Modulation of infant stool microbiota by bovine milk derived - and human milk oligosaccharides

Norbert Sprenger
Milk oligosaccharides in Microbiota & Host Interactions

Microbiota growth, function & establishment

Protection from Infection

Immune competence - Allergy

Brain - ENS

Glyconutrient?
Outline

Human milk oligosaccharides
Bovine milk oligosaccharides

Sourcing of milk oligosaccharides

Human infant intervention trials with
- bovine milk derived oligosaccharides
- Synthetic human milk oligosaccharides
19 structures characterized
Total 0.1 – 0.3 g/L

ca. 130 structures characterized
Total 5 – 15 g/L

TIGG 16: 135-142
Proportions of Major HMOs

- Summary of data from ~13 articles
- Data from multiple stages of lactation
- Used medians to construct chart

Name; conc. (mg/L)
Levels and Evolution of Selected HMOs in Breast Milk

Austin et al. 2016 Nutrients
Expression of key non-fucosylated HMO according to FUT2 status

Sprenger et al. 2016, unpublished
OS in milk of major farmed animals

<table>
<thead>
<tr>
<th></th>
<th>Cow</th>
<th>Goat</th>
<th>Sheep</th>
</tr>
</thead>
<tbody>
<tr>
<td>2'FL</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3FL</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hex3</td>
<td>+</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>GalNAcLac</td>
<td>+++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3'Neu5Ac-Lac</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>6'Neu5Ac-Lac</td>
<td>++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>3'Neu5Gc-Lac</td>
<td>-</td>
<td>+++</td>
<td>++++</td>
</tr>
<tr>
<td>6'Neu5Gc-Lac</td>
<td>-</td>
<td>+++</td>
<td>++++</td>
</tr>
</tbody>
</table>

(Austin et al., unpublished)
Sourcing of OS structurally and functionally identical to HMOs

Cow milk

Whey Permeate

• Cow milk whey permeate as source for sialyllactoses, but not for fucosylated and neutral non-fucosylated HMOs like LNnT or LNT

(Austin et al., unpublished)
BMOS, bovine milk derived oligosaccharides

Synthetic processes for fucosylated- and neutral non-fucosylated LNT-LNnT-type HMOs

Downstream processing  →  Pure HMO

Companies proposing synthetic HMOs

- Chem-tech Bio-tech
- GeneChem Inc.
- Jennevein Biotechnologie GmbH
- inbiose
- Glycosyn
- GLYCOM
Infant microbiota establishment and bovine milk derived oligosaccharides
Gut microbiota analysis reveals a marked shift to bifidobacteria by a starter infant formula containing a synbiotic of bovine milk-derived oligosaccharides and Bifidobacterium animalis subsp. lactis CNCM I-3446

Umberto Simeoni,1† Bernard Berger,2*† Jana Junick,3 Michael Blaut,3 Sophie Pecquet,4 Enea Rezzonico,2 Dominik Grathwohl,2 Norbert Sprenger,2 Harald Brüssow,2** The Study Team,† Hania Szajewska,5 J.-M. Bartoli,6 V. Brevaut-Malaty,7 M. Borszewska-Kornacka,5 W. Feleszko,5 P. François,6 C. Gire,7 M. Leclaire,8 J.-M. Maurin,6 S. Schmidt,6 A. Skórka,5 C. Squizzaro6 and J.-J. Verdot6

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7 Hôpital Nord, Marseille, France
8 Hôpital de la Conception, Marseille, France

Study sponsor: Nestlé Nutrition

ClinicalTrials.gov Identifier: NCT01983072

(Simeoni et al., Env. Microbio. 2015)
**Study design**

CON (C) = Formula with 1.8 g protein /100 Kcal; whey/casein ratio 70/30

TEST = CON + *B. lactis* 2.10^7 cfu/g + BMOS 8 g/L

**Centers:** France (and Poland)

**Population:** Term-born healthy infants, C-section and Vaginal born

*(Simeoni et al., Env. Microbio. 2015)*
Bacterial counts in stool measured by qPCR

- Bifidobacterium increased in Test with most prominent effect on *B. longum*.

(Simeoni et al., Env. Microbio. 2015)
Microbiota analysis by 16S rRNA gene pyrosequencing

(Simeoni et al., Env. Microbio. 2015)
Microbiota diversity

- Intra-individual alpha-diversity of Test (versus Control) closer to BF reference

(Simeoni et al., Env. Microbio. 2015)
Principal Coordinate Biplot of Weighted UniFrac distances.

- Strong shift of Test to a bifidobacterium dominanted microbiota

(Simeoni et al., Env. Microbio. 2015)
Infant stools characteristics

- Reduced stool pH in Test and reduced hard stool, similar to BF

(Simeoni et al., Env. Microbio. 2015)
Effect of infant formula on growth, gut maturation and microbiota composition: A multicentric randomized controlled study

G. Putet¹, E. Mallet², J-M. Hascoet³, P. Steenhout⁹, B. Berger⁷, N. Sprenger⁷, D. Grathwohl⁷, J-C. Roze⁴, A. Pollak⁶, N. Haiden⁶, C. Costalos⁵, H. Bruessow⁷, N. De Groot⁸, S. Pecquet⁸, J. Benyacoub⁷, J-C. Picaud¹

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Study sponsor: Nestlé Nutrition

ClinicalTrials.gov Identifier: NCT00984230

(Unpublished)
**Study design**

CON = Control infant formula

TEST1 = IF formulation + probiotics

TEST2 = IF formulation + probiotics + BMOS 9.6 g/L

*Centers:* France, Greece, Austria

*Population:* vaginal delivery only

From 4-8w: **Standard starter formula**

<table>
<thead>
<tr>
<th>Age</th>
<th>Centres</th>
<th>N (N microbiota)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;2d</td>
<td>40 (28-16-12)</td>
<td>44 (25-16-12)</td>
</tr>
</tbody>
</table>
Microbiota analysis by 16S rRNA gene pyrosequencing

(Unpublished)

<table>
<thead>
<tr>
<th>1w</th>
<th>4w</th>
<th>8w</th>
</tr>
</thead>
<tbody>
<tr>
<td>BF</td>
<td>CON</td>
<td>TEST1</td>
</tr>
</tbody>
</table>

- **Bifidobacterium longum**, **B. pseudocatenulatum**, and **B. bifidum** were the dominant bifidobacteria identified in TEST2 infants

(Unpublished)
### Microbiota analysis by 16S rRNA gene pyrosequencing

(Unpublished)

<table>
<thead>
<tr>
<th>Week</th>
<th>BF</th>
<th>CON</th>
<th>TEST1</th>
<th>TEST2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1w</td>
<td>BF</td>
<td>CON</td>
<td>TEST1</td>
<td>TEST2</td>
</tr>
<tr>
<td>4w</td>
<td>BF</td>
<td>CON</td>
<td>TEST1</td>
<td>TEST2</td>
</tr>
<tr>
<td>8w</td>
<td>BF</td>
<td>CON</td>
<td>TEST1</td>
<td>TEST2</td>
</tr>
</tbody>
</table>

**Bifidobacterium p=0.001 (rank test)**

- **Shift of Test2 to a bifidobacterium dominanted microbiota during the intervention period**

* = P<0.05
** = P<0.01

(Unpublished)
Global differences between the bacterial communities by Canonical Correspondence Analysis at genus level

- TEST2 most similar to BF group, but only during the intervention period

(Unpublished)
Infant microbiota establishment and synthetic human milk identical oligosaccharides
Term infant formula supplemented with human milk oligosaccharides (2’Fucosyllactose and Lacto-N-neotetraose) shifts stool Microbiota and Metabolic Signature Closer to That of Breastfed Infants

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Study sponsor: Nestlé Nutrition

ClinicalTrials.gov Identifier: NCT01715246
Study Objectives

**Overall objective:**
- Assess the effect of term infant formula supplemented with HMOs (2’FL 1g/L + LNnT 0.5g/L) on growth, tolerance, stool microbiota and metabolic signature in healthy infants in a randomized, double-blind, controlled clinical trial

**Microbiota related objectives:**
- To compare stool microbiota profile and metabolic signature among infants fed Control formula\(^1\), infants fed Test formula\(^2\), and non-randomized exclusively breastfed (BF) infants
- **Hypothesis:** stool microbiota profile & metabolic signature in infants fed Test (vs. Control) will be closer to that of BF infants

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1. Control formula: intact protein, cow’s-milk-based, whey-predominant infant formula
2. Test formula: identical to Control except for the addition of 2’FL (1.0 g/L) and LNnT (0.5 g/L)
**Study Design**

**Centers:** Italy and Belgium  
**Population:** Term-born healthy infants, C-section and Vaginal born

2. Test: identical to Control except for the addition of 2’FL (1.0 g/L) and LNNt (0.5 g/L).  
3. A non-randomized reference group of exclusively breastfed infants was enrolled at 3 months of age.
Sample Size for Microbiota-related Analysis

Breastfed infants

- Non-randomized
- BF (n=38)

3-mo Stool Sampling

- Bacterial abundance and diversity
  - for 16S (n=33)
- Specific GI pathogens
  - for GPP (NA)
- Stool metabolic signature
  - for NMR (n=32)

Formula-fed infants

- Randomized
- Control (n=87)
- Test (n=88)

- for 16S (n=63)
- for GPP (NA)
- for NMR (n=64)

- for 16S (n=58)
- for GPP (n=54)
- for NMR (n=57)

Sample size is the per-protocol population.
16S: 16S rRNA sequencing; GPP: Luminex xTAG Gastrointestinal Pathogen Panel (GPP); NMR, Nuclear Magnetic Resonance Spectroscopy
NA: analysis was not conducted due to insufficient samples
Baseline Characteristics of Subsets

<table>
<thead>
<tr>
<th>Characteristics¹</th>
<th>BF (n=36)</th>
<th>Control (n=64)</th>
<th>Test (n=58)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, n (%) girls</td>
<td>10 (28%)</td>
<td>31 (48%)</td>
<td>29 (50%)</td>
</tr>
<tr>
<td>Gestational age, weeks</td>
<td>39.3 (38.9-39.6)</td>
<td>39.2 (38.9-39.4)</td>
<td>39.3 (39.0-39.6)</td>
</tr>
<tr>
<td>Delivery mode, n (%) C-section</td>
<td>13 (36.1%)</td>
<td>23 (35.9%)</td>
<td>19 (32.8%)</td>
</tr>
<tr>
<td>Siblings, yes n (%)</td>
<td>21 (58.3%)</td>
<td>43 (67.2%)</td>
<td>35 (60.3%)</td>
</tr>
<tr>
<td>Weight, kg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>girls</td>
<td>3.4 (3.1-3.7)</td>
<td>3.3 (3.2-3.5)</td>
<td>3.3 (3.1-3.5)</td>
</tr>
<tr>
<td>boys</td>
<td>3.5 (3.3-3.6)</td>
<td>3.4 (3.3-3.6)</td>
<td>3.4 (3.3-3.5)</td>
</tr>
<tr>
<td>Length, cm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>girls</td>
<td>49.9 (48.6-51.1)</td>
<td>49.6 (48.9-50.4)</td>
<td>49.4 (48.8-50.1)</td>
</tr>
<tr>
<td>boys</td>
<td>50.4 (49.9-51.0)</td>
<td>50.4 (49.9-50.9)</td>
<td>50.4 (49.7-51.1)</td>
</tr>
<tr>
<td>HC, cm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>girls</td>
<td>34.5 (33.7-35.3)</td>
<td>34.1 (33.6-34.6)</td>
<td>33.9 (33.4-34.4)</td>
</tr>
<tr>
<td>boys</td>
<td>34.5 (33.9-35.1)</td>
<td>34.5 (34.1-35.0)</td>
<td>34.2 (33.7-34.7)</td>
</tr>
</tbody>
</table>

• Baseline characteristics of the subsets for stool microbiota/metabolic signature analysis are comparable among Control, Test, and BF groups

1. Data are presented as count (%) or mean (95% confidence interval)  
   HC: head circumference.
**Bacterial Composition**

- Distinctive stool bacterial profile was observed in BF vs. formula-fed infants.
- Stool bacterial profile in Test (vs. Control) appeared to be closer to BF infants.
Bacterial Diversity

**Alpha diversity:** intra-individual diversity

**Beta diversity:** inter-individual diversity

Diversity of BF significantly lower than formula groups ($p<0.01$); Diversity of Test significantly lower than Control ($p<0.05$) and therefore closer to BF

- Distinctive diversity pattern was observed in BF vs. formula-fed infants
- Both Alpha and Beta diversity of Test (vs. Control) was closer to BF infants
Specific Bacterial Abundance

Wilcoxon rank sum test at genus level, significant difference indicated by *p<0.05, **p<0.01, and ***p<0.001; False Discovery Rate (FDR) adjusted P-values between Test and Control groups are 0.16 for *Bifidobacterium* & *Escherichia*, and 0.26 for *Peptostreptococcaceae uncl*.

- Test significantly differed from Control on three bacterial genera, closer to BF
- HMOs promote the colonization of potentially beneficial *Bifidobacterium* and reduce potentially pathogenic *Escherichia* and *Peptostreptococcaceae uncl*
Microbiota at genus level by Dirichlet partitions

main partitions at 3 months

Partitions at genus level generated from 3 and 12 months microbiota data (unsupervised).
Microbiota at genus level by Dirichlet partitions

7 microbiota community types by Dirichlet partitioning of microbiota at genus level:

- * p<0.05; ** p<0.01

- Test increased number of infants breastfed typical microbiota community type and decreased those with formula specific community type

@ 3 months

@ 12 months

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### Specific Pathogen Load

**Number of infants with presence of at least one pathogen in stool at 3 months of age**

<table>
<thead>
<tr>
<th>Pathogens</th>
<th>Control</th>
<th>Test</th>
<th>Odds ratio (95% CI) of Test vs. Control</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viral</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenovirus 40/41</td>
<td>0/54</td>
<td>4/50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Norovirus GI/GII</td>
<td>15/54</td>
<td>10/50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rotavirus A</td>
<td>2/54</td>
<td>1/50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacterial</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. difficile toxin A/B</td>
<td>12/54</td>
<td>7/50</td>
<td>0.465 (0.170 – 1.270)</td>
<td>0.149</td>
</tr>
<tr>
<td>Campylobacter</td>
<td>1/54</td>
<td>0/50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli O157</td>
<td>1/54</td>
<td>0/50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ETEC LT/ST</td>
<td>0/54</td>
<td>0/50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>STEC stx1/stx2</td>
<td>0/54</td>
<td>0/50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vibrio cholerae</td>
<td>0/54</td>
<td>0/50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yersinia enterocolitica</td>
<td>0/54</td>
<td>0/50</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- Pathogens were detectable in a relatively small proportion of infants, as expected in a healthy infant population.
- A numerically smaller proportion of infants in Test (vs. Control) had detectable pathogens (no statistically significant difference).

1. Odds ratio and two tailed p-value were calculated by Fisher Exact Probability Test.
Stool Metabolic Signature

Relative concentration of influential metabolites derived from $^1H$ NMR spectroscopic analysis.

*Indicate significant difference by Wilcoxon rank sum test ($p<0.05$)

- Stool metabolic signature of Test was closer to BF, consistent with the findings in bacterial composition and diversity
- Altered bacterial composition may result in reduced protein fermentation in Test vs. Control
Conclusions

• The intestinal microbiota can be modulated by nutritional intervention in such a way that the diversity and composition of the microbiota becomes closer to those of breastfed infants

• Infant formula supplemented with 8 g/L BMOS and $10^7$ or $5 \times 10^4$ cfu/L *B. lactis* strongly shifts microbiota to a dominance of endogenous bifidobacteria

• Infant formula supplemented with 1.5 g/L HMOs (2’FL+LNnT) shifts the stool microbiota and metabolic signature towards those of breastfed infants

• Further studies are warranted to evaluate whether such shifts in gut ecology of formula-fed infants towards the breastfed standard leads to benefits
ACKNOWLEDGMENTS

Many thanks to the investigators and their study teams for their major contributions to these clinical trials

Many thanks also to the families and caregivers who consented to their infants’ participation in these trials

The team members at Nestlé Research Center, Nestlé Nutrition, Nestlé Product Technology Center and Nestlé Institute of Health Sciences

The Glycom team for collaboration on HMO production
Study flow chart

Enrolled N=203

Excluded from study before randomization n=1

Randomized N=202

CON n=40

Dropouts n=3 (7.5%)
- Reasons:
  - SAE n=2
  - Violation of inclusion criteria n=1

n=37

TEST1 n=44

Dropouts n=5 (11.4%)
- Reasons:
  - SAE n=1
  - Violation of inclusion criteria n=2
  - Medication intake n=1
  - SAE and violation of inclusion criteria n=1

n=39

TEST2 n=43

Dropouts n=8 (18.6%)
- Reasons:
  - SAE n=1
  - Violation of inclusion criteria n=2
  - Medication intake n=4
  - SAE and violation of inclusion criteria n=1

n=35

BF n=75

Dropouts n=14 (14.8%)
- Reasons:
  - Violation of inclusion criteria n=12
  - Medication intake n=1
  - SAE and violation of inclusion criteria n=1

n=61

Completed the Study
Study flow chart

Infants enrolled in the study (N=115)

REFERENCE GROUP

Group B: Breastfed (n = 39)
- Discontinued (n=10)
  - Voluntary withdrawal (n=5)
  - Other n=5

Primary analysis (n=29)
- Major protocol deviation (n=6)
  - Per protocol analysis (n=23)
  - Per protocol 16S analysis (n=13)

RANDOMIZATION

Group C: Control Formula (n = 37)
- Discontinued* (n=7)
  - GI symptoms (n=2)
  - Voluntary withdrawal (n=1)
  - Lost to follow up (n=2)

Primary analysis (n=30)
- Major protocol deviation (n=12)
  - Per protocol analysis (n=18)
  - Per protocol 16S analysis (n=13)

Group T: Test Formula (n = 39)
- Discontinued* (n=7)
  - GI symptoms (n=3)
  - Voluntary withdrawal (n=2)
  - Lost to follow up (n=3)

Primary analysis (n=32)
- Major protocol deviation (n=11)
  - Per protocol analysis (n=21)
  - Per protocol 16S analysis (n=14)
Study Methods

Stool microbiota profile

– 16S rRNA sequencing (16S)
  – High-throughput sequencing of the 16S V3-V4 region
  – Determine bacterial relative abundance & diversity at genus level

– Luminex xTAG Gastrointestinal Pathogen Panel (GPP)¹
  – PCR amplification of well-established marker genes
  – Detect the presence of specific GI pathogens

– Metagenomic Sequencing (MS)
  – Comprehensive sequencing of all genes in all organisms (bacterial, fungi, viral)
  – Analysis ongoing, preliminary findings confirm results from 16S and GPP

Stool metabolic signature

– Established Proton Nuclear Magnetic Resonance Spectroscopy (1H NMR)
  – Metabolites including organic acids and amino acids

¹ Luminex Molecular Diagnostics, Inc., Canada.