Non-Invasive Methods for Assessing Host-Microbe Interactions in the Infant

Sharon M. Donovan, PhD, RD

Department of Food Science & Human Nutrition
University of Illinois, Urbana, IL, 61801, USA
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• Dr. Donovan has received grant funding and has served on advisory boards for the Dairy Research Institute
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

• Future Directions
  – STRONG Kids 2 cohort
• The intestinal tract of the newborn undergoes marked structural and functional adaptation in response to feeding

• The response to human milk exceeds that of formula, suggesting that human milk components contribute to this response

• Data from germ free animals show that a microbiota is essential for normal GI development

• Human milk contains bioactive components that influence intestinal development and shape the intestinal microbiota
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

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

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

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

What components in the infant diet affect the intestinal gene expression & microbiota?

Intestinal Microbiome

What bacteria and their genes are involved in the interaction?

Bacterial Components & Metabolites

Host Genome

Which human genes are involved in the interaction and respond to bacterial signals?

Health Disease

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

We hypothesized that the epithelial cell transcriptome of breastfed infants would differ from formula-fed and would provide insight into the developmental pathways that are modulated by diet.

Exfoliated intestinal cells may provide a means to 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).

We hypothesized that the epithelial cell transcriptome of breastfed infants would differ from formula-fed and would provide insight into the developmental pathways that are modulated by diet.
Overall Experimental Approach


Host-Microbe Interactions in the Neonate

What components in the infant diet affect the intestinal gene expression & microbiota?

What bacteria and their genes are involved in the interaction?

Which human genes are involved in the interaction and respond to bacterial signals?

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

<table>
<thead>
<tr>
<th></th>
<th>Breastfed (BF)</th>
<th>Formula-fed (FF)</th>
</tr>
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<tbody>
<tr>
<td>N</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>Maternal Age (years)</td>
<td>29.5 ± 4.2</td>
<td>29.8 ± 4.9</td>
</tr>
<tr>
<td>Infant Birth Weight (kg)</td>
<td>3.78 ± 0.56</td>
<td>3.51 ± 6.2</td>
</tr>
<tr>
<td>Infant Birth Length (cm)</td>
<td>52.5 ± 5.5</td>
<td>51.0 ± 2.8</td>
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</tbody>
</table>

Milk Intake & Infant Growth

- No significant difference in intake or body weight gain
Stool Sample Collection

• 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
mRNA Isolation & Microarray

• Poly A+ RNA was isolated from sloughed epithelial cells to enrich mammalian RNA using established methods (U.S. Patent 6258541)

• Previous studies have shown that mRNA isolated from colonic mucosa or stool hybridizes to biotinylated oligo dT, whereas bacterial RNA or DNA from stool do not (Davidson et al., 1995)

• mRNA samples analyzed using the Human Whole Genome Expression Bioarray (CodeLink™, Applied Microarray, Tempe, AZ) (Davidson et al., 1995)
These 146 genes were subjected to further analyses

- **Linear Discriminant Analysis (LDA)**
  - What genes or combinations of genes provide the best “classifiers” of a breast-fed vs. formula-fed infant?

- **Gene Networks (Metacore™, GeneGo, St. Joseph, MI)**
  - What gene networks are involved based on known relationships between genes?

Linear Discriminant Analysis (LDA)

2-Gene Combination

- Uncoupling Protein 2
- Endothelial PAS Domain Protein 1

Formula
- Fed
- Breast-fed

3-Gene Combination

- Synaptophysin
- Forkhead box protein E3
- Endothelial PAS Domain Protein 1

Formula
- Fed
- Breast
- Fed
## Best Genes from LDA For Classifying BF vs FF

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

- Signal transduction
  - WNT
  - NOTCH
  - TGF-β

- Cytoskeleton remodeling
  - Cell migration

- Cell adhesion
  - Barrier function

- Immune response
  - Inflammation
  - Histamine

From: Metacore™, Thomson Reuters, 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

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Host-Microbe Interactions in the Neonate

What components in the infant diet affect the intestinal gene expression & microbiota?

- Intestinal Microbiome
- Host Genome
- Bacterial Components & Metabolites

What bacteria and their genes are involved in the interaction?

Which human genes are involved in the interaction and respond to bacterial signals?

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.

Fecal Microbiota of BF and FF Infants

- 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

- 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?
SEED level 1 functional categorization via MG-RAST revealed that:

- A larger proportion of genes involved in CHO metabolism in FF
- A larger proportion of genes were involved in AA and protein metabolism in BF
- **virulence characteristics** differed between FF and BF babies
Multivariate Analysis of Host Transcriptome and Functionally-Profiled Microbiome Data

11 Host Immunity Genes Most Related to Microbial Virulence Genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Expression</th>
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<tbody>
<tr>
<td>TACR1</td>
<td>neurokinin (NK) 1 receptor; member of the tachykinin family of G-protein-coupled receptors</td>
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<tr>
<td>VAV2</td>
<td>Guanine-nucleotide exchange factor</td>
<td></td>
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<tr>
<td>ALOX5</td>
<td>Lipoxygenase gene; synthesis of leukotrienes from arachidonic acid</td>
<td></td>
</tr>
<tr>
<td>NDST</td>
<td>GlcNAc N-deacetylase/N-sulfotransferase-1; heparin sulfate synthesis</td>
<td></td>
</tr>
<tr>
<td>REL</td>
<td>Member of Rel/NFKB family</td>
<td></td>
</tr>
<tr>
<td>BPI1</td>
<td>Bactericidal/permeability-increasing protein-like 1; LPS binding protein</td>
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<tr>
<td>AOC3</td>
<td>Mediates the binding of lymphocytes to vascular endothelial cells in an L-selectin-independent fashion</td>
<td></td>
</tr>
<tr>
<td>KLRF1</td>
<td>NK Cell Receptor; stimulates natural kill cell cytotoxicity</td>
<td></td>
</tr>
<tr>
<td>DUOX2</td>
<td>NADPH oxidase; lactoperoxidase-mediated antimicrobial defense</td>
<td>Up-regulated in BF vs FF</td>
</tr>
<tr>
<td>IL1A</td>
<td>Cytokine secreted by activated macrophages, IL-1 stimulates thymocyte proliferation</td>
<td>Down-regulated in BF vs FF</td>
</tr>
<tr>
<td>SP2</td>
<td>transcription factor required for expression of cell cycle- and developmentally-regulated genes</td>
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</table>
A multivariate structure relating the host immune system and microbiome virulence characteristics exists in the infant gut.

The virulence properties of the microbiota were the most responsive characteristics with respect to BF vs. 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.
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## STRONG Kids 2 (SKP2): 2012-2019

- **A Cells-to-Society Approach to Nutrition in Early Childhood**
- Supported by the Dairy Research Institute (Rosemont, IL), $1.6M
- Birth to 5 years of age (n=440)
- Recruited from hospitals in third trimester of pregnancy

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- Systematic reviews reveal that rapid growth in early infancy is a risk factor for childhood obesity\(^1\).
- Exclusive breastfeeding (**BF**) is modestly protective against excessive infant gain and later obesity, whereas exclusive formula feeding (**FF**) increases obesity risk\(^2,3\).
- In the U.S., 30-40% of infants are fed both breastmilk and infant formula (combined feeding, **CF**)\(^4\), however, few studies have examined how CF affects the risk of childhood obesity\(^5\).

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SKP 2 Data Collection

SKP Parent Survey:
- Nutrition Practices & Intake
- Day Care Practices
- Family Mealtimes
- Family Physical Activity
- Media Exposure
- Sleep patterns
- Attachment

Biometric Measures (Mom and Child):
- Height and weight (all time points)
- Stool sample (child all time points; mom at 6 weeks)
- Saliva sample (6 weeks)
- Human milk sample and milk intake (6 weeks and 6 months)
Feeding Mode in the First Year

- **Cow-milk (13%)**

% of Infants

Exclusively Breastfed

Exclusively Formula-fed

Combined-fed

Postnatal Age

1 week 6 weeks 3 months 6 months 9 months 12 months

(N=295)
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- Dairy Research Institute
Breastfeeding:
A Balance of Art and Science