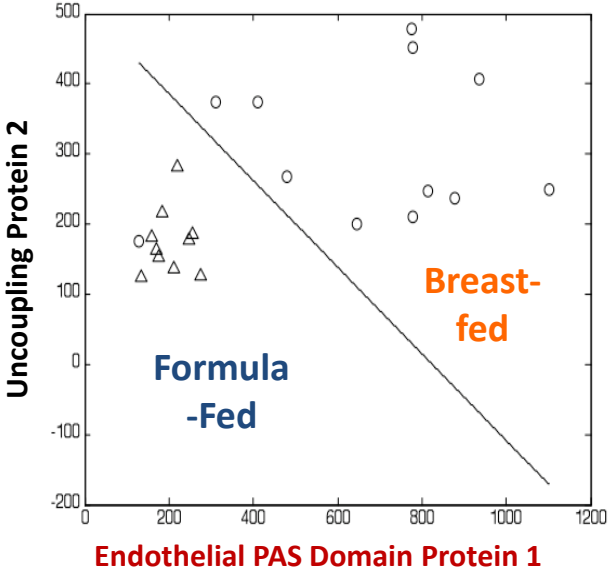
- Sample was collected at 3 months postnatal age by the parent
- Freshly voided stool (~10 g) was placed into a sterile tube containing Trizol reagent (Ambion, Austin, TX)
- Poly A+ RNA was isolated to from sloughed epithelial cells to enrich mammalian RNA using established methods (**U.S. Patent 6258541)**



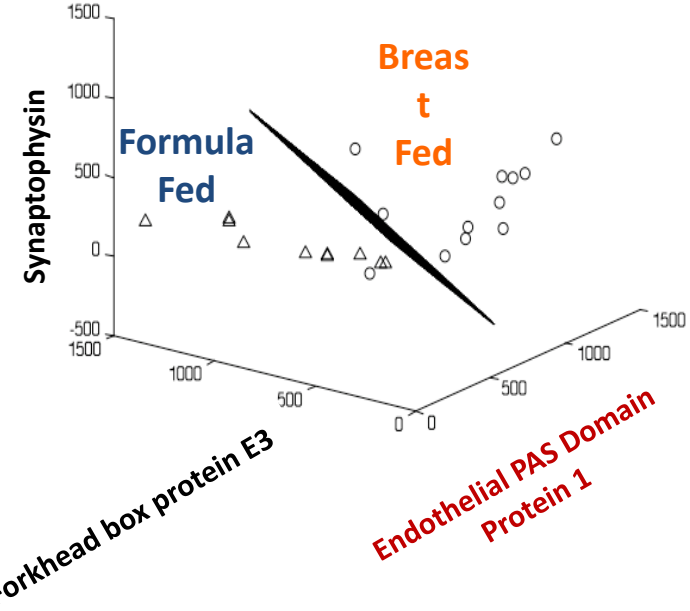
- These 146 genes were subjected to further analyses
  - **Linear Discriminant Analysis (LDA) – Best Classifiers of BF vs FF**
  - **Gene Networks (Metacore™, GeneGo, St. Joseph, MI) - Networks**

BF vs FF Infants (Gene Classifications) "Linear Discriminant Analysis"

2-Gene Combination



3-Gene Combination

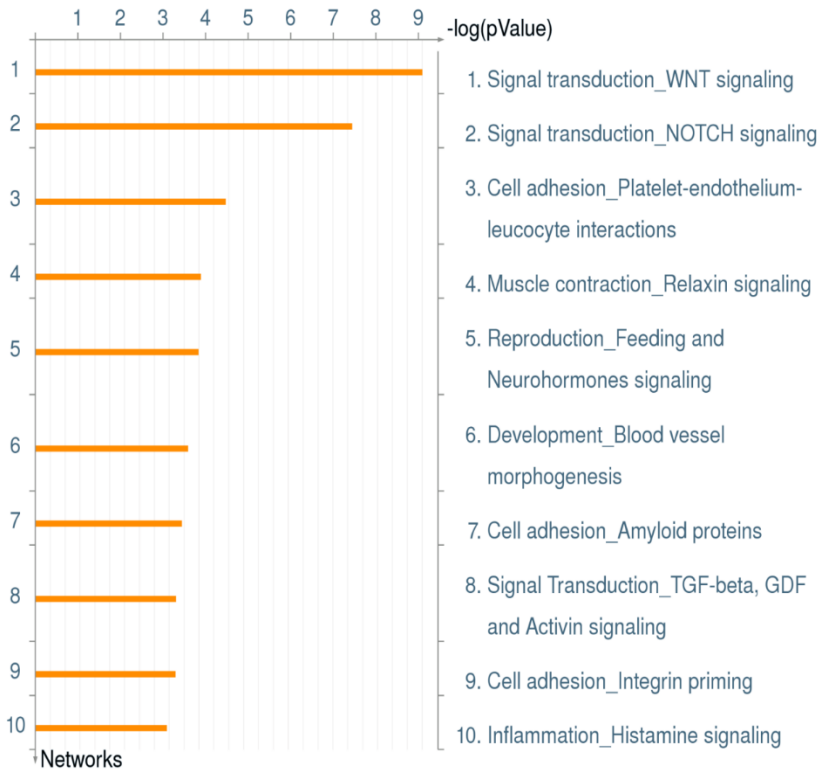


LDA - Best Genes For Classifying BF vs FF

Gene Name	Function	Fold Change (BF/FF)
EPAS1	Transcription Factor (TF); cellular response to hypoxia	3.3
NR5A2	TF, encodes liver receptor homolog-1 (LRH-1); development	2.8
NR3C1	Encodes glucocorticoid receptor	5.5
PCDH7	Encodes protocadherin-7; membrane protein	3.9
ITGB2	Encodes integrin beta-2 (CD18); ICAM-1 receptor	2.5
FGF5	Encodes fibroblast growth factor 5; mitogenesis & cell survival	2.0
TJP1	Encodes ZO-1; intercellular tight junctions	2.2
MYB	TF, transcriptional transactivation; proto-ongogene	2.8
EPIM	Syntaxin 2/Epimorphin; epithelial cell morphogenesis	2.5
BAD	BCL2-associated agonist of apoptosis	4.0

Metacore™ Gene Networks – BF vs FF Infants

- **Signal transduction**
  - **WNT**
  - **NOTCH**
  - **TGF-ß**
- **Cytoskeleton remodeling**
  - **Cell migration**
- **Cell adhesion**
  - **Barrier function**
- **Immune response**
  - **Inflammation**
  - **Histamine**



From: Metacore™, GeneGo, St. Joseph, MI

Summary of Intestinal Gene Expression

- The relationships between diet and host gene expression can be assessed non-invasively in the human infant
  - 2- and 3-gene combinations were shown to distinguish BF from FF infants
- Provides insight into potential mechanisms whereby human milk regulates intestinal development and represent potential targets for manipulation of infant formula composition
- In preterm infants, this approach has shown developmental differences in gene expression compared to term infants (Knight et al. 2014)
  - Lower expression of genes in LCPUFA synthesis
  - Lower proliferation/cell cycle gene expression
  - Greater inflammatory gene expression

Knight JM, Davidson LA, Herman D, Martin CR, Goldsby JS, Ivanov IV, Donovan SM, Chapkin RS. Non-invasive analysis of intestinal development in preterm and full term infants using RNA-SEQ: A pilot study. *Nature Scientific Reports* 2014; 4: 5453.

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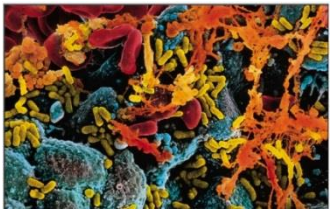
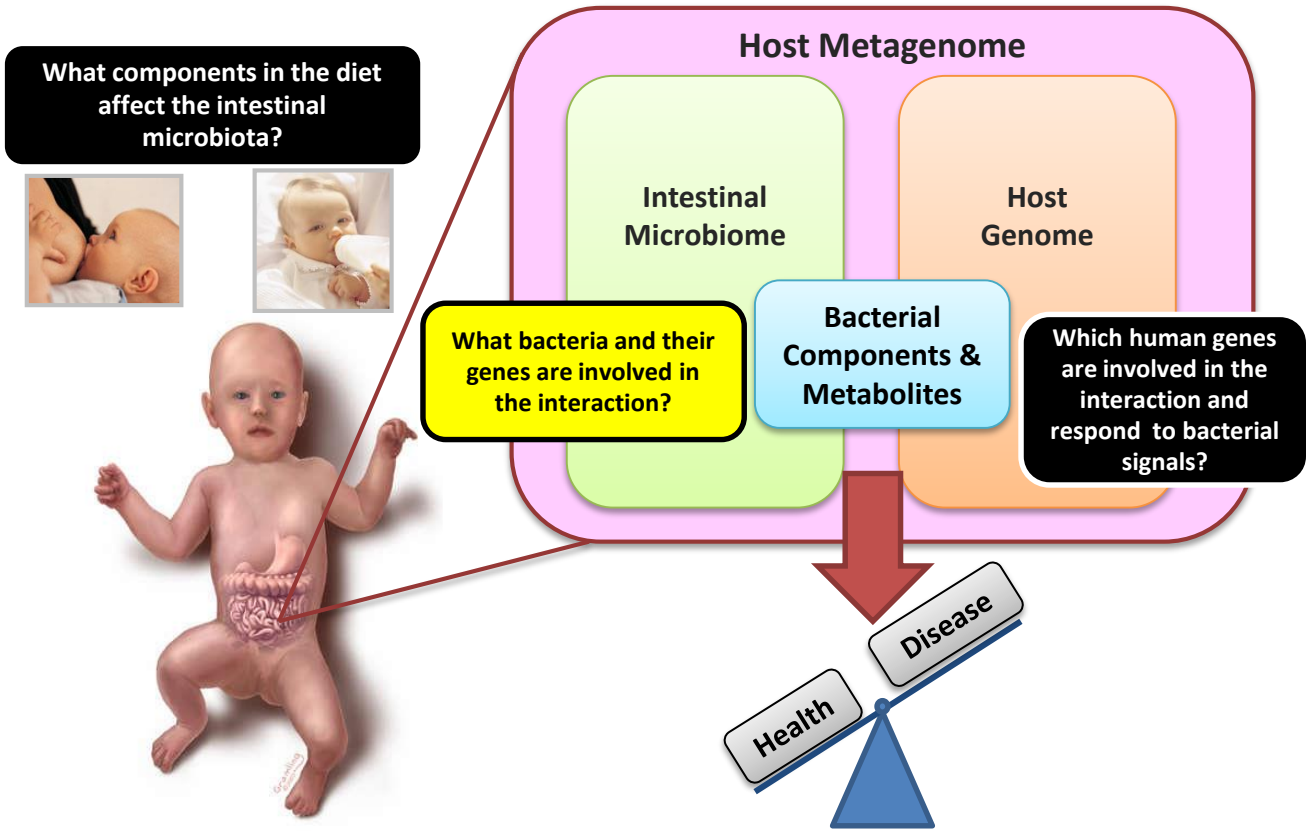


Host-Microbe Interactions in the Neonate



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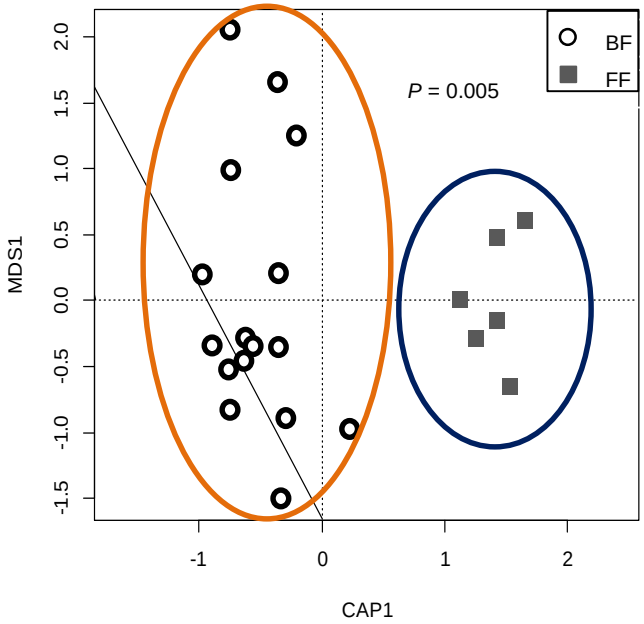
Fecal Microbiota of BF and FF Infants

- Pyrosequencing of V1-V3 region of 16s rRNA gene amplicons
- 321,822 sequences (10,743 per sample)

- Distance based redundancy analysis (dbRDA) showed that the overall structure of the microbiome differed between BF and FF infants.



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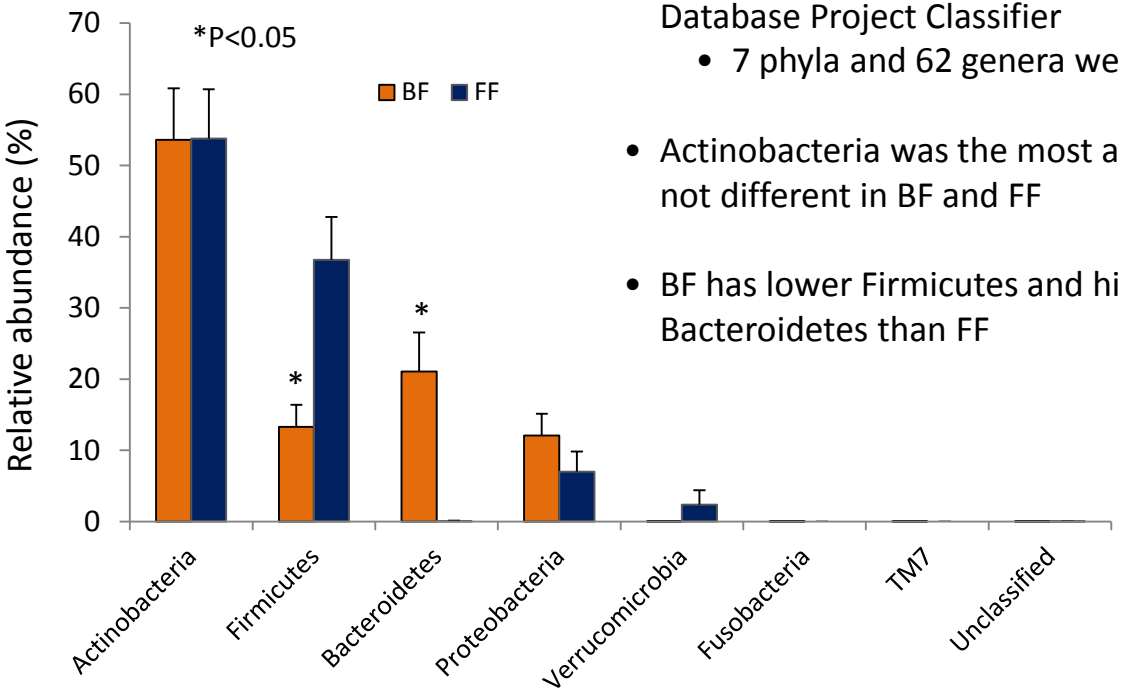
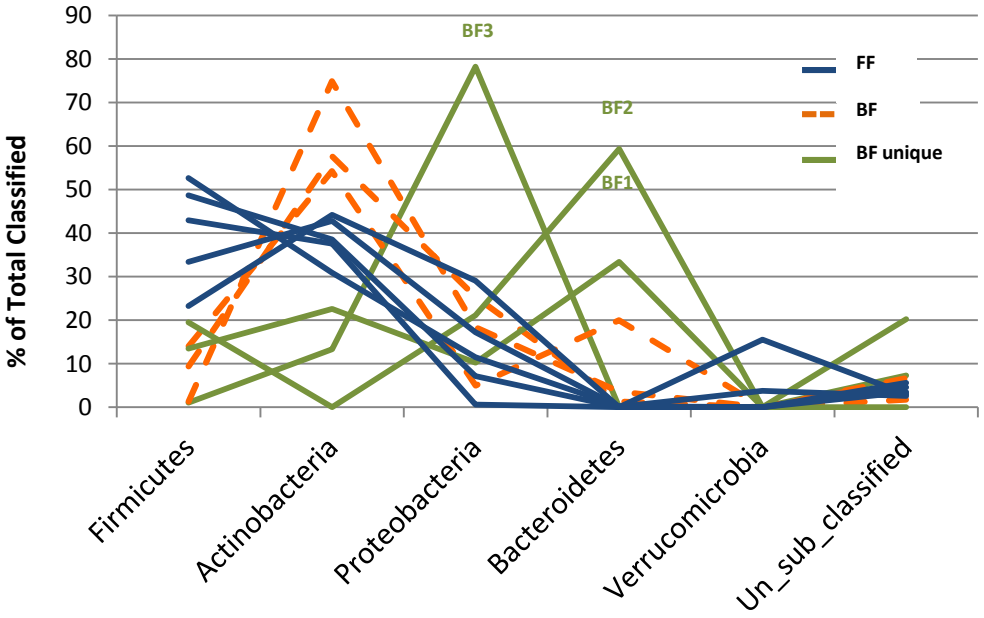


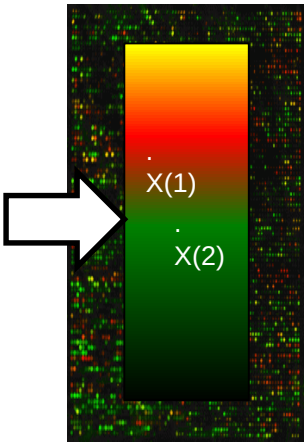
- Sequences classified using Ribosomal Database Project Classifier
  - 7 phyla and 62 genera were identified
- Actinobacteria was the most abundant, but not different in BF and FF
- BF has lower Firmicutes and higher Bacteroidetes than FF

Wang M, Li M, Wu S, Lebrilla CB, Chapkin RS Ivanov I, Donovan SM. Fecal microbiota composition of breast-fed infants is correlated with human milk oligosaccharides consumed. *JPGN* 2015; 60:825-33.

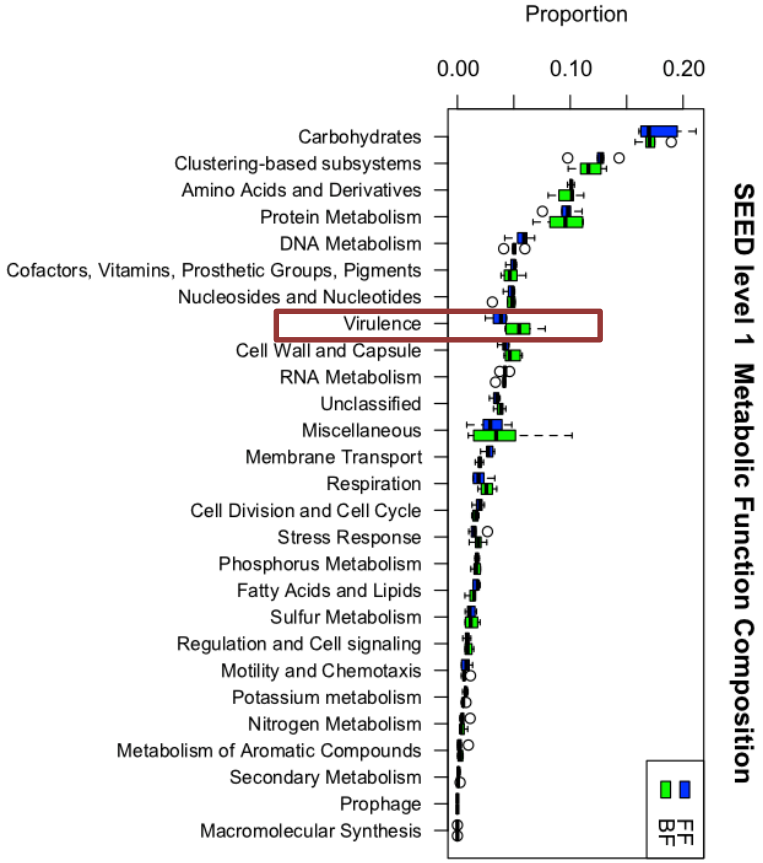
Variation in Microbiome Composition

- 5 distinct signatures: **FF**, **BF (3 infants)**, **BF1**, **BF2**, **BF3**
- Can we use differences in microbiota of BF and FF infants to predict differences in host gene expression?



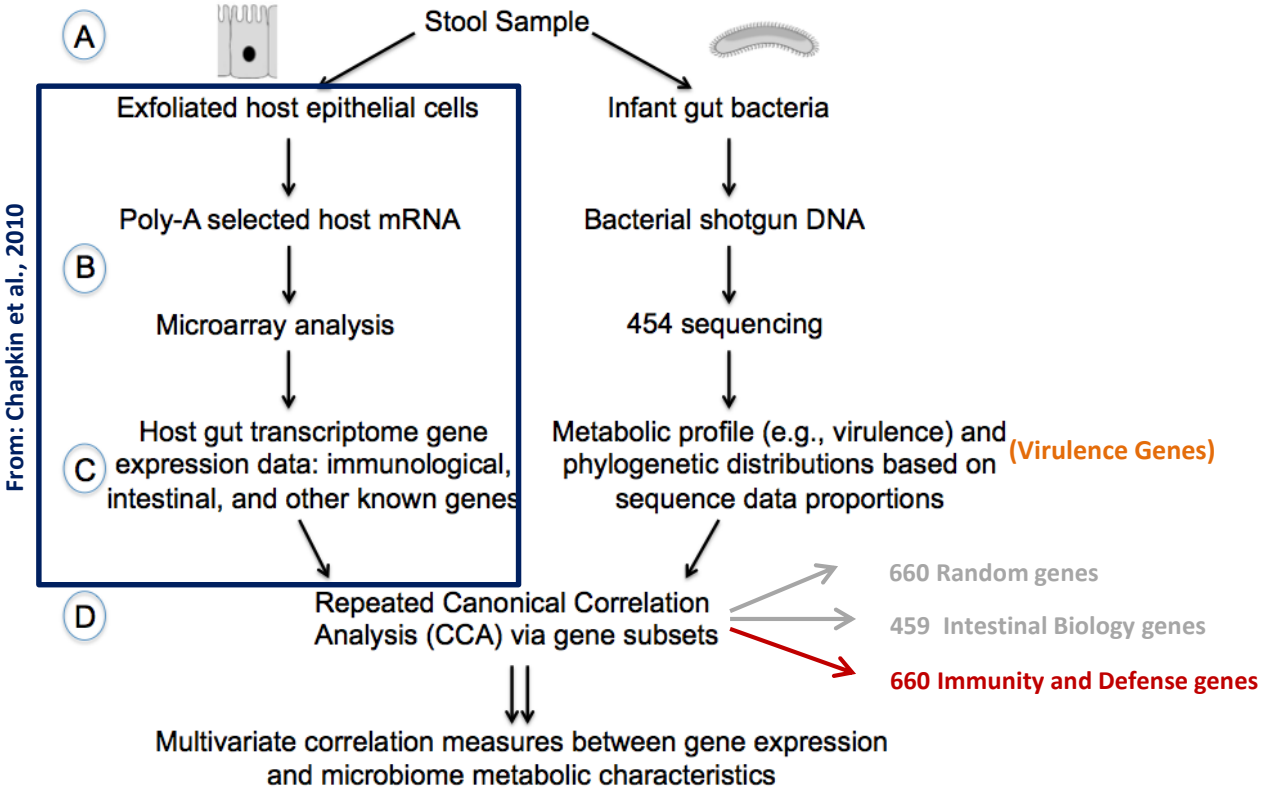


Bacterial Metagenomics



- SEED level 1 functional categorization via MG-RAST revealed
  - A larger proportion of genes involved in *CHO metabolism* in FF
  - Lower proportion of genes involved in *AA and protein metabolism* in FF
  - that ***virulence characteristics*** differed between FF and BF babies

Multivariate Analysis of Host Transcriptome and Functionally-Profiled Microbiome Data



Schwartz et al. A metagenomic study of diet-dependent interaction between gut microflora and host in infants reveals differences in developmental and immune responses. *Genome Biology* 2012; 13:R32.

11 Baby Immunity & Defense Genes Most Related to Microbial Virulence Genes

TACR1 *neurokinin (NK) 1 receptor; member of the tachykinin family of G-protein-coupled receptors*	**VAV2** *Guanine-nucleotide exchange factor*	**ALOX5** *Lipoxygenase gene; synthesis of leukotrienes from arachidonic acid*	**NDST** *GlcNAc N-deacetylase/N-sulfotransferase-1; heparin sulfate synthesis*
REL *Member of Rel/ NFKB family*	**BPILI** *Bactericidal/perme-ability-increasing protein-like 1; LPS binding protein*	**AOC3** *Mediates the binding of lymphocytes to vascular endothelial cells in an L-selectin-independent fashion*	**KLRF1** *NK Cell Receptor; stimulates natural kill cell cytotoxicity*
DUOX2 *NADPH oxidase; lactoperoxidase-mediated antimicrobial defense*	**IL1A** *Cyotkine secreted by activated macrophages, IL-1 stimulates thymocyte proliferation*	**SP2** *transcription factor required for expression of cell cycle- and developmentally-regulated genes*	

Up-regulated in BF

Down-regulated in BF

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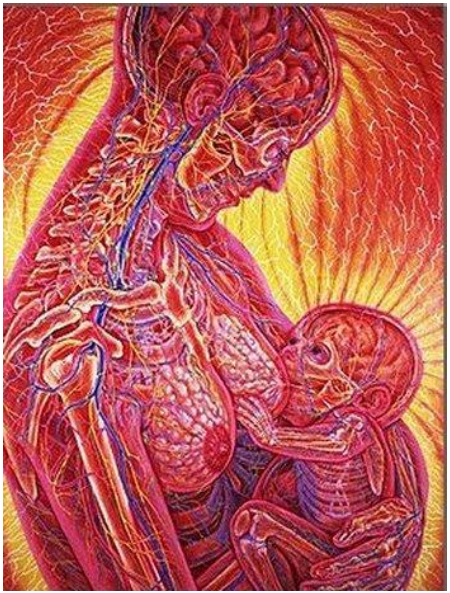
Summary of Host-Microbe Gene Expression

- We found evidence of multivariate structure relating the host immune system and microbiome virulence characteristics.
- The virulence properties of the microbiota were the most responsive characteristics with respect to BF versus FF, but probably do not reflect an infection.
  - BF babies had a larger complement of gram-negative bacteria than FF.
  - Gram-negative bacteria have genes that, although classified as 'virulent,' can activate the immune system but not cause an infection in the process.
- The relative abundance of CHO and protein metabolizing genes differed in the microbiota of FF and BF infants.
- These data suggest linkages between early nutrition and the functional characteristics of the neonatal microbiota.

Acknowledgments

- **Robert Chapkin PhD , Texas A&M University**
- **Term Infant Study:**
  - Rose Ann Mathai, MS, RD
  - Marcia Monaco PhD
- **Metagenomics and Bioinformatics**:
  - Mei Wang PhD and Min Li PhD
  - Scott Schwartz PhD, Ivan Ivanov PhD and Iddo Friedberg PhD
- **HMO Analyses:**
  - Shuai Wu and Carlito Lebrilla, PhD
- NIH CA59034, NIH CA129444, NIH DK71707, NIH P30ES09106
- DNS Vision 20/20
- Mead Johnson Nutrition

Questions?



Breastfeeding:
A Balance of Art and Science

Stool Sample Processing

- Sample was collected at 3 months postnatal age by the parent
- Freshly voided stool (~10 g) was placed into a sterile tube containing Trizol reagent (Ambion, Austin, TX)
- Samples were mixed by hand to create a homogenous sample and were immediately frozen at -20 °C
- Samples were held at –80 °C until shipped on dry ice to Texas A&M University
- An additional aliquot was immediately frozen for microbial and SCFA analyses