Human Milk: Role of Indigenous Prebiotics and Probiotics

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Presentation Outline

• Introduction

• Indigenous Human Milk Prebiotics

• Indigenous Human Milk Probiotics

• On-going Research
  • NIH Funded Research
    • Fermentation potential & anti-rotaviral activity
  • Research in the Piglet Model
    • Microbiome and Metagenome
  • Human Infant Studies
    • Gene expression in exfoliated epithelial cells
Considerations for Additions to Infant Formula on Neonatal Health

“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”


Factors Impacting Establishment of the 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
- Bacteria in milk
- Bacteria on maternal skin
- Type of formula
- Prebiotics/Probiotics

**Neonatal Microbiome**

**First colonizers:**

- Facultative anaerobes, such as streptococci, staphylococci, enterococci, lactobacilli or enterobacteria
- Together with some strictly anaerobic ones, especially bifidobacteria

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**Microbiota of Breastfed and Formula-fed Infants**

**Breast-fed:**

- Strong prevalence of *Lactobacilli* and *Bifidobacteria*, with predominant species *B. longum*, *B. infantis* and *B. breve*.

**Formula-fed:**

- more diverse and prone to changes
- contains higher counts of *Bacteroides*, *Clostridium*, and *Enterobacteriaceae*

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Questions

- Why do differences exist between breast- and formula-fed infants?

- Are differences attributable to components present in human milk or those absent in infant formula?

- Do HMO and probiotics in human milk contribute to these differences?
  - Is human milk a synbiotic?

- How do we assess the bioactivity of human milk in neonates?
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Prebiotics in Human Milk

• Human milk contains a remarkable content and structural diversity of oligosaccharides (HMO) that act as a component of the infant’s innate immune system.

• Oligosaccharides (HMO) are the 3rd most abundant component of human milk (Kunz et al., 2000).

<table>
<thead>
<tr>
<th>(g/L)</th>
<th>Human Milk</th>
<th>Bovine Milk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Lactose</td>
<td>65</td>
<td>45</td>
</tr>
<tr>
<td>Oligosaccharides</td>
<td>10-15</td>
<td>0.05-0.08</td>
</tr>
<tr>
<td>Neutral</td>
<td>6-10</td>
<td>trace</td>
</tr>
<tr>
<td>Acidic</td>
<td>1.5-5</td>
<td>0.03-0.06</td>
</tr>
<tr>
<td>Protein</td>
<td>10</td>
<td>35</td>
</tr>
</tbody>
</table>
Complexity of HMO

- Structural complexity of HMO is not observed in any other species
- Up to 200 different forms with masses up to 6000 Da have been identified in HM (Ninonuevo et al. 2006)
- Core structures are elongated and modified by fucosyl- and or sialyltransferases
  - Length
  - Branching (β1-3 vs. β1-6)
  - Charge (neutral and acidic)

Lactosamine

Lactose

Gal β1-4 GlcNAc

β1-3 Gal β1-4 Glc

Lacto-N-Tetraose (LNT)

Elongation (n=1-15 units):
- Lactosamine: Gal β1-4 GlcNAc
- Lacto-N-biose: Gal β1-3 GlcNAc

Modification:
- Fucose: Fuc (α 1-2, 1-3, α1-4)
- N-Acetylneuraminic acid: NeuAc (α2-3, α2-6)

Structure of an HMO
Proposed Synthetic Pathways of Human Milk Fucosyloligosaccharides

Lewis Blood Group & HMO Quantity

<table>
<thead>
<tr>
<th></th>
<th>Fucosyltransferase (FucT)</th>
<th>% Population</th>
<th>Forms of HMO</th>
<th>HMO (g/L)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Neutral</td>
<td>Acidic</td>
</tr>
<tr>
<td>Lewis a\text{b}^+</td>
<td>α1-2 FucT</td>
<td>70</td>
<td>&gt;200</td>
<td>9.51\text{b}</td>
<td>2.23</td>
</tr>
<tr>
<td>Secretor</td>
<td>α1-4 FucT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lewis a\text{b}^-</td>
<td>α1-4 FucT</td>
<td>20</td>
<td>50-100</td>
<td>5.58\text{a}</td>
<td>2.17</td>
</tr>
<tr>
<td>Non-Secretor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lewis a\text{b}^- (independent of secretor status)</td>
<td>α1-3 FucT</td>
<td>10</td>
<td>50</td>
<td>10.53\text{c}</td>
<td>2.48</td>
</tr>
</tbody>
</table>

Different letter superscripts, p<0.05
Adapted from:
Lewis Blood Group & HMO Composition

Three patterns of neutral HMO are observed using the high-pH anion-exchange chromatographic (HPAEC) method


*LNDFH: lacto-N-difucohexaose
**LNFP: lacto-N-fucopentaose

Implications for Infant Health?

Potential Physiological Functions

- **Systemic effects:** less than 1% of HMO are absorbed
  - Antiinflammatory
  - Reduce leukocyte extravasation (movement of leukocytes from circulation into tissues) by inhibiting binding to P-selectin (Bode et al. 2004)
  - Reduce formation of platelet-neutrophil complexes (Bode et al., 2004)

- **Local effects within the intestine:** approximately 90% of HMOs are found intact in infant’s feces.
  - Antiinflammatory (Kuntz et al., 2008)
    - 3’SL and 6’SL reduce cytokine-induced IL-8 secretion and signaling through MAP Kinase in HT-29 cells
  - Glycome modifying (Angeloni et al., 2005; Bode and colleagues)
    - Exposing CaCo-2 cells to 3’SL diminished content of cell surface sialic acid, fucose and galactose
  - Prevent attachment of pathogens (Newburg et al., 2005)
    - Acting as a receptor mimetic
  - Serve as prebiotics and stimulate growth of biofidobacteria (Ward et al., 2007; LoCasio et al., 2007)
Host-Pathogen Interactions through Oligosaccharides

HMO as Receptors for Microbes

<table>
<thead>
<tr>
<th>Receptors</th>
<th>Microorganisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mannose-containing glycoproteins</td>
<td><em>Escherichia coli</em> (type 1 fimbriae)</td>
</tr>
<tr>
<td>Fucosylated oligosaccharides</td>
<td><em>E. coli</em> (heat-stable enterotoxin)</td>
</tr>
<tr>
<td>Fucosylated tetra- and pentasaccharides</td>
<td><em>E. coli</em></td>
</tr>
<tr>
<td>Sialyl((α2-3)lactose and glycoproteins</td>
<td><em>E. coli</em> (S-fimbriae)</td>
</tr>
<tr>
<td>Sialyl((α2-3)galactosides in mucus</td>
<td><em>E. coli</em> (S-fimbriae)</td>
</tr>
<tr>
<td>Neutral oligosaccharides (LNT, neo-LNT)</td>
<td><em>Streptococcus pneumoniae</em></td>
</tr>
<tr>
<td>Galβ(1–4)GlcNAc or Galβ(1–3)GlcNAc</td>
<td><em>Pseudomonas aeruginosa</em></td>
</tr>
<tr>
<td>Fucα1–2Gal epitopes</td>
<td><em>Candida albicans</em></td>
</tr>
<tr>
<td>Sialyl-lactose</td>
<td><em>Helicobacter pylori</em></td>
</tr>
<tr>
<td>Sialyl-lactose</td>
<td><em>Streptococcus suis</em></td>
</tr>
<tr>
<td>Sialyl-lactose and sialylated glycoproteins</td>
<td><em>H. pylori</em></td>
</tr>
<tr>
<td>Sialylated glycoproteins (α2-3-linked)</td>
<td><em>Mycoplasma pneumoniae</em></td>
</tr>
<tr>
<td>Sialylated poly-N-acetylactosamine</td>
<td><em>M. pneumoniae</em></td>
</tr>
<tr>
<td>Sialylated (α2-3)poly-N-acetylactosaminoglycans</td>
<td><em>Streptococcus suis</em></td>
</tr>
<tr>
<td>Sialyl((α2-6)lactose)</td>
<td><em>Influenzavirus A</em></td>
</tr>
<tr>
<td>Sialyl((α2-3)lactose)</td>
<td><em>Influenzavirus B</em></td>
</tr>
<tr>
<td>9-O-Ac of NeuAc((α2-3)R)</td>
<td><em>Influenzavirus C</em></td>
</tr>
</tbody>
</table>

Are HMO Prebiotics?

• Three criteria for a carbohydrate to be classified as a prebiotic:
  1) resistant to absorption and hydrolysis by gastric enzymes,
  2) fermentable by gut microflora, and
  3) selectively stimulate the growth and/or activity of beneficial intestinal bacteria

HMO Utilization by Bifidobacterium

• Research conducted by Robert Ward and Riccardo LoCascio, in collaboration with Bruce German, David Mills and Carlito Lebrilla

• Demonstrated preferential HMO consumption by select bifidobacterial strains
  • B. longum bv. infantis (ATCC 15697) grew best on HMO
  • B. longum bv. infantis consumed 64% of HMO; mostly fucosylated; DP<7
  • B. longum bv. longum and B. breve consumed 35 and 24%, respectively, of LNT, a non-fucosylated HMO

HMO Utilization by Bifidobacterium

- Enzymatic assays showed that B. longum bv. infantis had 16.6 and 33.7-fold higher sialidase activity than B. longum bv. longum and B. breve when grown on lactose.

- In addition, fucosidase activity was only present in B. longum bv. infantis and was only detected when grown on HMO.

- Thus, the ability to consume HMO is reflected in differences in growth rates and is supported by their inherent enzymatic capacities.

- Small mass HMOs are selectively metabolized by select bifidobacterial strains and represent a potential new class of bioactive molecules functioning as prebiotics to facilitate a protective gut colonization in breast-fed newborns.
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Bacteria in Human Milk

• Culture-dependent analyses of human milk have shown that it contains $10^3$ to $10^6$ cfu/ml live:
  • Staphylococci, Streptococci, Lactic Acid Bacteria, Enterobacteria and Bifidobacteria (Heikkila et al; 2003; Martin et al., 2007; 2009)

• Culture-independent analyses of have shown that human milk contains mRNA for 16s rDNA for many bacterial species
  • Dominant genera: Streptococcus, Staphylococcus and Corynebacterium

• Potential sources of bacteria in milk:
  • Maternal skin
  • Reflux from infant during suckling
  • Maternal PBMC/milk cells

• Do they have a function in the neonate?
Human Milk Microbiota based on 16s rDNA

- Isolated bacterial DNA was used to amplified the V6-V8 regions of rDNA by PCR
- Amplicons were separated by Temporal Temperature Gradient Gel Electrophoresis (TTGE) and selected bands were sequenced.
- Samples:
  - Lactating mothers
  - peripheral blood mononuclear cells (PBMC)
  - milk cells
  - fecal samples
  - Infant fecal samples
  - PBMC from age-matched, non-pregnant, non-lactating women


HM Microbiota based on 16s rDNA

![Diagram showing microbiota](image)

Milk contained <10³ cfu/ml

Perez et al. *Pediatrics* 2007;119:e724
Summary of Perez Study

- Some bacterial signatures were common to infant samples and maternal samples.

- Data from mice demonstrated bacterial translocation from MLN to mammary gland during late pregnancy/early lactation.

- Proposed that intestinally-derived bacterial components are transported into milk within mononuclear cells.

- Exposure to specific bacterial molecular patterns programs the neonatal immune system to recognize and respond appropriately to pathogens and commensals.

Perez et al. *Pediatrics* 2007;119:e724

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Host-Microbe Interactions

Mechanisms by which HMO Protect against RV (R01 HD061929)

- Objective is to determine how HMO influence activation of dendritic cells (DC) and lymphocyte responses to vaccination against rotavirus (RV) and a subsequent RV challenge in the piglet model.

- Our central hypothesis is that HMO will enhance immune function by two inter-related mechanisms, by directly interacting with dendritic cells and, indirectly, by altering the intestinal microbiota.
  - First, we postulate that HMO glycans interact with c-type lectin receptors on DC, which induce in DC activation and activation of B- and T-lymphocytes.
  - Second, we hypothesize that HMO will promote the growth of Lactobacillus and Bifidobacterium, leading to fermentation of oligosaccharides and enhanced mucosal resistance to RV infection.
Inhibition of RV Infectivity by HMO

**FFU Assay:**
- MA-104 cells in MEM + 5% FBS
- Pre-incubate Oligo + OSU (P9[7], G5) RV at RT for 30 min
- Inoculate cells with HMO + RV and further incubate for 30 min at 37°C
- Rinse cells with PBS and incubate overnight at 37°C in MEM alone
- RV-infected cells detected by immunohistochemistry

- No inhibition by 2 FL, LnNT, Polydextrose or Galactooligosaccharides

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**Effect of Age and Diet on In Vitro Fermentation of Oligosaccharides**

- In collaboration with Dr. George Fahey (University of Illinois)
- Ascending colon contents of
  - Formula-fed and sow-reared piglets
  - Days 9 and 17 postnatal age
- Substrates (80 mg/tube):
  - scFOC, GOS/PDX (2:1), LNnT and HMO (isolated)
- Pull times: 0, 2, 4, 8 and 12h
- Analyses:
  - pH and total gas production completed
  - SCFAs and Lactate (on-going)
pH Change by Substrate

Gas Production by Substrate
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Which Bacteria and Their Genes are Involved in the Interactions?

• Microbial DNA and RNA were harvested from cecal contents of 21-day-old sow-reared or formula-fed piglets (n=4 group).

• Pyrosequencing-based whole transcriptome shotgun sequencing was used to evaluate community wide gut microbial gene expression in cDNA libraries and 16S rDNA amplicons were sequenced on the Roche 454 GS-FLX Titanium system.

• Screened cDNA sequences were assigned functional annotations by the MG-RAST annotation pipeline and based upon best-BLASTX-hits to the NCBI COG database.

Microbial Community Structure in Cecal Contents from d21 Sow-reared and Formula-fed Piglets

- 16S rDNA amplicon sequences classified to the highest taxonomic level to which they could be confidently assigned using the RDP classification algorithm and taxonomic hierarchy.


A Core Microbial Metatranscriptome in the Piglet Cecum

A) Mean relative abundances of annotated sequences within cDNA libraries from all 8 piglets. Displayed are the automated SEED Level 1 Subsystem assignments, as determined by MG-RAST.

B) Projection of the global metabolic profiles onto the KEGG pathways using the iPath tool. Metabolic pathways common to both diets are shown in blue. Pathways unique to sow-reared piglets are represented in green, and pathways unique to the formula-fed are represented in red.
Conclusions

- Communities were similar at the level of phylum, but were dissimilar at the level of genus
  - *Prevotella* was the dominant genus in sow-reared samples and *Bacteroides* was most abundant in formula-fed samples.

- Patterns of gene expression were very similar in sow-reared and formula-fed piglets.
  - All were enriched for carbohydrate and protein metabolizing enzymes and proteins involved in stress response, binding to host epithelium and LPS metabolism.

- The abundance of enzymes involved in several pathways related to amino acid metabolism (e.g. arginine metabolism) and oxidative stress response differed in sow-reared and formula-fed animals.

- Abundant transcripts identified in this study may contribute to a core microbial metatranscriptome in the distal intestine.

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Noninvasive Stool-based Detection of Infant Gastrointestinal Development

<table>
<thead>
<tr>
<th></th>
<th>Breastfed (BF)</th>
<th>Formula Fed (FF)</th>
</tr>
</thead>
<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>
</tr>
</tbody>
</table>


Microarray Analysis

- Poly A+ RNA was isolated to enrich mammalian RNA and were analyzed using the Human Whole Genome Expression Bioarray according to CodeLink™ Gene Expression Assay protocols (Applied Microarray, Tempe, AZ) (Davidson et al., 1995)

- Median expression for 33 housekeeping genes were calculated and used to normalize the expression of the 4,250 common "good" spots present on all 22 arrays.
  - 1214 had p-values < 0.05.
  - Using prior knowledge of 529 genes involved in intestinal biology, 146 genes differentially expressed between BF and FF were identified.

- These genes were subjected to further analyses
  - Linear Discriminant Analysis (LDA)
  - Coefficient of Determination (CoD)
  - Gene Networks (Metacore™, GeneGo, St. Joseph, MI)
LDA - Best Genes 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; immunomodulatory</td>
<td>5.5</td>
</tr>
<tr>
<td>PCDH7</td>
<td>Encodes protocadherin-7; membrane protein; cell adhesion</td>
<td>3.9</td>
</tr>
<tr>
<td>ITGB2</td>
<td>Encodes integrin beta-2 (CD18); ICAM-1 receptor; cell adhesion</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-oncogene</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>

BF vs FF Infants (2-Gene Classification)

![BF vs FF Infants (2-Gene Classification)](image)
BF vs FF Infants (3-gene Classification)

Metacore™ Gene Networks – BF vs FF Infants

- Signal transduction
  - WNT
  - NOTCH
  - TGF-β

- Cytoskeleton remodeling
  - Cell migration

- Cell adhesion
  - Barrier function

- Immune response
  - Inflammation
  - Histamine

(Metacore, GeneGo, St. Joseph, MI)
Summary & Conclusions

- The content and structural diversity of HMO confer a vast array of physiological functions to human milk
  - It will likely be impossible to recapitulate this activity in infant formula with prebiotics
  - An understanding of the structure-function relationships for HMO will facilitate the selection/synthesis of prebiotics for addition to infant formula

- Gene expression biomarkers that are sensitive to nutrition can be used to
  - Understand HMO bioactivities
  - Evaluate the efficacy of novel ingredients towards optimizing intestinal function and provide therapeutic targets for future formula additives.

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