A Milk-Oriented Microbiota (MOM) in Infants—How Babies Find their MOMs

Insights into next generation prebiotics & probiotics

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Peter J. Shields Endowed Chair
Dept. Food Science & Technology
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The probiotic concept

“the Bulgarian bacillus became a rage, companies were formed, and their directors grew rich off selling these silly bacilli.”

Paul de Kruif “The Microbe Hunters” 1926

Proposed fermented milks would contain factors (microbes) that prevent putrification → they should also help prevent putrification in the gut
Probiotics & prebiotics

Definitions and misperceptions

**Probiotics** – “live microorganisms that **when administered in adequate amounts** confer a health benefit on the host” (UNFAO/WHO 2001).

**Prebiotics** – “a prebiotic is a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora, that **confer benefits upon host well-being and health**” (J. Nutr. 2007 137:830S-837S)

**Synbiotics** – combinations of prebiotics and probiotics
Two consumers...infants and the infant gut microbiota

“…to be constituted, by microscopic examination, of only one species, *Bacterium bifidus*, a strictly anaerobic bacterium…”


“The bifidus factor contains in various proportion, lactose, galactose, fucose, N-acetylglucosamine and N-acetylneuraminic acid (sialic acid)”
What factors in milk shape the microbiota?
Human milk composition

- Macro- and micronutrients
  - Lactose
  - Water

- Milk
  - Macro-/Micronutrients
    - HMOs
    - Protein
      - Immunoglobulins
      - Lysozyme
      - Lactoferrin
        → Lactoferricin
      - Triglycerides
        → Free fatty acids

- Microbiota shaping
  - Immunoglobulins
  - Lysozyme
  - Lactoferrin
  → Lactoferricin
  - Triglycerides
  → Free fatty acids

Food!

Newburg 2005, 2009
Human Milk Glycans

HMOs
Protein
Lipids
Lactose

A. HMO
- α1-2 Fucosidase
- α1-3/4 Fucosidase
- β1-3 Galactosidase
- β1-4 Galactosidase
- α2-6 Sialidase
- α2-3 Sialidase
- β1-3 N-Acetylglucosaminidase
- β1-6 N-Acetylglucosaminidase
- β1-4 Galactosidase (lactase)
- Lacto-N-biosidase

B. O-linked glycans
- Core 1
- Core 2
- Core 3

C. Glycolipids
- Galactose
- Glucose
- N-Acetylglucosamine
- Fucose
- Sialic acid
- Ceramide

D. Complex N-glycan

Garrido et al Microbiology 2013
Human milk oligosaccharides

- HMOs
- Lactose
- Lipids
- Protein

Chain Length
- 4
- 5
- 6
- 7
- 8
- 9
- 10

Other HMOs of longer lengths

Nature 468 S5-S7 (23 December 2010)
Garrido et al Microbiology (2013)

- Human indigestible and highly variable
- Higher proportion of fucosylated (40-70%) than sialylated (4-38%)
- Nearly 200 species in pooled human milk
Why Make Glycoconjugates/Free Glycans?
(if they are not consumed by the infant)

- Pathogen deflection
  - Immune development
  - Neural development
  - Enrich specific microbes

Bode 2009
HMO Structural Diversity

150 – 200 different HMO

Courtesy of Lars Bode
Breast milk enriches bifidobacterial populations

Fallani et al. Microbiology 2011

Grzeskowiak et al. JPGN 2012

South Eastern African and Northern European
Malawi
N=44

Finland
N=31

Bifidobacterium

Penders et al. Pediatrics 2006

Quantitative PCR
Breast milk enriches bifidobacterial populations

“Most shotgun and 16S rRNA V4 sequences (75 ± 20%) in all babies mapped to members of the Bifidobacterium genus.”
n=110 babies
1 month of age
Measured by qPCR

Influence of Maternal Bifidobacteria on the Establishment of Bifidobacteria Colonizing the Gut in Infants

KATSUNAKA MIKAMI, HIDENORI TAKAHASHI, MOTO KIMURA, MITSUHIRO ISOZAKI, KUNIO IZUCHI, RUMIKO SHIBATA, NOBUYUKI SUDO, HIDEO MATSUMOTO, AND YASUHIRO KOGA

Laboratory for Infectious Diseases [K.M., H.T., M.K., Y.K.], Department of Psychiatry and Behavioral Science [K.M., H.M.], and
Department of Human Genetics, Fukuoka 812, Japan.

Mikami et al. Pediatrics Research 2009
Lewis et al (in prep)

48 breast fed infants
4 time points
(Day 6, 21, 71, 120)

High Bifs

<10^7.6/g feces, average 10^6.1

Low Bifs

>10^8.9/g feces, average 10^{10.3}

Zach Lewis
Why bifidobacteria?
Bifidobacteria can protect from enteropathogenic infection through production of acetate

Shinji Fukuda, Hidehiro Toh, Koji Hase, Kenshiro Oshima, Yumiko Nakanishi, Kazutoshi Yoshimura, Toru Tobe, Julie M. Clarke, David L. Topping, Tohru Suzuki, Todd D. Taylor, Kikuji Itoh, Jun Kikuchi, Hidetoshi Morita, Masahira Hattori, Hiroshi Ohno

Mouse survival after O157 infection

BA = B. animales
BL = B. longum
If bifidobacteria can grow well on a targeted sugar \textit{in situ}, growth and accompanying production of acetate is protective.
Why bifidobacteria?

Bangladesh Infant Vitamin A Study

International Center for Diarrhoeal Disease Research (Bangladesh)

Western Human Nutrition Research Center (Davis, CA)

Huda, Stephensen et al In Prep
Positive correlation between Actinobacteria and vaccine response

Huda, Stephensen et al In Prep
Do different mother’s milk glycan types influence different microbiota populations?

Secretor vs non-secretor

FUT2 ("Secretor" gene)
• Produces the 2’ fucosylated precursor to the A, B, H, and Lewis b antigens in secretions, including breast milk
• 20% of U.S. population are non-secretors
Secretor vs. non-secretor milk protects differently

Incidence of diarrhea per 100 child-months

Morrow et al J. Pediatrics 2004
48 breast fed infants
4 time points
(Day 6, 21, 71, 120)

<10^{7.6}/g feces, average 10^{6.1}

>10^{8.9}/g feces, average 10^{10.3}

Low Bifs

High Bifs
• 48 breast fed infants
• 4 time points
• (Day 6, 21, 71, 120)
Do different mothers milk glycan types influence different microbiota populations?

% Babies with bifidobacteria established (>10^9)

- **B. longum**
  - Secretor n=15 babies
  - Non-secretor n= 8 babies

- **B. breve**

Poster 24

Lewis et al (in prep)
Are specific glycans consumed by bifidobacteria *in situ*?

- Feces from breast fed infant
  - Glycoprofile
  - Microbial ecology

Are oligos missing in feces in which bifidobacteria are dominant?
Feces Oligosaccharides of Term Infant Vary With Bacterial Population

**Fecal HMO Profile**

**FULL TERM INFANT**

**Fecal Bacterial Profile**

**B. longum/infantis**

**Bacteria**
- Other
- Staphylococcaceae
- Enterobacteriaceae
- Coriobacteriaceae
- Streptococcaceae
- Bifidobacteriaceae
- Bacteroidaceae

**HMO neutral mass**
- 709
- 855
- 1001
- 1074
- 1148
- 1221
- 1367
- 1440
- 1513
- 1586
- 1732
- 1805
- 1878
- 1951
- 2097

**Lorna de Leoz**
Specific oligosaccharides are consumed in the gut

Fecal HMO Profile

TERM INFANT

Glycan Specific Changes

Carlito Lebrilla
Lorna de Leoz
UCD Chemistry
(Submitted)
We need to be cautious on associations.
HMOs as “prebiotics” for Bifidobacteria

Which bifidobacterial species grow on HMO?
Which bifidobacteria grow on HMO sugars?

40 breast-fed infant (3-4 month old) stool samples
300 isolates → 74 characterized

<table>
<thead>
<tr>
<th>Growth</th>
<th>Structure</th>
<th>B. infantis</th>
<th>B. bifidum</th>
<th>B. longum</th>
<th>B. breve</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMO</td>
<td>22/22</td>
<td>14/14</td>
<td>8/17</td>
<td>10/23</td>
<td></td>
</tr>
<tr>
<td>Lacto-N-tetraose</td>
<td>22/22</td>
<td>14/14</td>
<td>17/17</td>
<td>23/23</td>
<td></td>
</tr>
<tr>
<td>Lacto-N-neotetraose</td>
<td>22/22</td>
<td>14/14</td>
<td>2/17</td>
<td>23/23</td>
<td></td>
</tr>
<tr>
<td>2'-fucosyl lactose</td>
<td>22/22</td>
<td>13/14</td>
<td>1/17</td>
<td>2/23</td>
<td></td>
</tr>
<tr>
<td>3-fucosyl lactose</td>
<td>22/22</td>
<td>14/14</td>
<td>1/17</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>6-sialyl lactose</td>
<td>22/22</td>
<td>10/14</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Multilocus sequence tagging

Santi Ruis Moyano

Bifidobacteria vs Bacteroides *in situ*

Lacto-N-neotetrose supplementation of gnotobiotic mice with *Bifidobacterium infantis* and *Bacteroides thetaiotaomicron*.
Several small MW oligosaccharides consumed by B. infantis

Single HMO composition consumed by other bifidobacteria

Locascio et al JAFC 2007
NanoLC separation of individual HMO compositions

m/z 611.2387
### Bacteria can be characterized by their HMO consumption profile.

- Orange circles are sized proportionally to the percent consumption.

What genome features are required to utilize human milk oligosaccharides?
HMO utilization by Bifidobacteria

![Graph showing OD600 over time for different strains of Bifidobacteria.](image)

- B. longum subsp. infantis ATCC15697
- B. longum subsp. longum DJO10A
- B. adolescentis ATCC15703
Comparative Bifidobacterium Genomics

B. longum subsp. infantis ATCC15697
2,832,748 bp

B. longum subsp. longum DJO10A
2,389,526 bp

Adult derived strain
BMC Genomics 2008

Infant derived strain
PNAS 2008
What’s Needed to Deconstruct HMOs?

- Transport systems for oligo & monosaccharides
- Glycosyl hydrolases
B. infantis HMO cluster

All 4 glycosyl hydrolases Array of oligosaccharide transporters

0 10 kb 20 kb 30 kb 40 kb

permease permease permease permease SBP permease

Genes unique to milk-associated bifidobacteria are uniquely expressed during growth on milk sugars (PLoS One 2013, unpublished)

- ATP hydrolysis prompts transport of oligosaccharides across membrane
- Intracellular glycolytic enzymes deconstruct oligosaccharide

Sela PNAS 2008
Characterization of the glycosidases and transporters from *B. infantis*?

Sialidases - ------------------ Sela et al JBC 2011
Fucosidases - --------------- Sela et al AEM 2012
Hexosaminidases - -------- Garrido et al Anaerobe 2012
Galactosidases - ----------- Garrido et al Food Micro 2012
Growth on milk oligosaccharides helps some bifidobacteria bind intestinal cells.

HMO vs Lac grown cells:
- Induce TJ proteins
- Induce anti-inflammatory cytokines (IL-10)

Chichlowski et al JPN 2012
Model for bifidobacterial enrichment in the infant GIT
Model for bifidobacteria enrichment in the infant GIT

Complex milk glycans enhance efficacy of specific bifidobacteria
Can this knowledge be translated?
Will synbiotic feeding HMO$^+$ *B. infantis* with HMOs help establish bifidobacteria?

![Graph showing OD vs Time for HMO$^+$ and HMO$^-$ B. infantis and B. lactis](graph.png)

Mark Underwood

UCD Med School
Neonatology

Prolacta BIOSCIENCE
Advancing the Science of Human Milk

National Institutes of Health
Eunice Kennedy Shriver
National Institute of Child Health & Human Development
Take home points….

• Milk provides a model of establishing (modulating) a microbiota
• Specificity of that modulation is driven in part by glycan complexity and cognate bacterial catabolism
• Exploiting that knowledge to partner specific glycans with specific cognate bifidobacteria can enable more persistent colonization in humans

…this took detailed mechanistic research….

…mechanism leads to translational diagnostics….
Gaps/Needs/Challenges

- Which bacteria and their genes are involved in the interactions?
- Which human genes are involved and respond to bacterial signals?
- What components of the diet affect the intestinal microbiota?

Diagram:
- Human metagenome
- Intestinal microbiome
- Human genome
- Interactions
- Bacterial components and metabolites
- Diet
- Health/Disease
Gaps/Needs/Challenges

• Mechanistic research needed (systems biology + strain level examination)

• Interdisciplinary teams (thanks Vince)

• Better (supported) animal models (thanks Gary)

• Continued tool development…
  • Metabolomic/Metagenomic
  • Genetic tools!!
  • Glycomics

• Ability to stratify clinical populations
Needs/Challenges

Harmonizing of protocols – are we settled on a DNA prep?

8F forward primer misses bifidobacteria (need to spike in a 8Fbif primer)

Lack of bead beating lowers bifidobacteria
Lack of accurate annotation/function will impede or misdirect microbiome work ....
Media/Public/Physician Confusion on Probiotics

Reality of the research so far
Probiotic action occurs at the level of strains

But the public perception is not at the strain level

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Table 1. Key genera and species of microbes studied and used as probiotics

<table>
<thead>
<tr>
<th>Genus</th>
<th>Species</th>
</tr>
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<tbody>
<tr>
<td>Lactobacillus</td>
<td>acidophilus</td>
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<tr>
<td></td>
<td>brevis</td>
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<tr>
<td></td>
<td>delbrueckii</td>
</tr>
<tr>
<td></td>
<td>fermentum</td>
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<td></td>
<td>gasseri</td>
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<tr>
<td></td>
<td>johnsonii</td>
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<td></td>
<td>paracasei</td>
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<tr>
<td></td>
<td>plantarum</td>
</tr>
<tr>
<td></td>
<td>reuteri</td>
</tr>
<tr>
<td></td>
<td>rhamnosus</td>
</tr>
<tr>
<td></td>
<td>salivarius</td>
</tr>
<tr>
<td>Bifidobacterium</td>
<td>adolescents</td>
</tr>
<tr>
<td></td>
<td>animals</td>
</tr>
<tr>
<td></td>
<td>bifidum</td>
</tr>
<tr>
<td></td>
<td>breve</td>
</tr>
<tr>
<td></td>
<td>infantis</td>
</tr>
<tr>
<td></td>
<td>longum</td>
</tr>
<tr>
<td>Streptococcus</td>
<td>thermophilus</td>
</tr>
<tr>
<td></td>
<td>salivarius</td>
</tr>
<tr>
<td>Enterococcus</td>
<td>faecium</td>
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<tr>
<td>Escherichia</td>
<td>coli</td>
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<tr>
<td>Bacillus</td>
<td>coagulans</td>
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<td></td>
<td>clausli</td>
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<tr>
<td>Saccharomyces</td>
<td>cerevisiae</td>
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</tbody>
</table>

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Our kombucha is raw meaning that it has never been pasteurized nor heat treated. As a living product, new cultures will continue to form even once bottled. Sometimes they are clear, like egg whites, and other times they are brownish in color. These cultures are harmless and indicate that the beverage is live and rich in probiotics. It’s completely edible (go ahead, be bold) but if you prefer, please strain.

What’s that floating in my kombucha?
Are you delivering the probiotic species and strain you think you are?

<table>
<thead>
<tr>
<th>Product</th>
<th>Microorganisms listed on the product label</th>
<th>T-RFLP</th>
<th>Species-specific PCR</th>
<th>Additional microbe T-RFLP patterns detected</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Lb</em> acidophilus</td>
<td>+</td>
<td>+</td>
<td><em>Lb</em> brevis, <em>Lb</em> plantarum*, <em>Lb</em> johnsonii†, <em>Lb</em> amylolyticus, Lactobacillus sp†</td>
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<tr>
<td></td>
<td><em>B</em> bifidum</td>
<td>−</td>
<td>+</td>
<td></td>
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<tr>
<td></td>
<td><em>L</em> helveticus</td>
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<td><em>Lb</em> brevis, <em>Lb</em> plantarum*, <em>Lb</em> amylolyticus, Lactobacillus sp†</td>
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<tr>
<td></td>
<td><em>B</em> bifidum</td>
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<td></td>
<td><em>Lb</em> helveticus</td>
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<td><em>B</em> bifidum</td>
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</tbody>
</table>

Marcobal et al. JPGN 2008
Barcoded Pyrosequencing Reveals That Consumption of Galactooligosaccharides Results in a Highly Specific Bifidogenic Response in Humans

Lauren M. G. Davis¹, Inés Martínez¹, Jens Walter¹, Caitlin Goin², Robert W. Hutkins¹*¹

¹ Department of Food Science and Technology, University of Nebraska, Lincoln, Nebraska, United States of America, ² School of Biological Sciences, University of Nebraska, Lincoln, Nebraska, United States of America

Subject 2

Subject 3

Subject 11

Subject 12

Subject 16

Subject 17
Understanding Responder Non-Responder Issues

Davis et al PLoS One 2011
Pls: Carlito Lebrilla, J. Bruce German, Xi Chen, Mark Underwood, Chuck Bevins, Helen Raybould

Students/Postdocs: David Sela, Maciej Chichlowski, Karen Kalanetra, Santiago Ruiz-Moyano, Milady Ninonuevo, Riccardo LoCascio, Yanhong Lin, Larry Lerno, Jae Han Kim, Mariana Barboza, Scott Kronewitter, Richard Siepert, Aaron Adamson, Daniel Garrido, Angela Marcobal, Robert Ward and Samara Freeman
Acknowledgements
Conflict of Interest Statement

Co-Founder – Evolve Biosystems Inc.

Co-Founder – MicroTrek Inc.
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What about bifidobacterial growth on milk glycoproteins?

N-linked exp.  
Lactoferrin  
Immunoglobulins

O-linked exp.  
Caseins (K)
What about bifidobacterial growth on milk glycoproteins?

RNaseB as proxy N-linked glycan

Garrido et al Molecular Cellular Proteomics 2012
Endoglycosidase genes in bifidobacteria

Endo-\(\beta\)-N-acetylglucosaminidases

EndoBI-1 and EndoBI-2 active on all N-linked milk glycoproteins

Garrido et al Molecular Cellular Proteomics 2012
**Whole cell proteomics of B. infantis grown on different prebiotic sugars**

Genes unique to milk-associated bifidobacteria are uniquely expressed during growth on milk sugars.

**HMO**
- Blon_2344
- Blon_2347

**GOS**
- Blon_2414

**FOS/Inulin**
- Blon_2061

HexNAc catabolism
- Blon_2336: α-fucosidase
- Blon_0732: β-hexosaminidase
- Blon_2016: β-galactosidase
- Blon_2334: β-galactosidase
- Blon_2416: β-galactosidase
- Blon_1740: GH, family 13

Leloir pathway
- Blon_2453: GH, family 13

Bifidus shunt & glycolysis
- Blon_2056: Exo-inulinase
- Blon_0787: Exo-inulinase
- Blon_0128: Suc phosphorylase
- Blon_2460: α-galactosidase

**PLoS One 2013**

Jae Han Kim
Milk Bioactives Project activities

Human milk research

Translation

Bovine milk research

Translation

Translation

Prebiotic milk oligosaccharides

Milk-enhanced Probiotics (bifidobacteria)

Milk processing enzymes

Glycoproteins, glycopeptides, glycolipids
Whey permeate oligosaccharides

BMMI Project

Bill & Melinda Gates Foundation

Jeff Gordon Wash U
“In the 20th century we determined what food is…

…but in the 21st century we need to determine what food does.”