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

Insights into next generation prebiotics & probiotics

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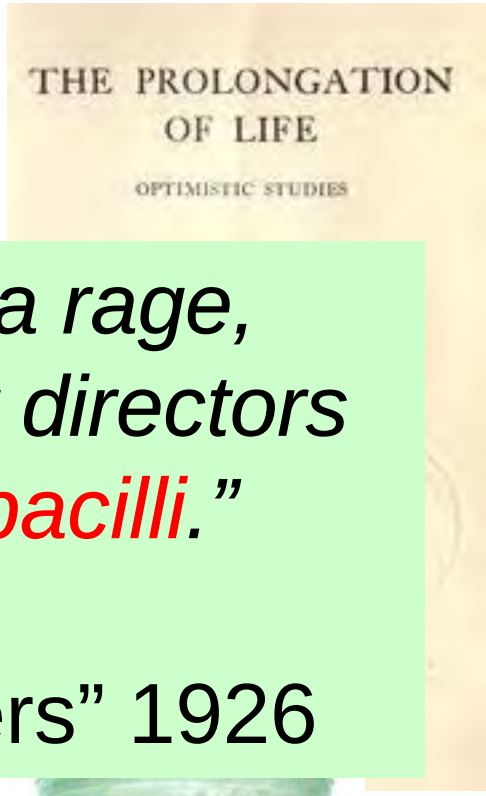
V&E Viticulture & Enology UNIVERSITY OF CALIFORNIA DAVIS

The probiotic concept

1906



"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 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

Tissier H. 1905. Repartition des microbes dans l'intestin du nourisson. Ann. de l'Institut de Pasteur 19:109

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

Gyorgy, P (1971) The uniqueness of human milk, biochemical aspects. AJCN 24 970.

"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?

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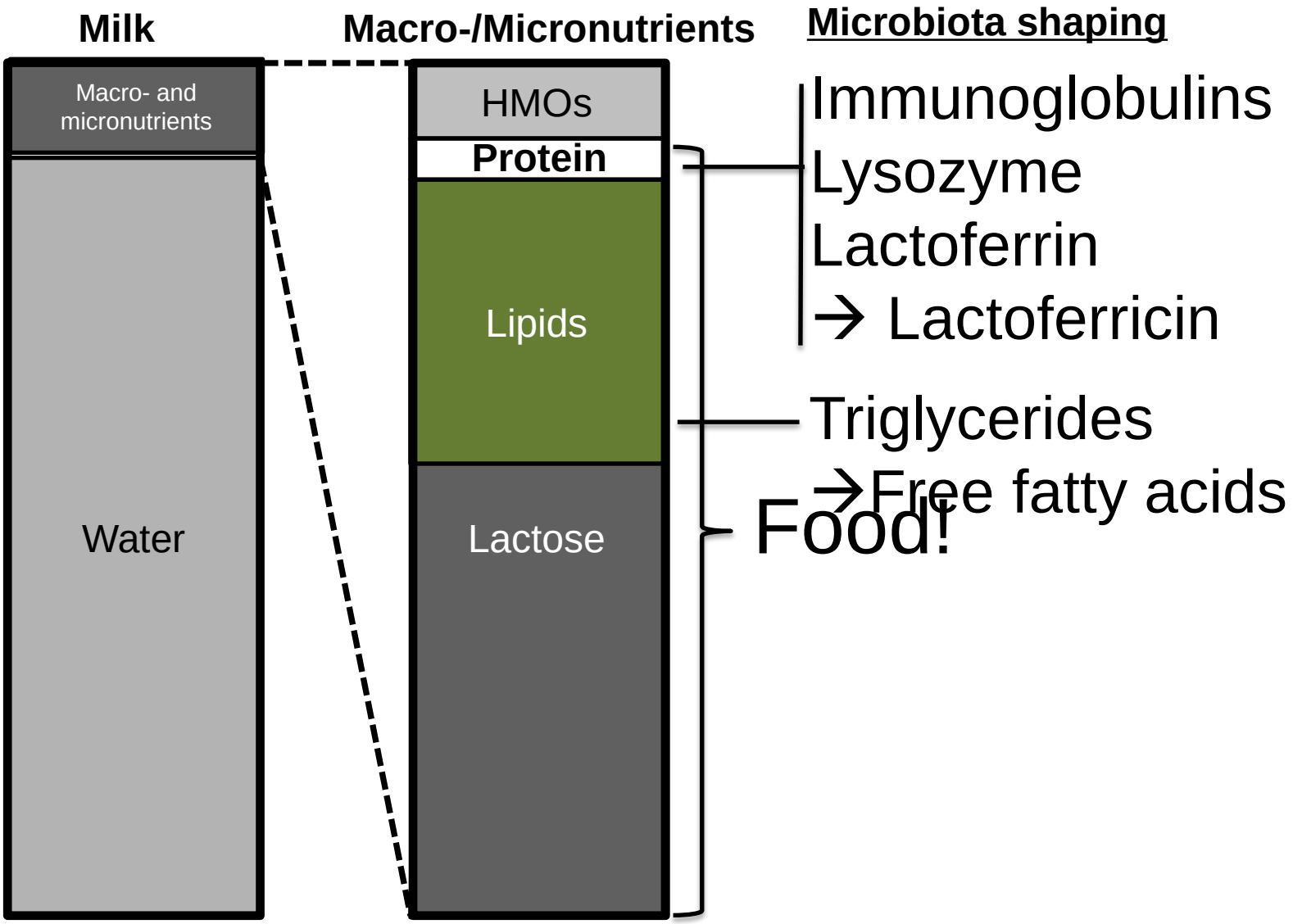
Human milk composition



Newburg 2005, 2009

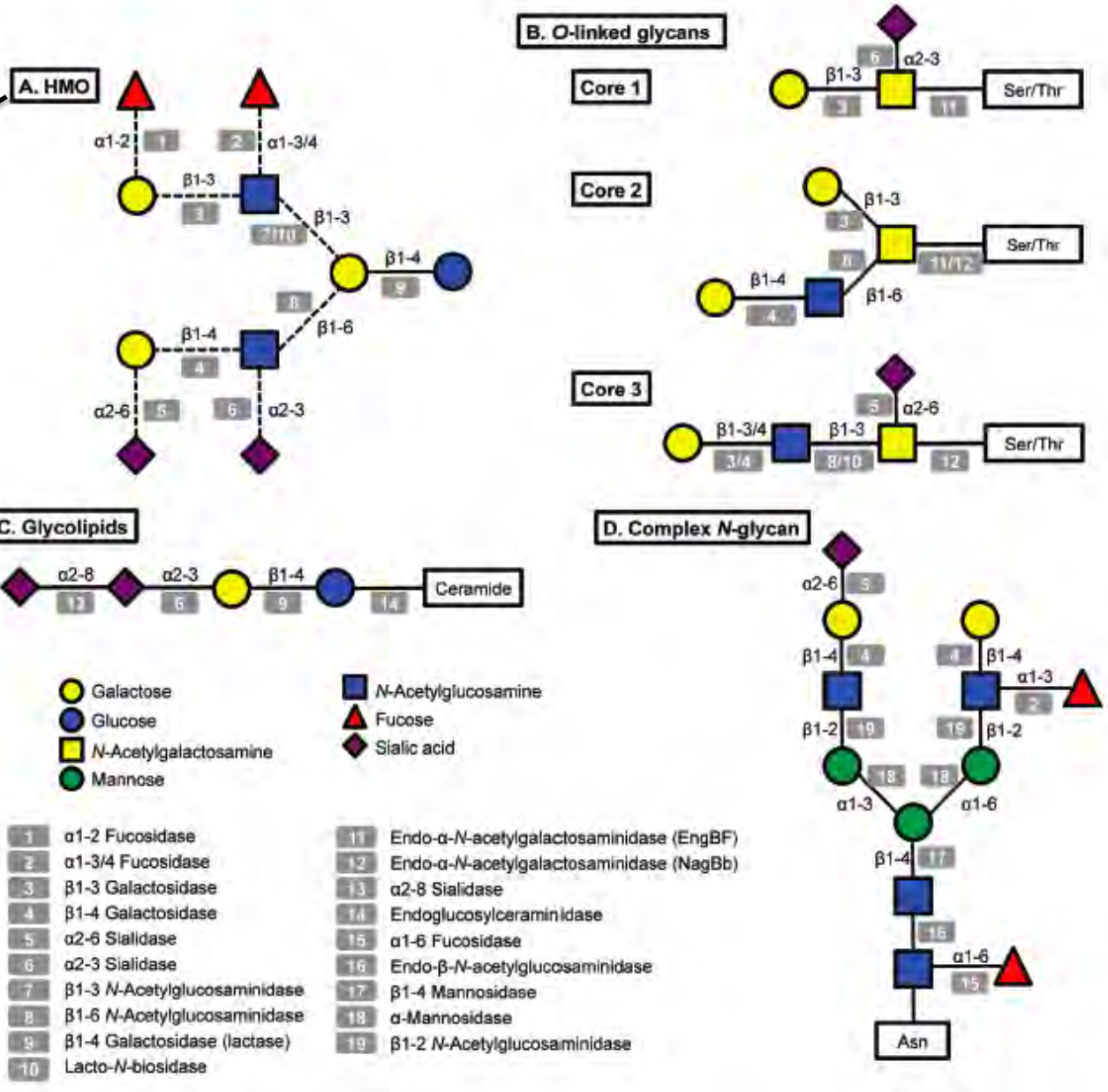
Human Milk Glycans

HMOs

Protein

Lipids

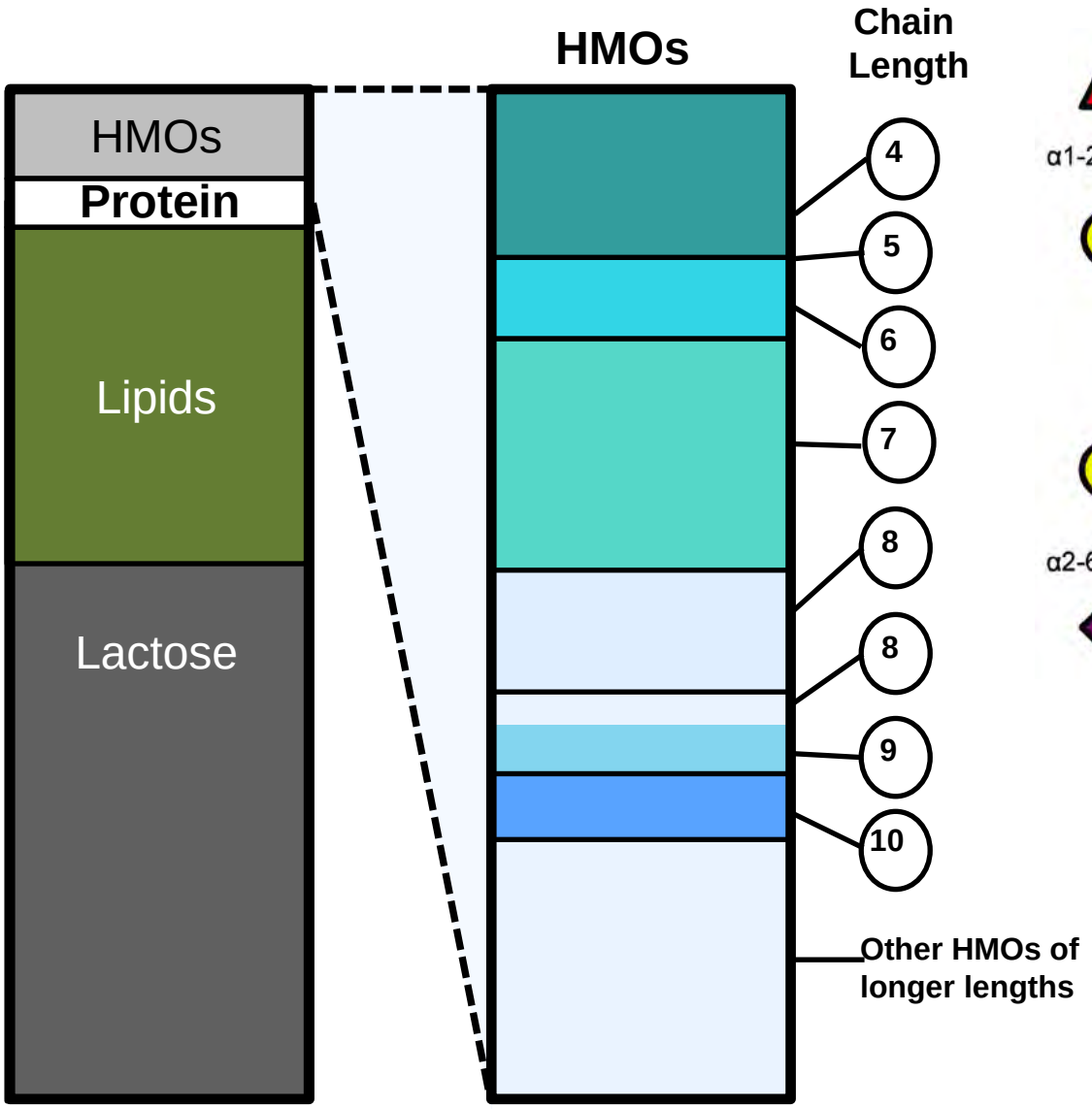
Lactose

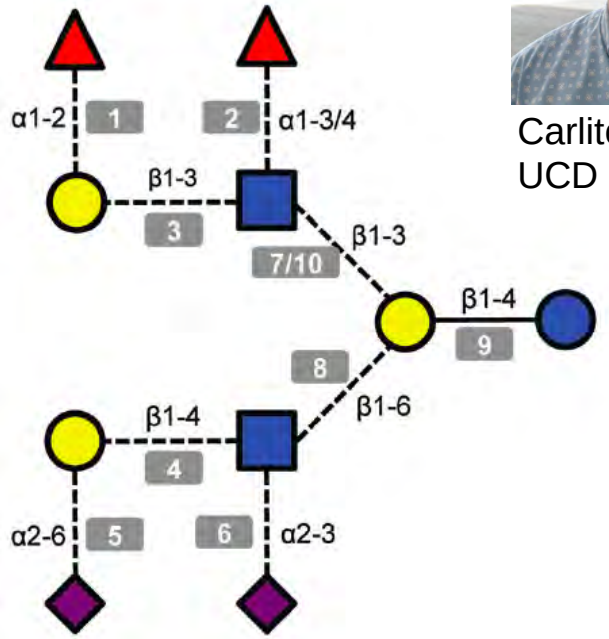


Garrido et al Microbiology 2013

Human milk oligosaccharides

Carlito Lebrilla
UCD Chemistry





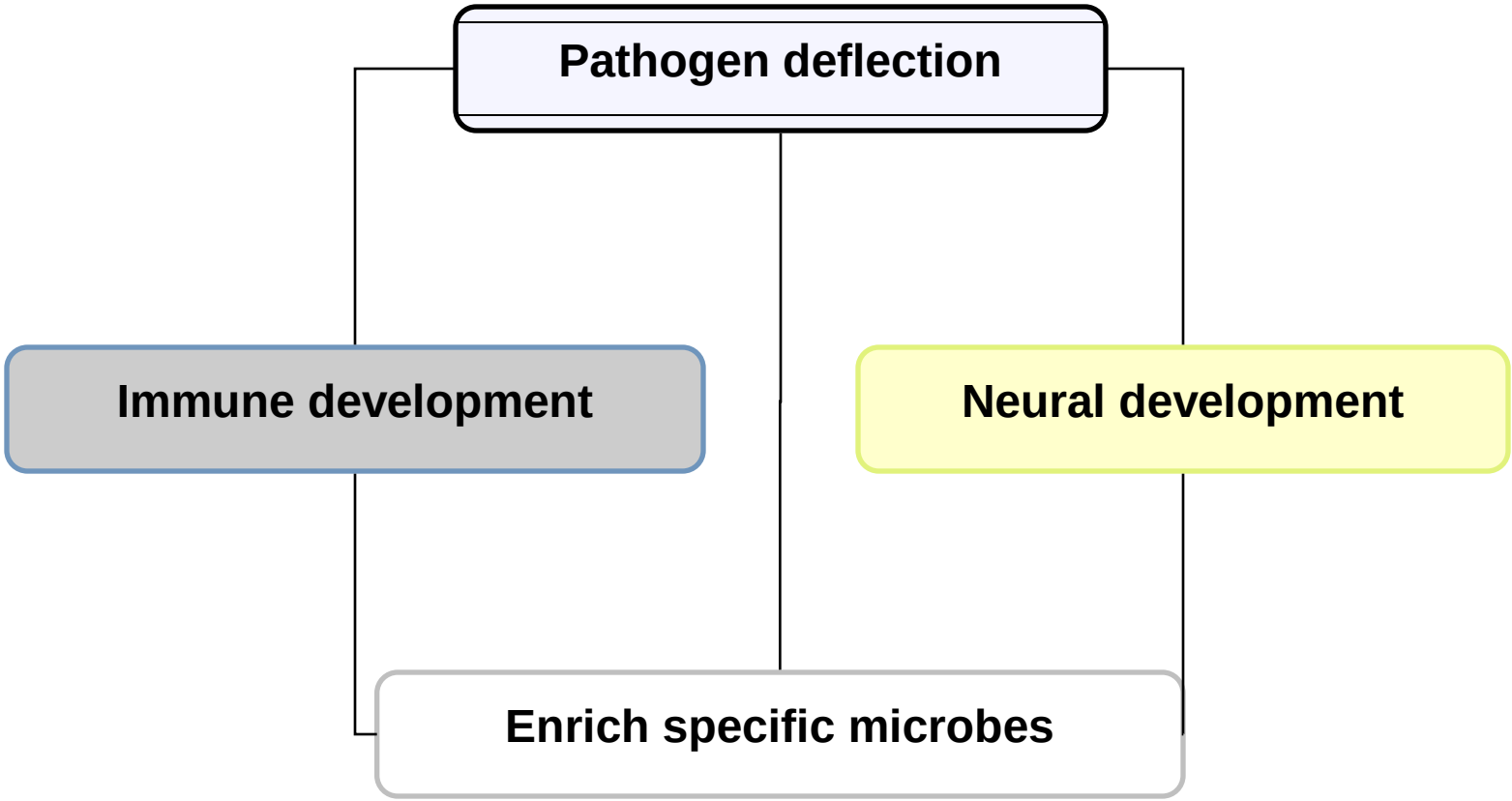
- **Human indigestible and highly variable**
- **Higher proportion of fucosylated (40-70%) than sialyated (4-38%)**
- **Nearly 200 species in pooled human milk**

Nature 468 S5-S7 (23 December 2010)

Garrido et al Microbiology (2013)

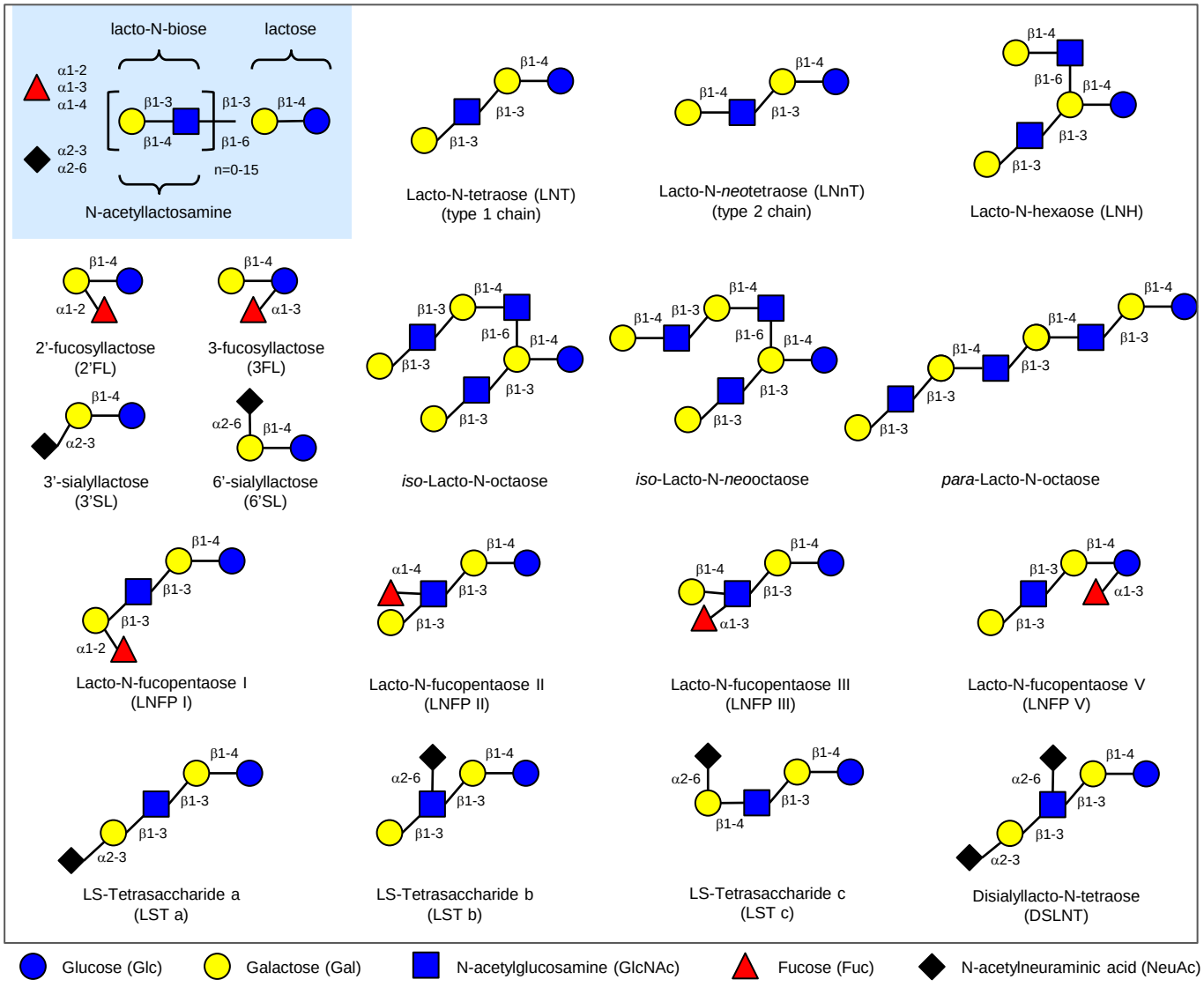
Why Make Glycoconjugates/Free Glycans?

(if they are not consumed by the infant)

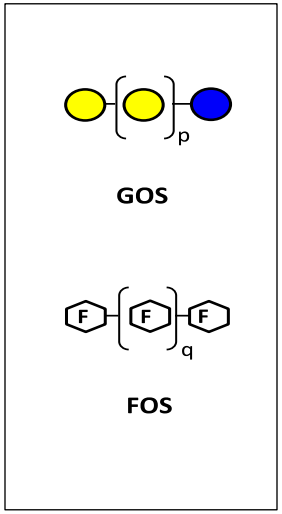


Bode 2009

HMO Structural Diversity



FOS/GOS



150 – 200 different HMO

Courtesy of Lars Bode

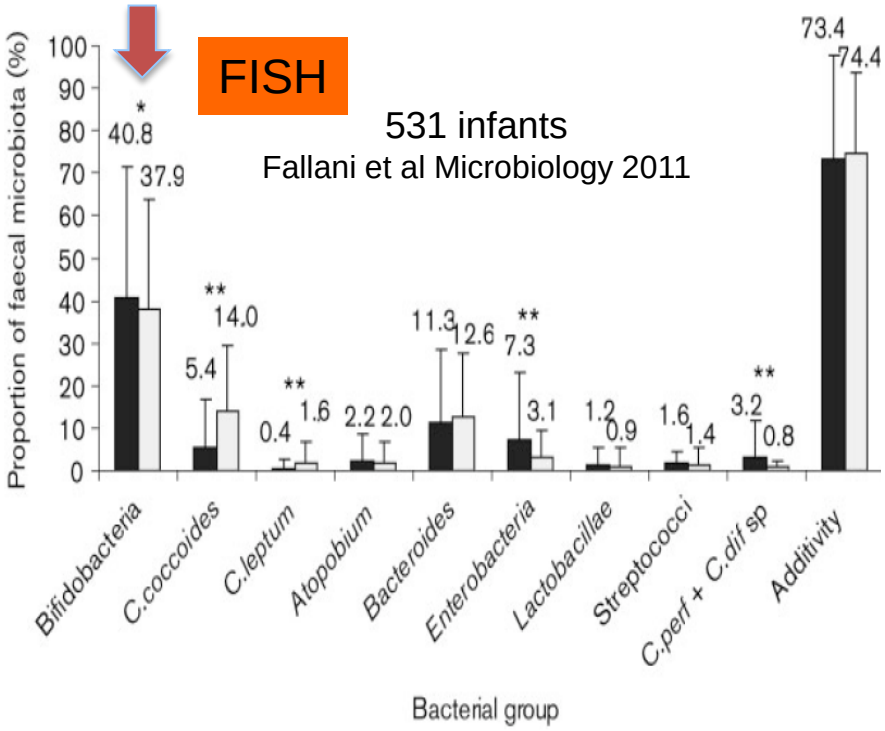
Breast milk enriches bifidobacterial populations

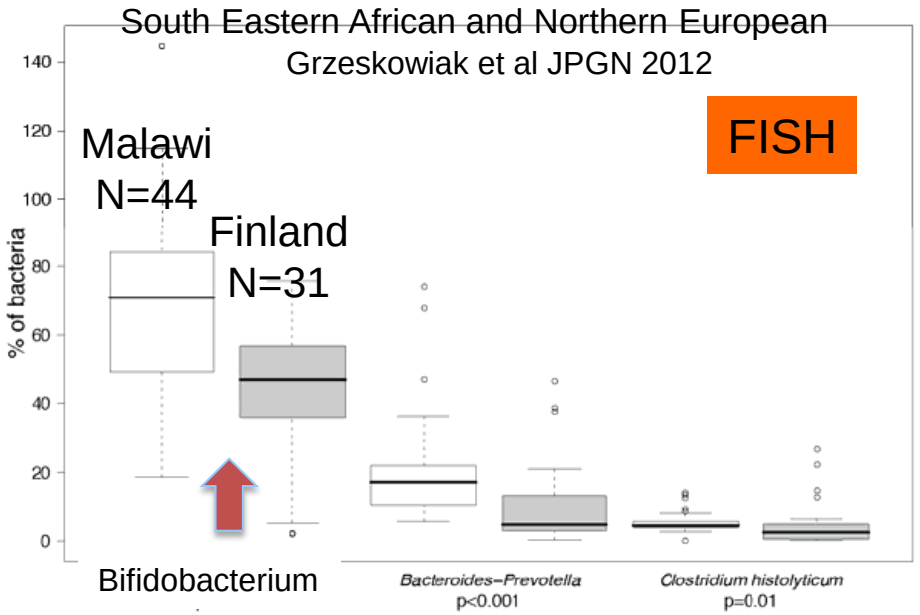
Penders et al. Pediatrics 2006

TABLE 2 Median Counts and Prevalence of Selected Gut Bacteria in Feces of Infants 1 Month of Age (n = 1032)

Quantitative PCR

	Bifidobacteria	*E coli*	*C difficile*	*B fragilis* Group	Lactobacilli	Total
Median counts (range), $\log_{10}$ CFU/g feces	10.68 (6.84–11.56)	9.35 (5.91–10.79)	5.32 (2.70–9.57)	9.28 (5.74–10.44)	8.66 (7.92–10.73)	11.12 (9.43–12.14)
Prevalence, %	98.6	87.7	25.0	81.6	32.4	100





Breast milk enriches bifidobacterial populations

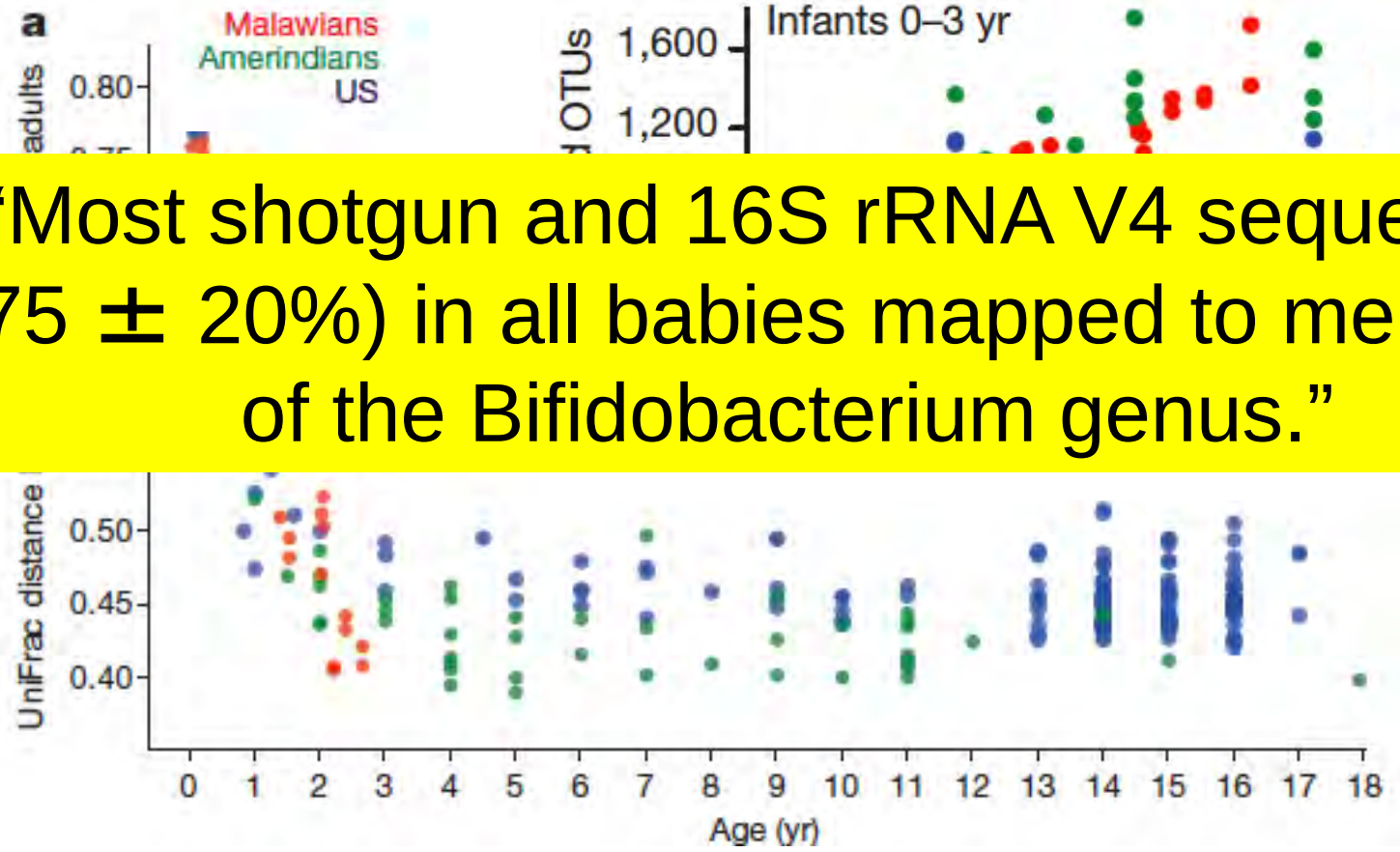
ARTICLE

doi:10.1038/nature11053

Human gut microbiome viewed across age and geography

Tanya Yatsunenko[1], Federico E. Rey[1], Mark J. Manary[2,3], Indi Trehan[2,4], Maria Gloria Dominguez-Bello[5], Monica Contreras[6], Magda Magris[7], Glida Hidalgo[7], Robert N. Baldassano[8], Andrey P. Anokhin[9], Andrew C. Heath[9], Barbara Warner[2], Jens Reeder[10], Justin Kuczynski[10], J. Gregory Caporaso[11], Catherine A. Lozupone[10], Christian Lauber[10], Jose Carlos Clemente[10], Dan Knights[10], Rob Knight[10,12] & Jeffrey I. Gordon[1]

N= 531 (16S) N=110 (metagenome)



"Most shotgun and 16S rRNA V4 sequences (75 ± 20%) in all babies mapped to members of the Bifidobacterium genus."

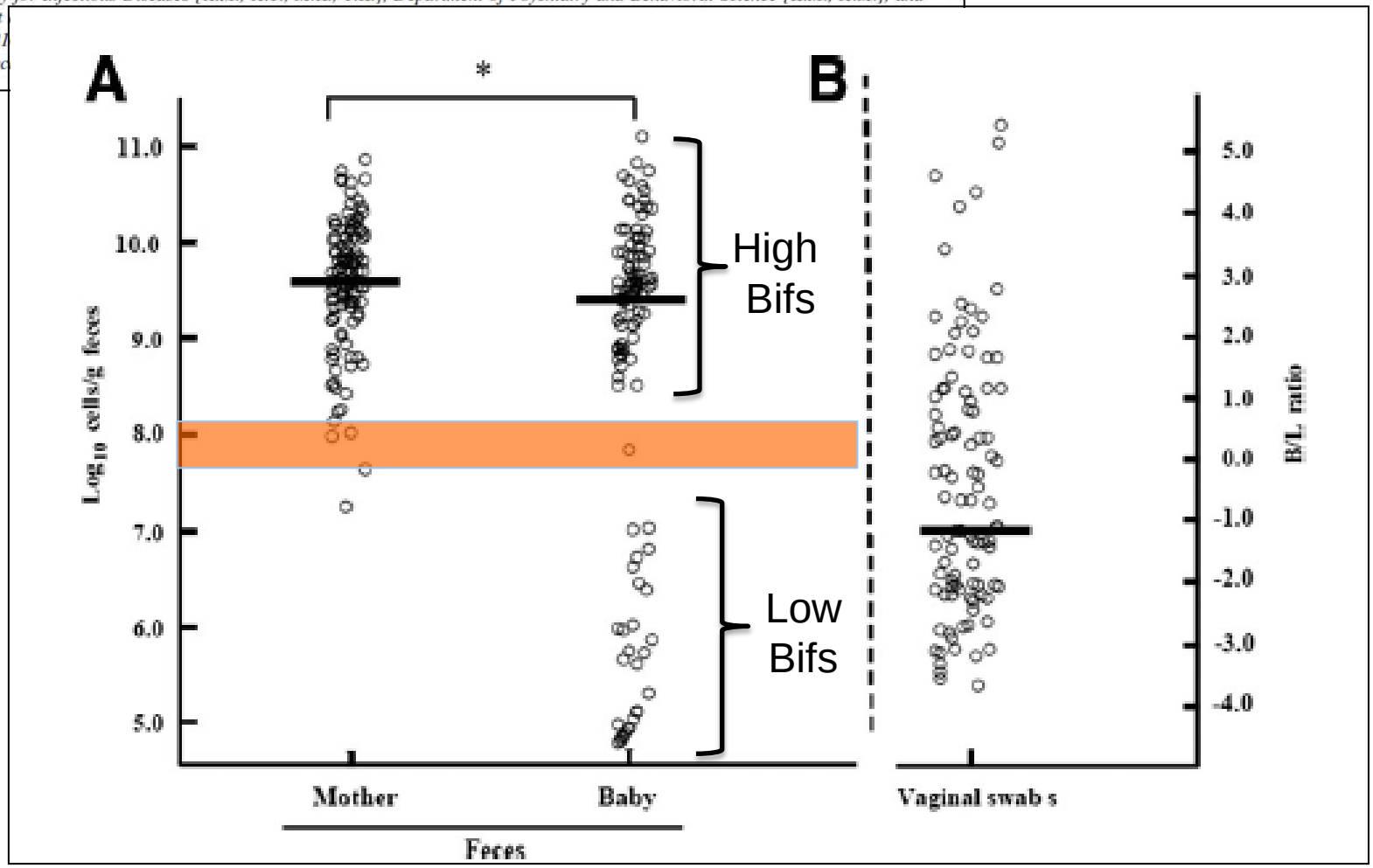
0031-3998/09/6506-0669
PEDIATRIC RESEARCH
Vol. 65, No. 6, 2009
Printed in U.S.A.

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
Fukuoka 81
Psyc

- n=110 babies
- 1 month of age
- Measured by qPCR



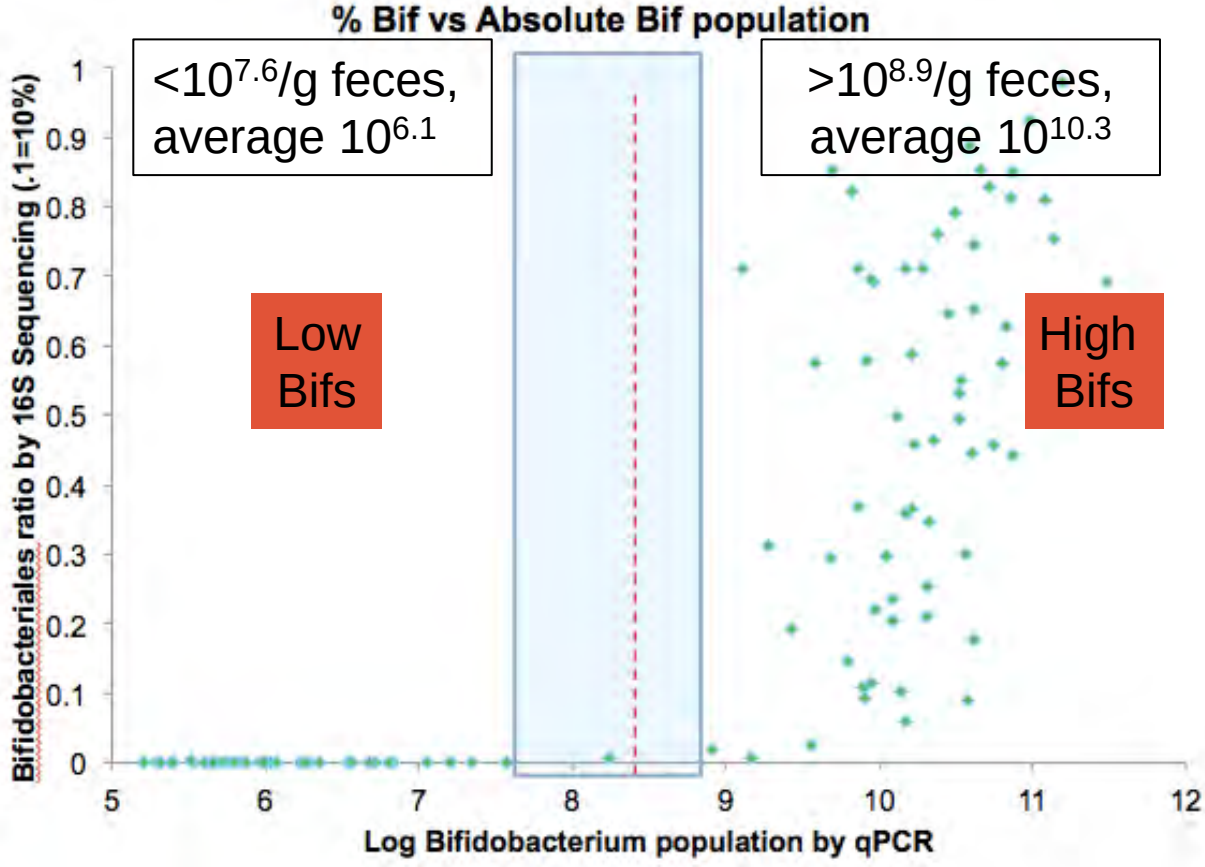
Mikami et al. Pediatrics Research 2009

Lactation Study

Who is conducting this study?

This clinical study led by J. Bruce German, Ph.D., and colleagues at the UC Davis Foods for Health Institute, and Food Science and Technology, Viticulture and Enology, and Chemistry departments is part of the **Milk Bioactives** and the **Functional Glycobiology Program**. This

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




Why bifidobacteria?
Acc.V Magn WD
10 µm
20.0 kV 3348x 8.9 UCD 272, 2 HMO

LETTER

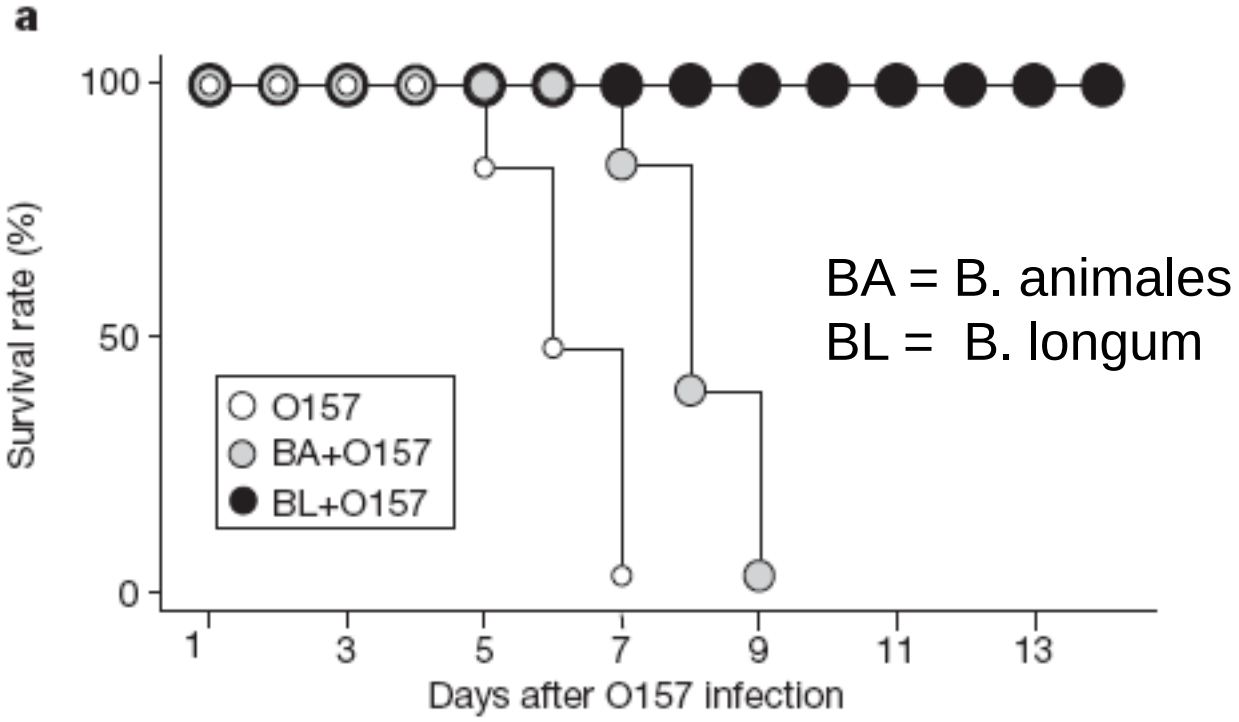
doi:10.1038/nature09646

Bifidobacteria can protect from enteropathogenic infection through production of acetate

Shinji Fukuda[1,2], Hidehiro Toh[3], Koji Hase[1], Kenshiro Oshima[4], Yumiko Nakanishi[1,2,5], Kazutoshi Yoshimura[6], Toru Tobe[7], Julie M. Clarke[8], David L. Topping[8], Tohru Suzuki[9], Todd D. Taylor[3], Kikuji Itoh[6], Jun Kikuchi[2,5,10], Hidetoshi Morita[11], Masahira Hattori[4] & Hiroshi Ohno[1,2,12]

Nature 2010

Mouse survival after O157 infection



BA = B. animales
BL = B. longum

LETTER

doi:10.1038/nature09646

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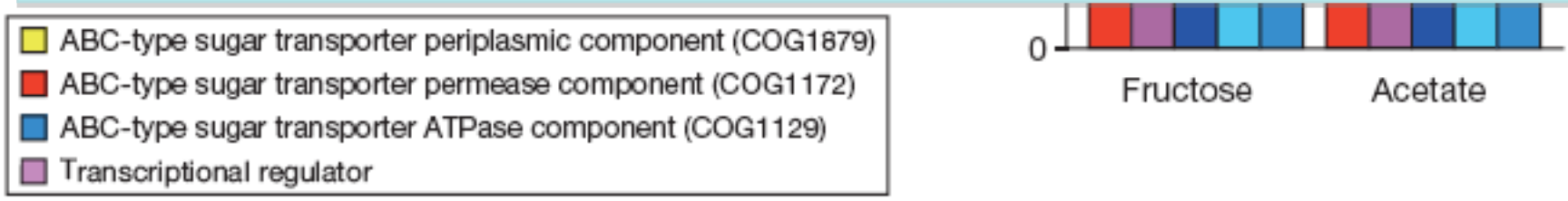
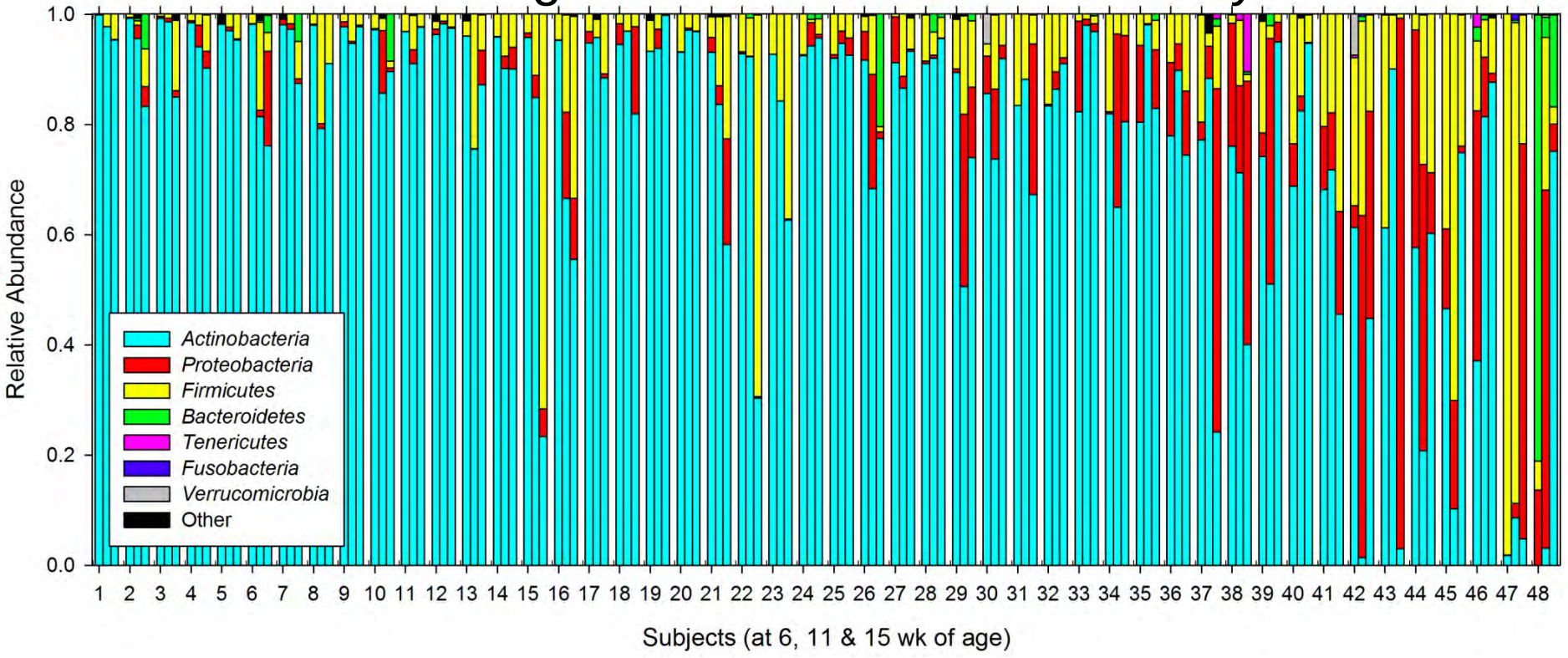
If bifidobacteria can grow well on a targeted sugar *in situ*, growth and accompanying production of acetate is protective



Nature 2010

Why bifidobacteria?

Bangladesh Infant Vitamin A Study



icddr,b
KNOWLEDGE FOR GLOBAL LIFESAVING SOLUTIONS

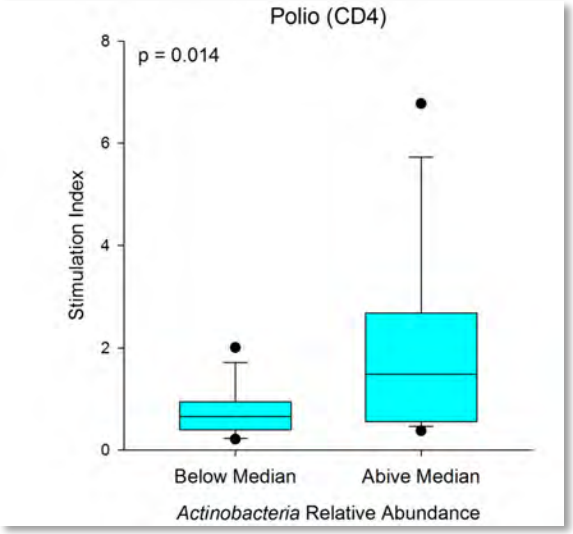
International Center for Diarrhoeal Disease Research (Bangladesh)

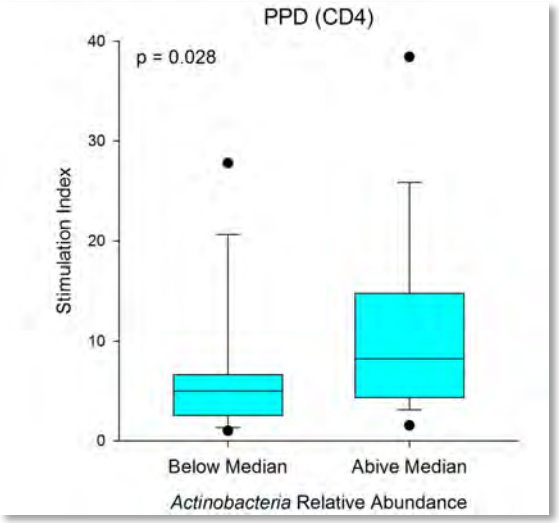
USDA ars United States Department Of Agriculture Agricultural Research Service

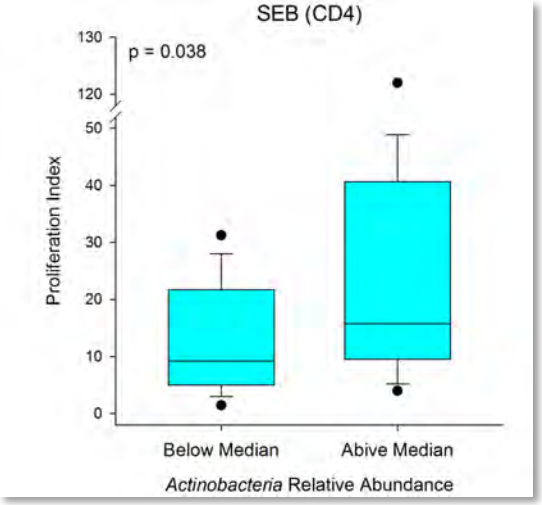
Western Human Nutrition Research Center (Davis, CA)

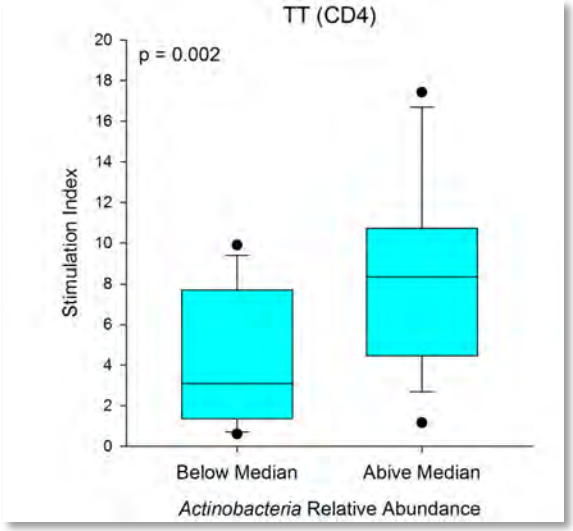
Huda, Stephensen et al In Prep

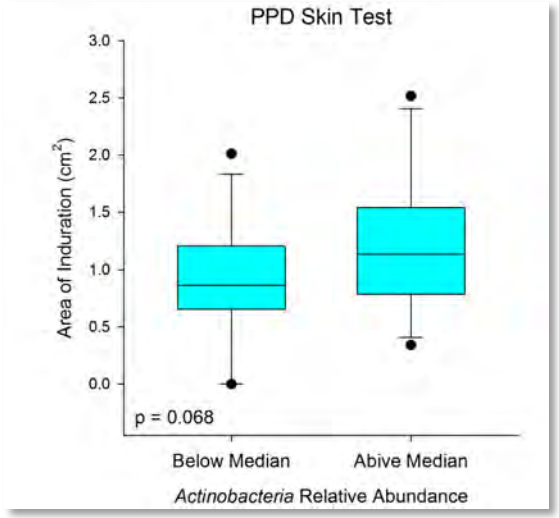
Positive correlation between Actinobacteria and vaccine response

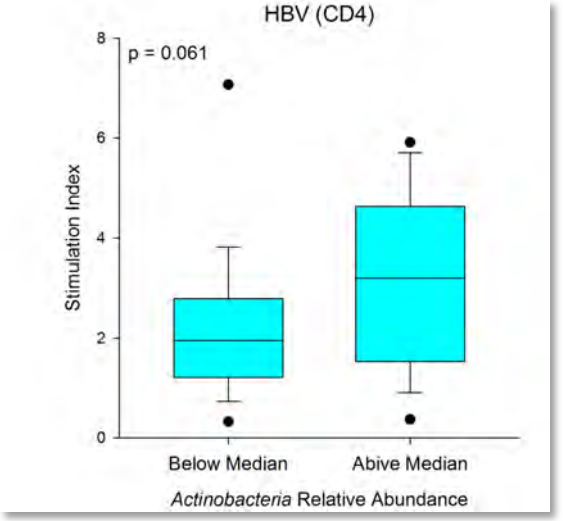












Huda, Stephensen et al In Prep

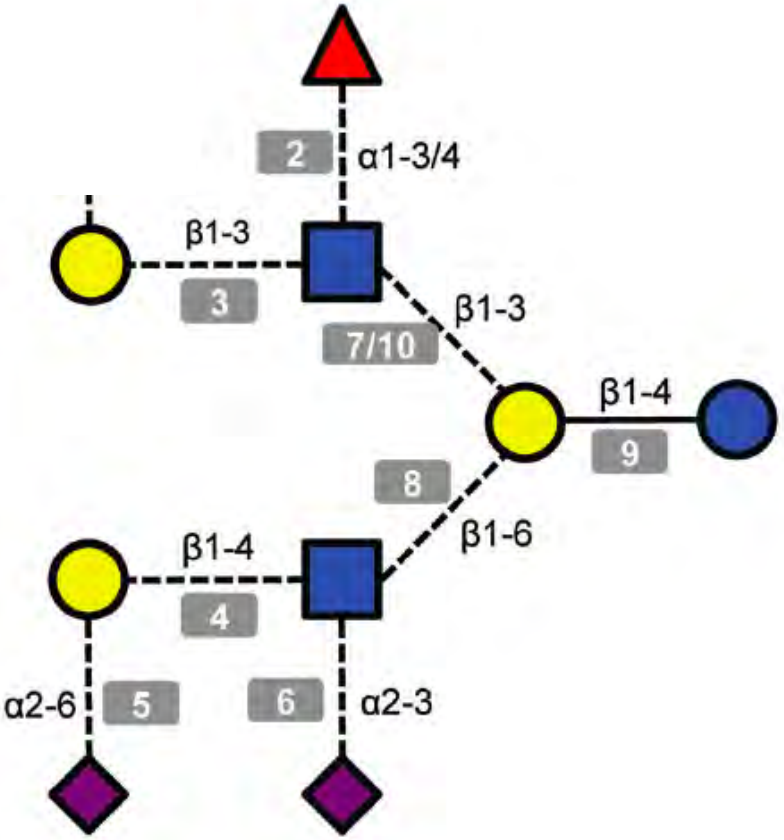
Do different mother's milk glycan types influence different microbiota populations?

Secretor vs non-secretor

FUT2 ("Secretor" gene)

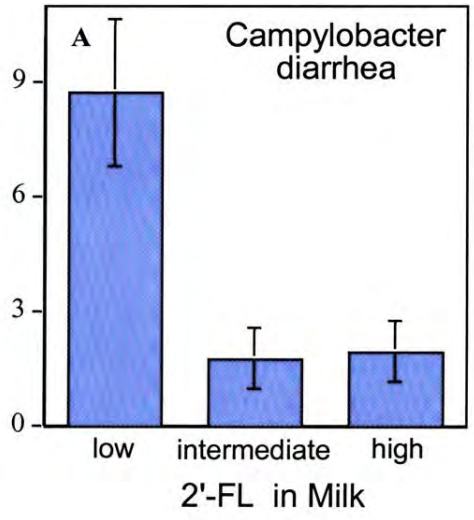
- Produces the 2' fucosylated precursor to the A, B, H, and Lewis b antigens in secretions, including breast milk

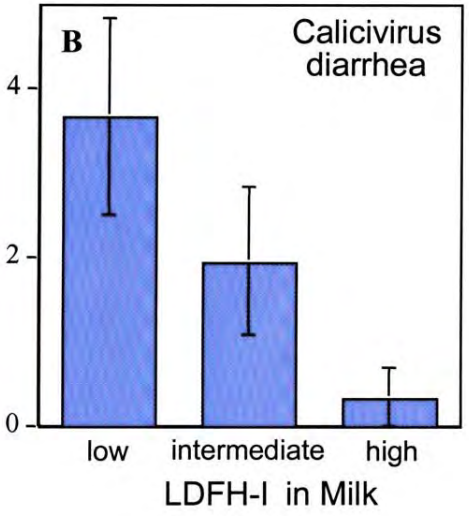
- 20% of U.S. population are non-secretors

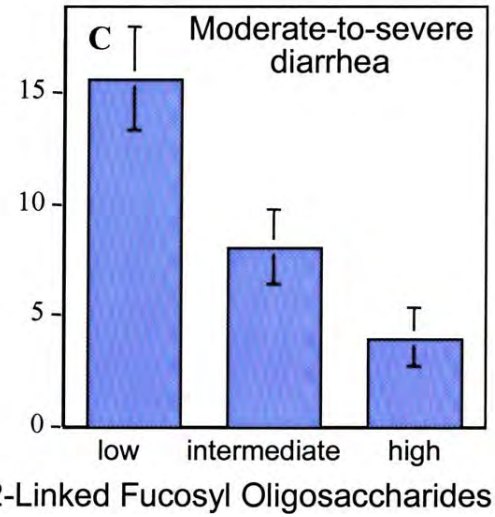


Secretor vs. non-secretor milk protects differently

Incidence of diarrhea per 100 child-months







Morrow et al J. Pediatrics 2004

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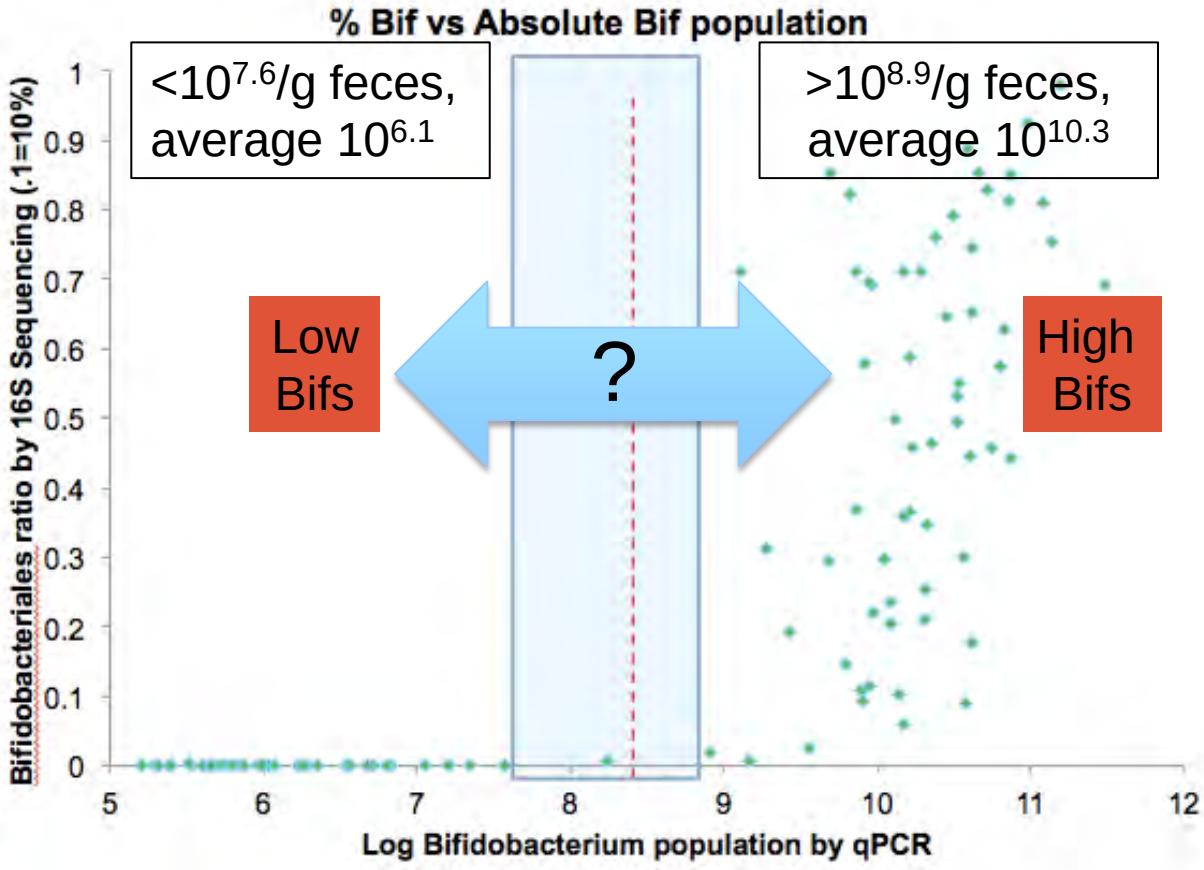
Human Studies > Lactation Study

Search...

Who is conducting this study?

This clinical study led by J. Bruce German, Ph.D., and colleague
Health Institute, and Food Science and Technology, Viticulture a
departments is part of the **Milk Bioactives** and the **Functional G**
study has been reviewed and approved by the UC Davis Commit
Subjects.

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



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Human Studies > Lactation Study

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

Red= Secretor Blue= Non-secretor

PCoA - PC3 vs PC2

Enterobacteriales

Clostridiales

Bifidobacteria

Lactobacillales (Mostly Streptococci)

PC2 - Percent variation explained 12.86%

PC3 - Percent variation explained 9.05%

0.6
0.4
0.2
0.0
−0.2
−0.4
−0.6
−0.8
−0.6
−0.4
−0.2
0.0
0.2
0.4
0.6

Poster 24

Lewis et al (in prep)

Do different mothers milk glycan types influence different microbiota populations?

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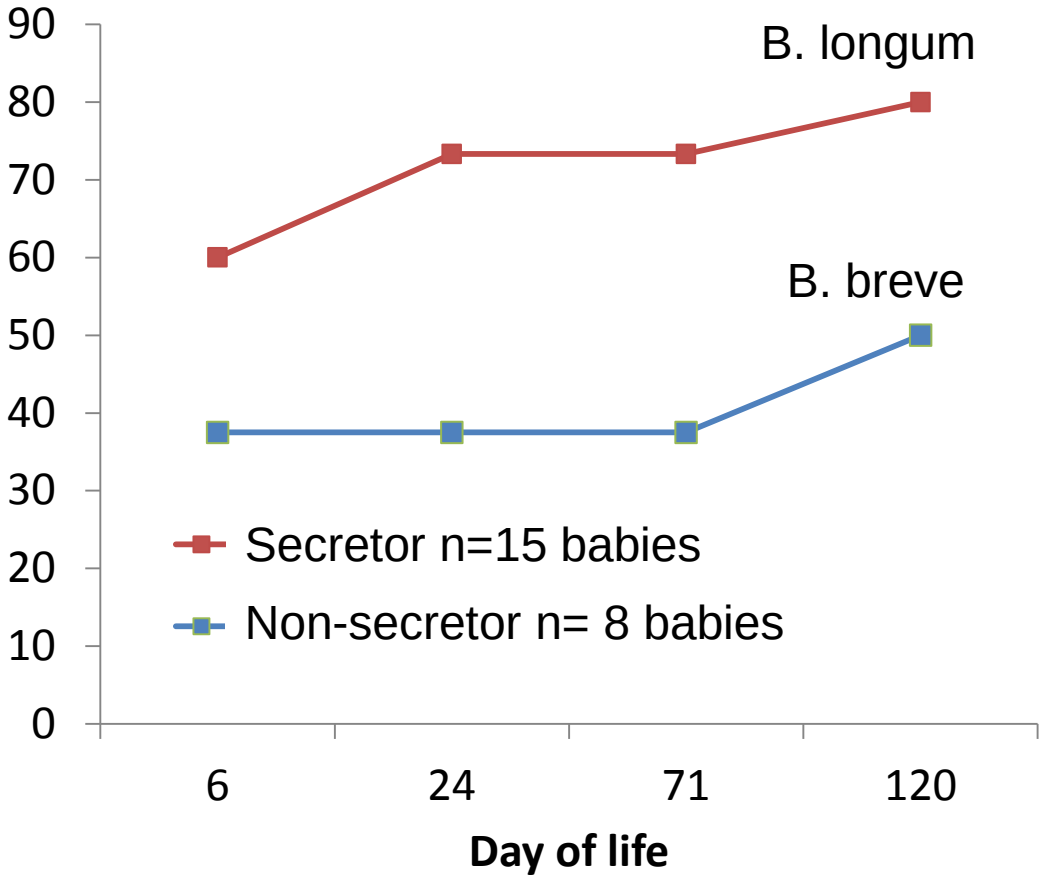
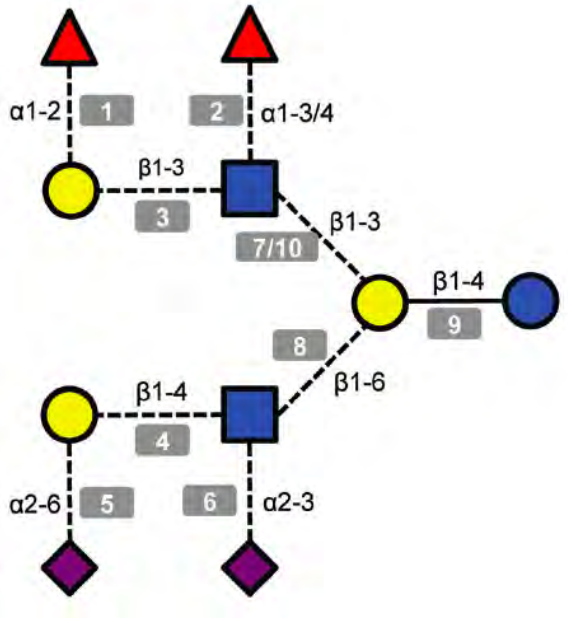
Human Studies > Lactation Study

% Babies with bifidobacteria established (>10^9)

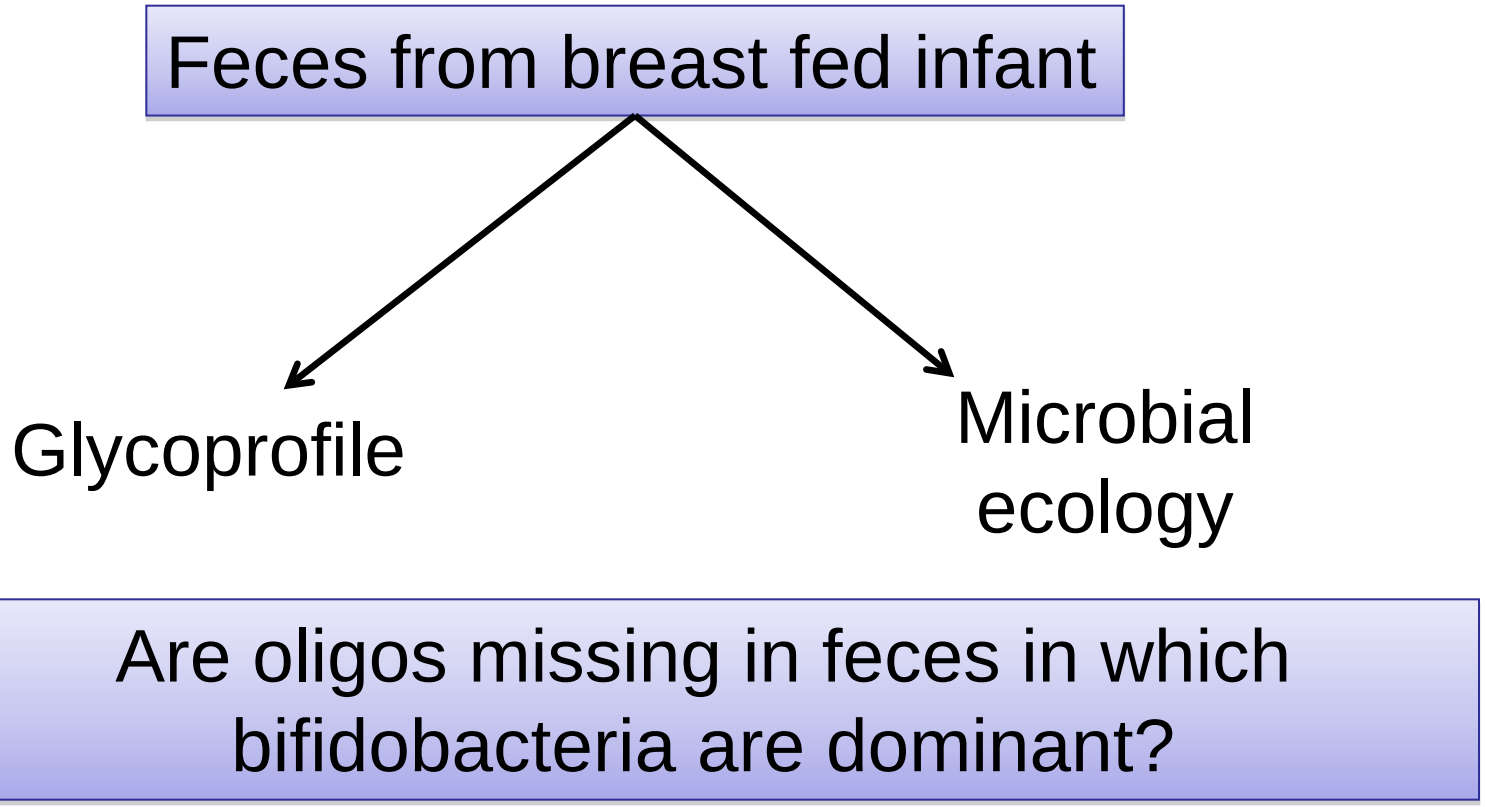




Poster 24

Lewis et al (in prep)

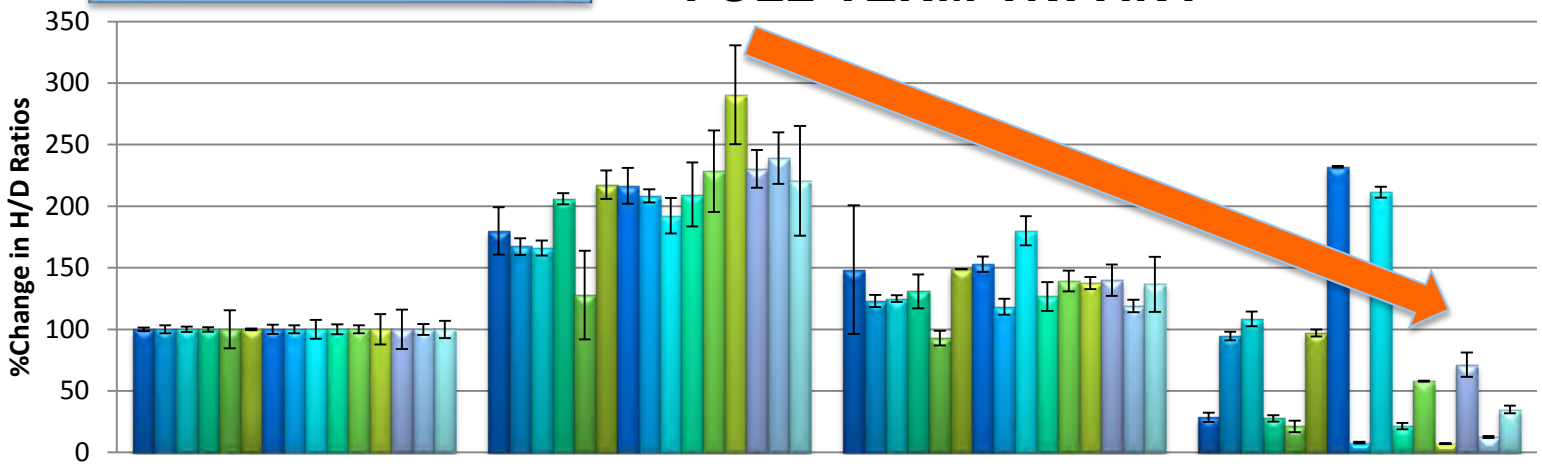
Are specific glycans consumed by bifidobacteria *in situ*?



Feces Oligosaccharides of Term Infant Vary With Bacterial Population

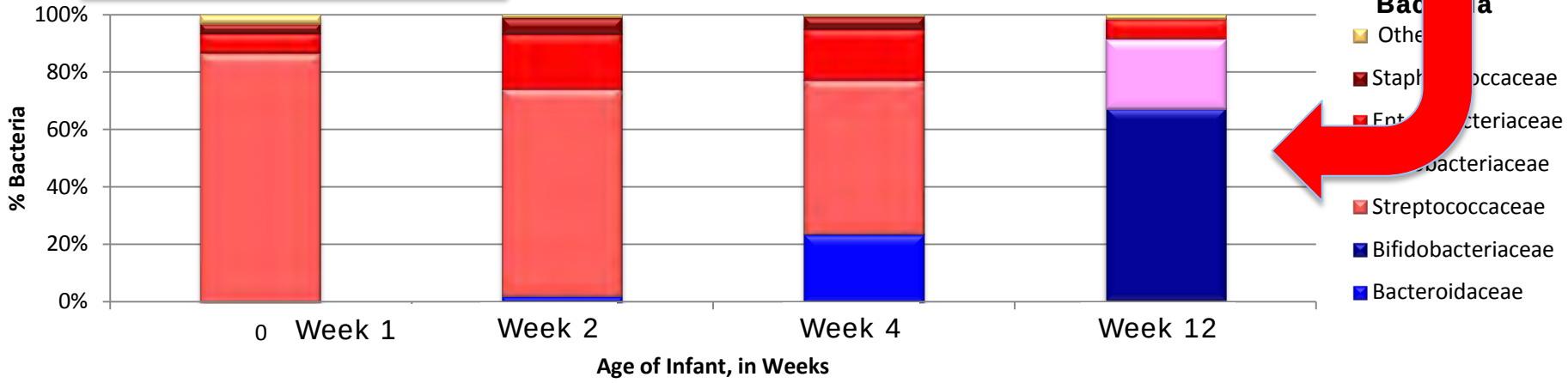
Lorna de Leoz

Fecal HMO Profile



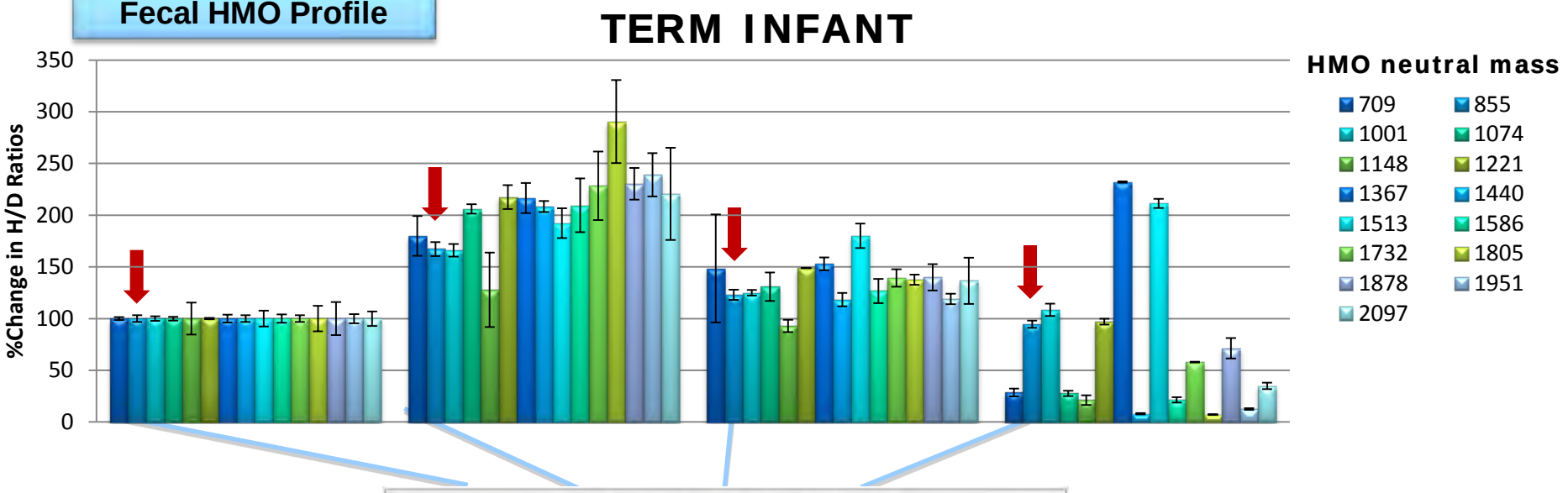
B. longum/infantis

Fecal Bacterial Profile

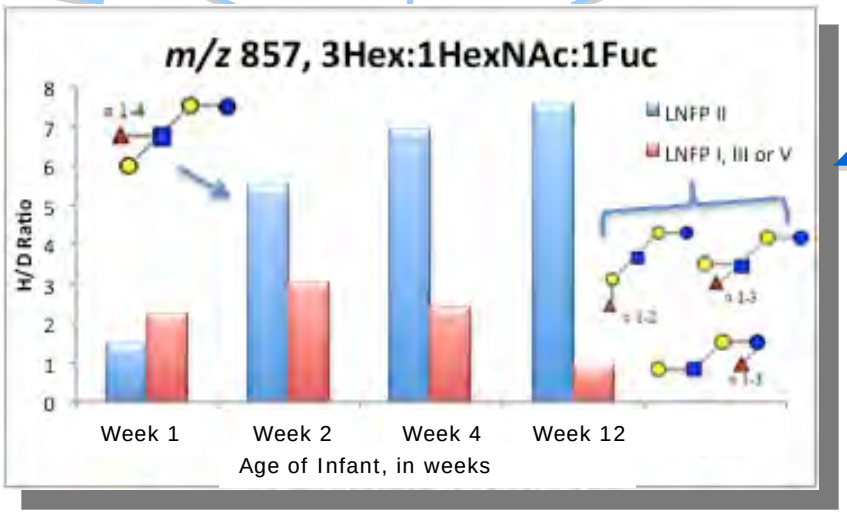


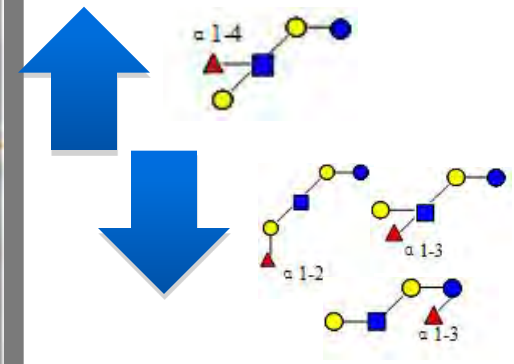
Specific oligosaccharides are consumed in the gut

Fecal HMO Profile



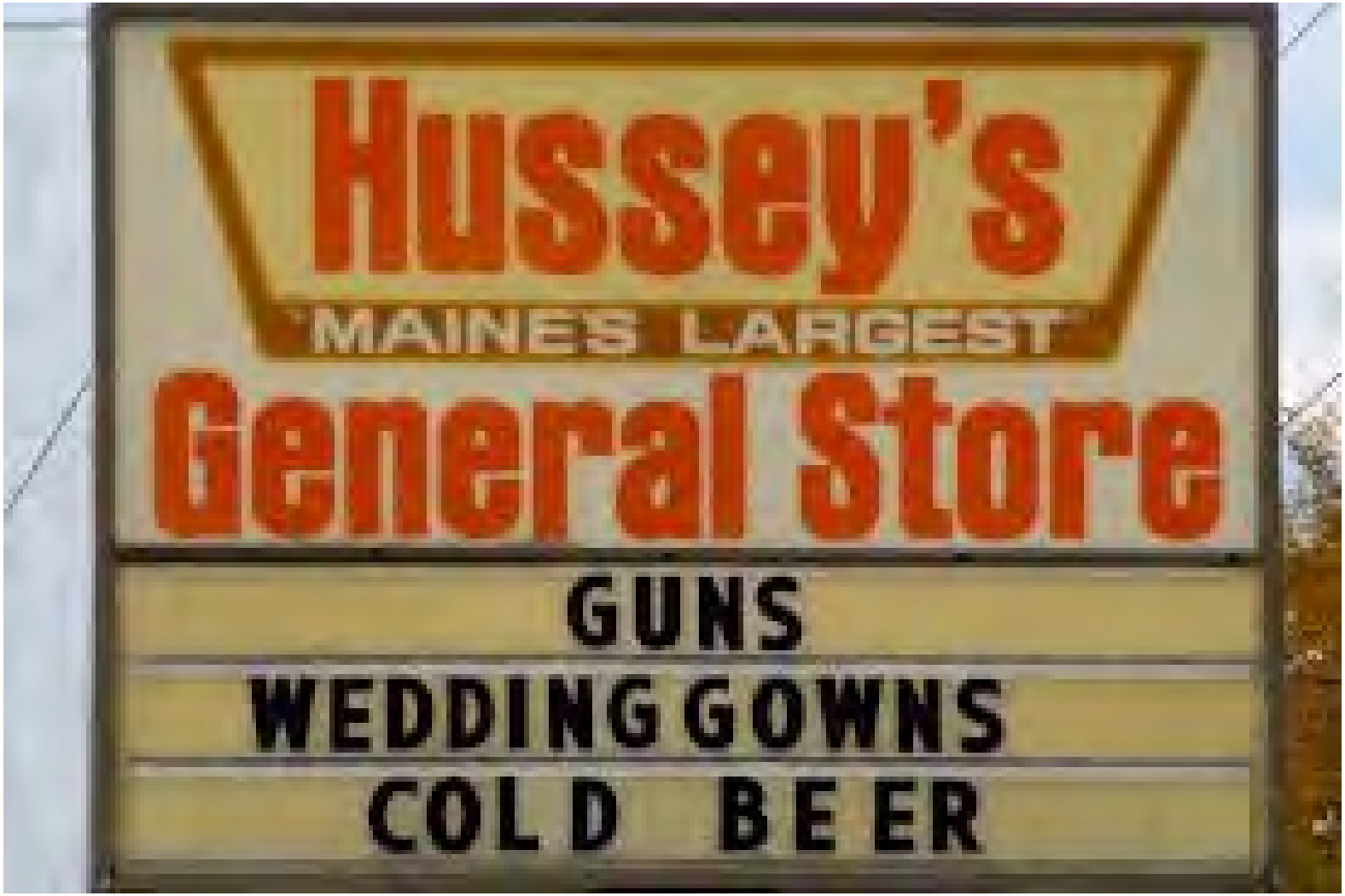
Glycan Specific Changes





Carlito Lebrilla
Lorna de Leoz
UCD Chemistry
(Submitted)

We need to be cautious on associations

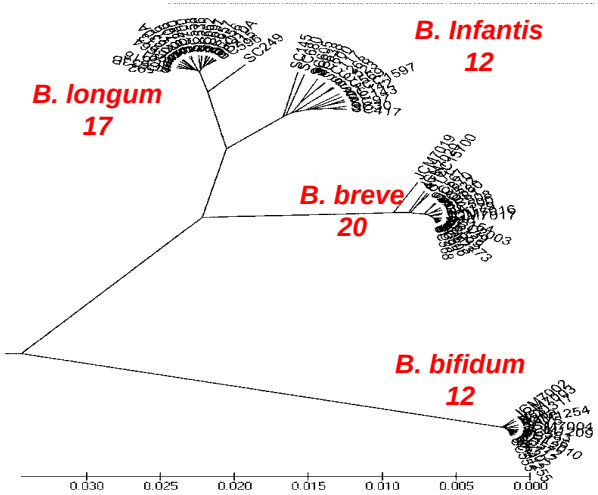


HMOs as “prebiotics” for Bifidobacteria

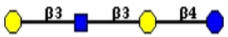
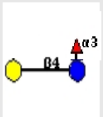
Which bifidobacterial species grow on HMO?

Which bifidobacteria grow on HMO sugars?

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



Multilocus sequence tagging

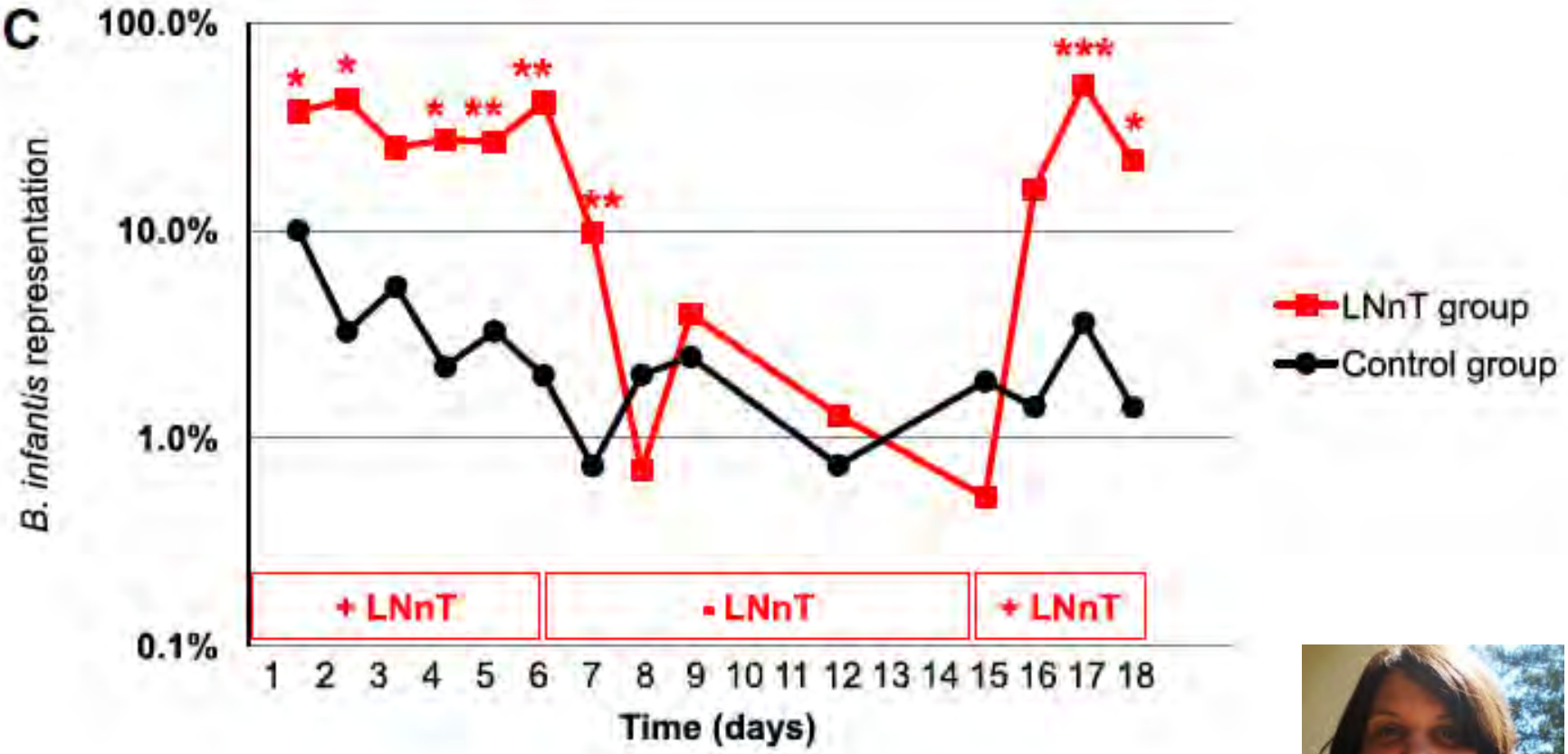
Growth	Structure	B. infantis	B. bifidum	B. longum	B. breve
HMO		22/22	14/14	8/17	10/23
Lacto-N-tetraose		22/22	14/14	17/17	23/23
Lacto-N-neotetraose		22/22	14/14	2/17	23/23
2'-fucosyl lactose		22/22	13/14	1/17	2/23
3-fucosyl lactose		22/22	14/14	1/17	0
6-sialyl lactose		22/22	10/14	0	0

Santi Ruis Moyano

AEM 2006, MicroBiotech 2009, AEM 2013

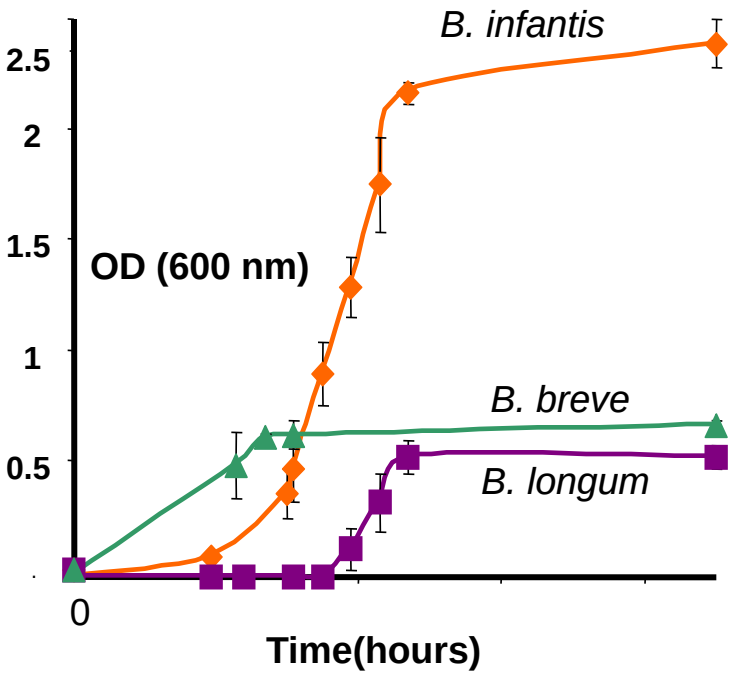
Bifidobacteria vs Bacteroides *in situ*

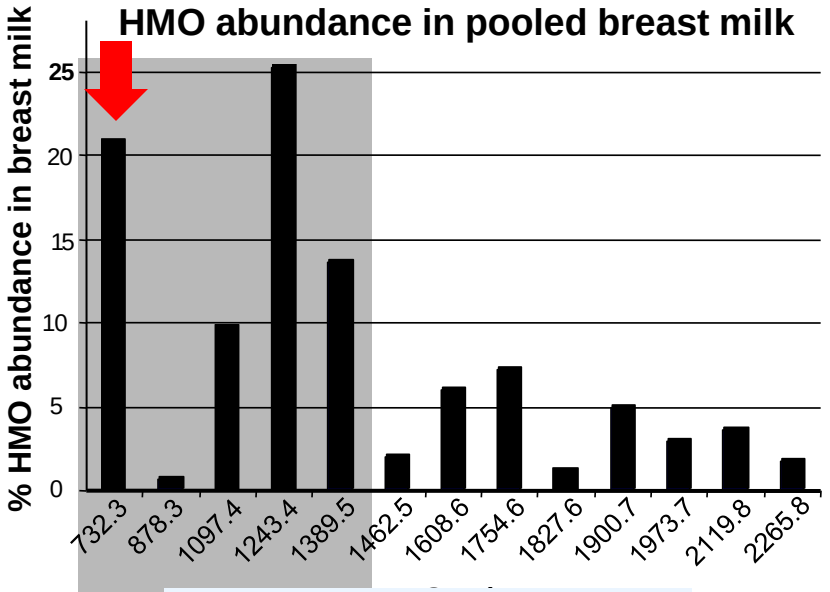
Lacto-N-neotetrose supplementation of gnotobiotic mice with Bifidobacterium infantis and Bacteroides thetaiotaomicron

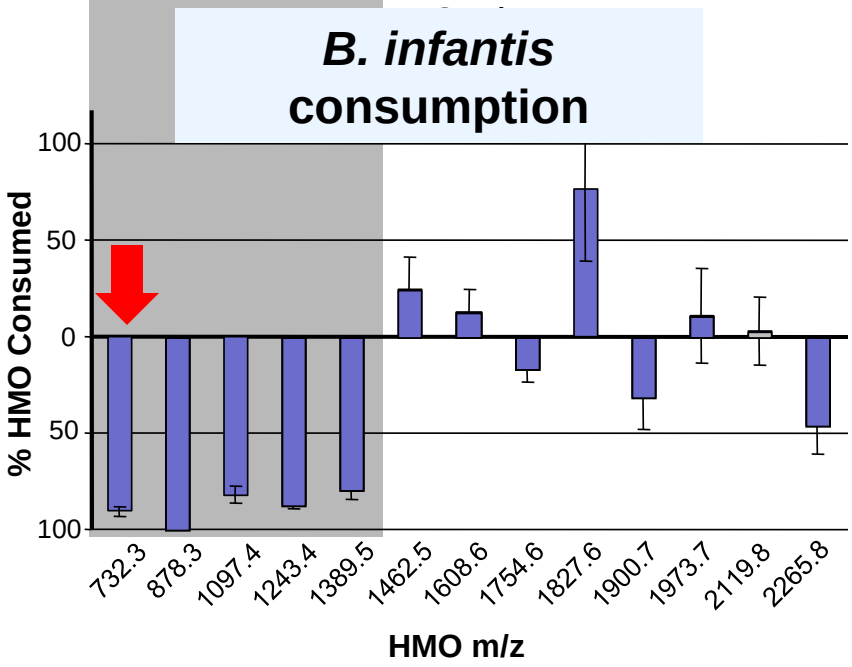


Marcobal… & Sonnenburg CHM 2011

Bifidobacterial HMO Glycoprofiling





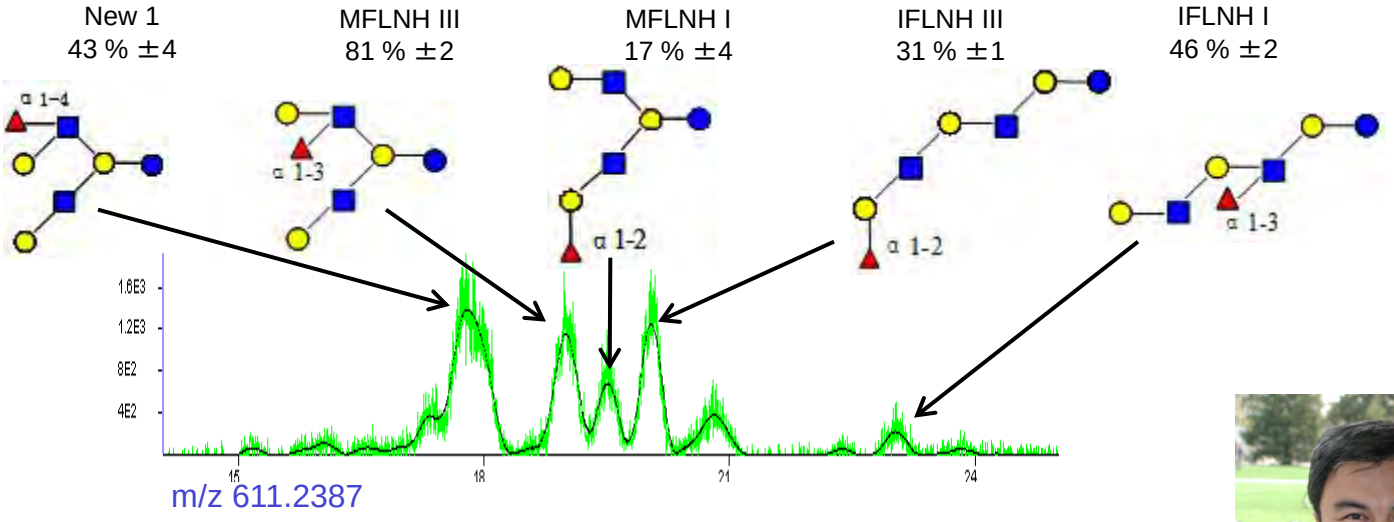


Several small MW oligosaccharides

Single HMO composition consumed by other bifidobacteria

Locascio et al JAFC 2007

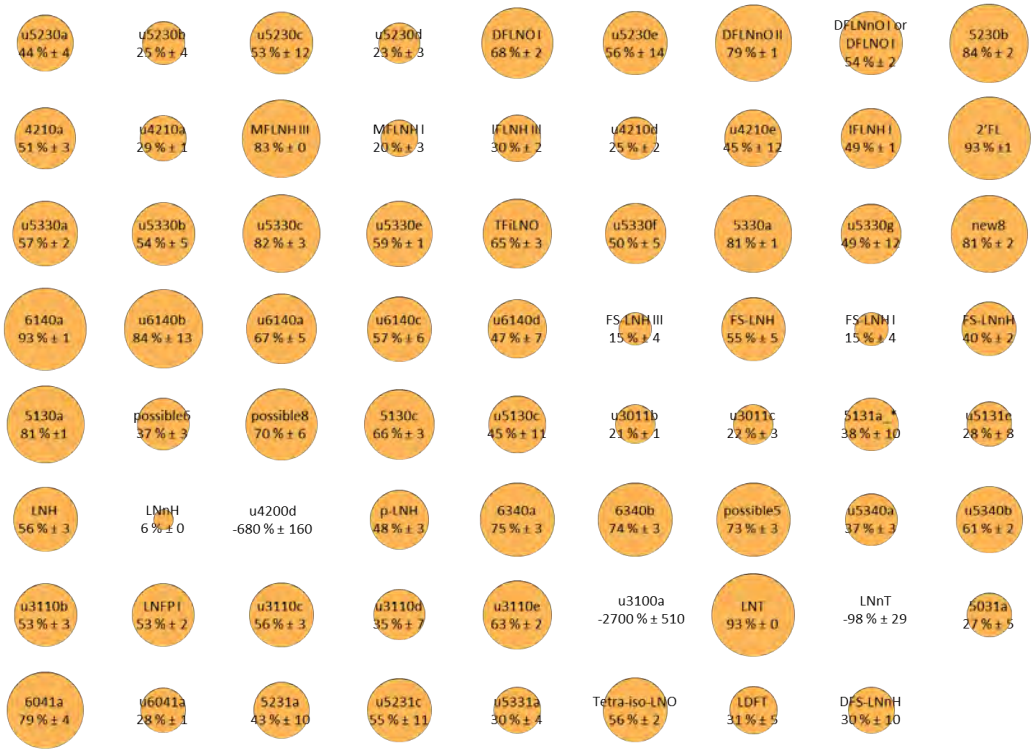
NanoLC separation of individual HMO compositions



Carlito Lebrilla
UCD Chemistry

Percent Consumption of HMO Structures

Consumption array of *B. infantis* ATCC 15697

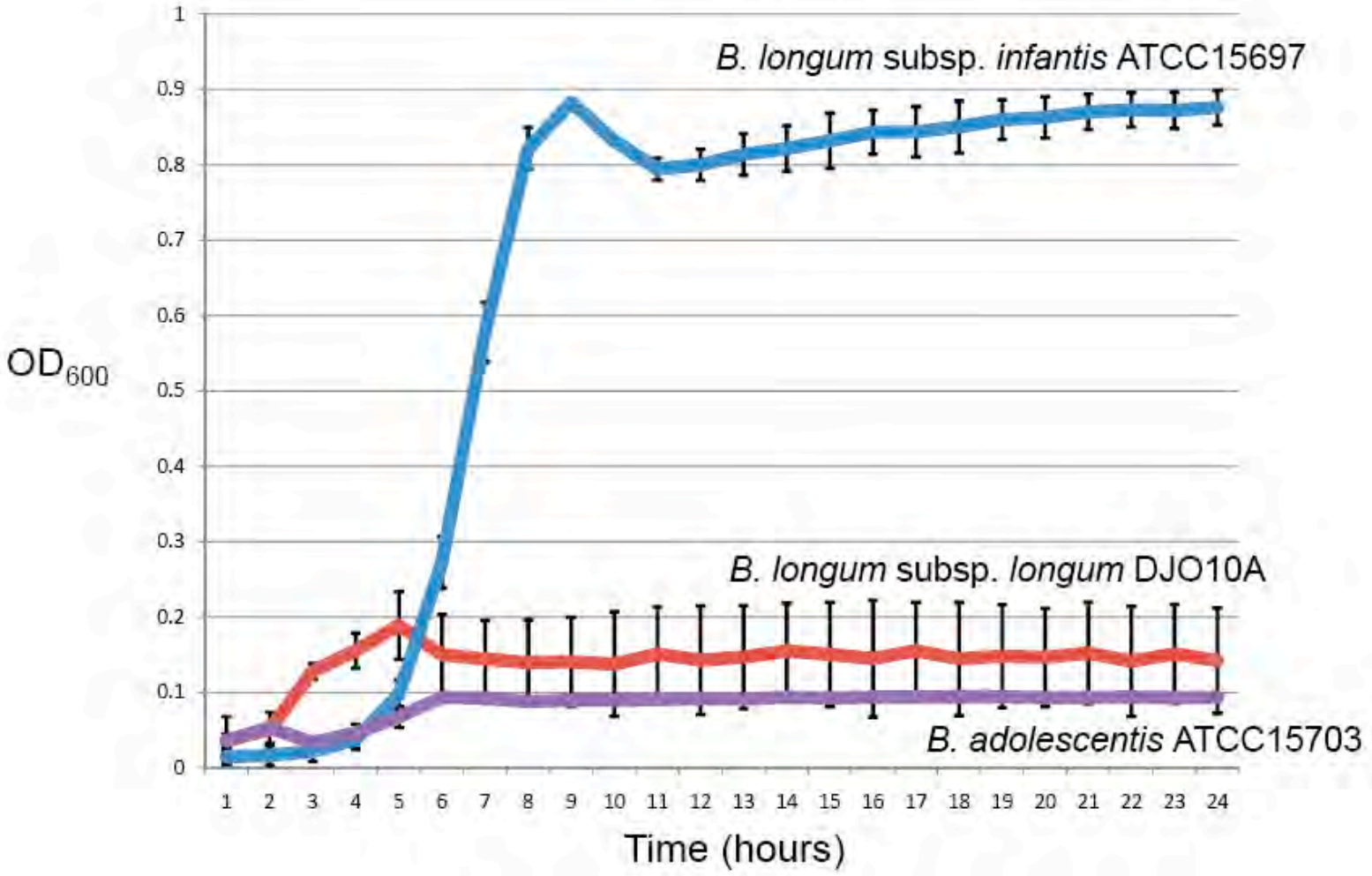


- Bacteria can be characterized by their HMO consumption profile.
- Orange circles are sized proportionally to the percent consumption.

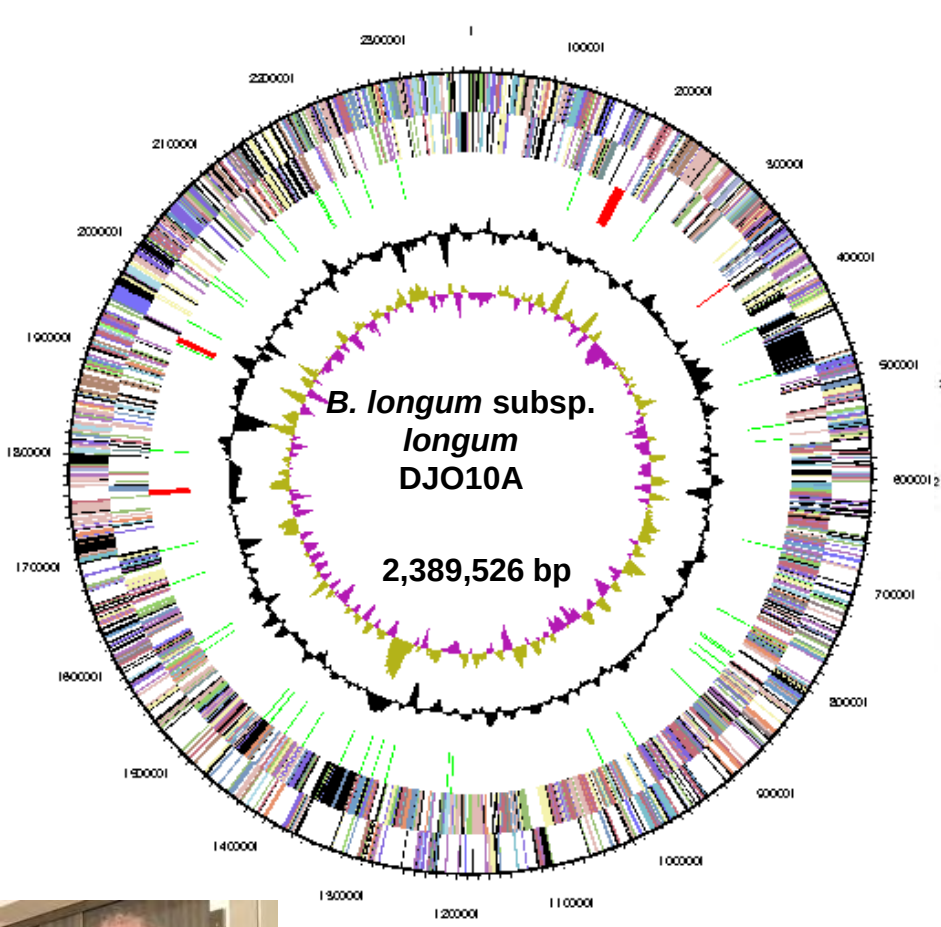
Strum, et al. *Anal. Chem.* **2013**

What genome features are required to utilize human milk oligosaccharides?

HMO utilization by Bifidobacteria

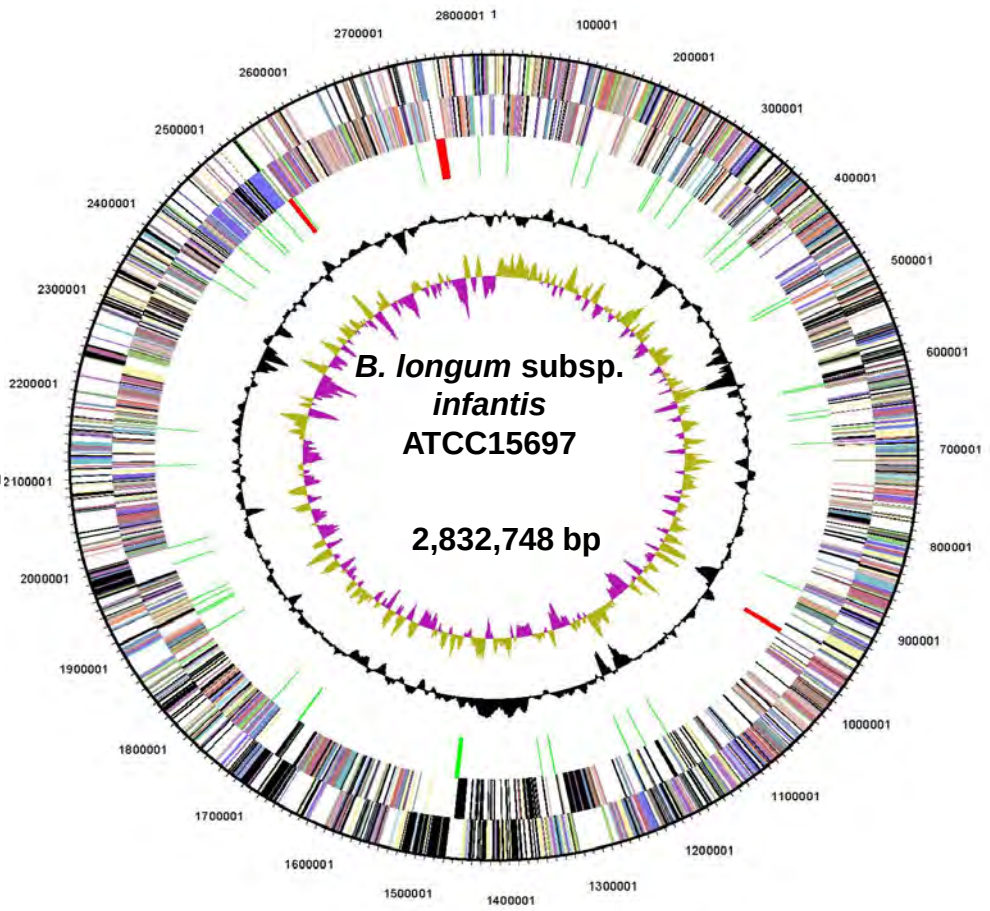


Comparative Bifidobacterium Genomics



Adult derived strain

BMC Genomics 2008

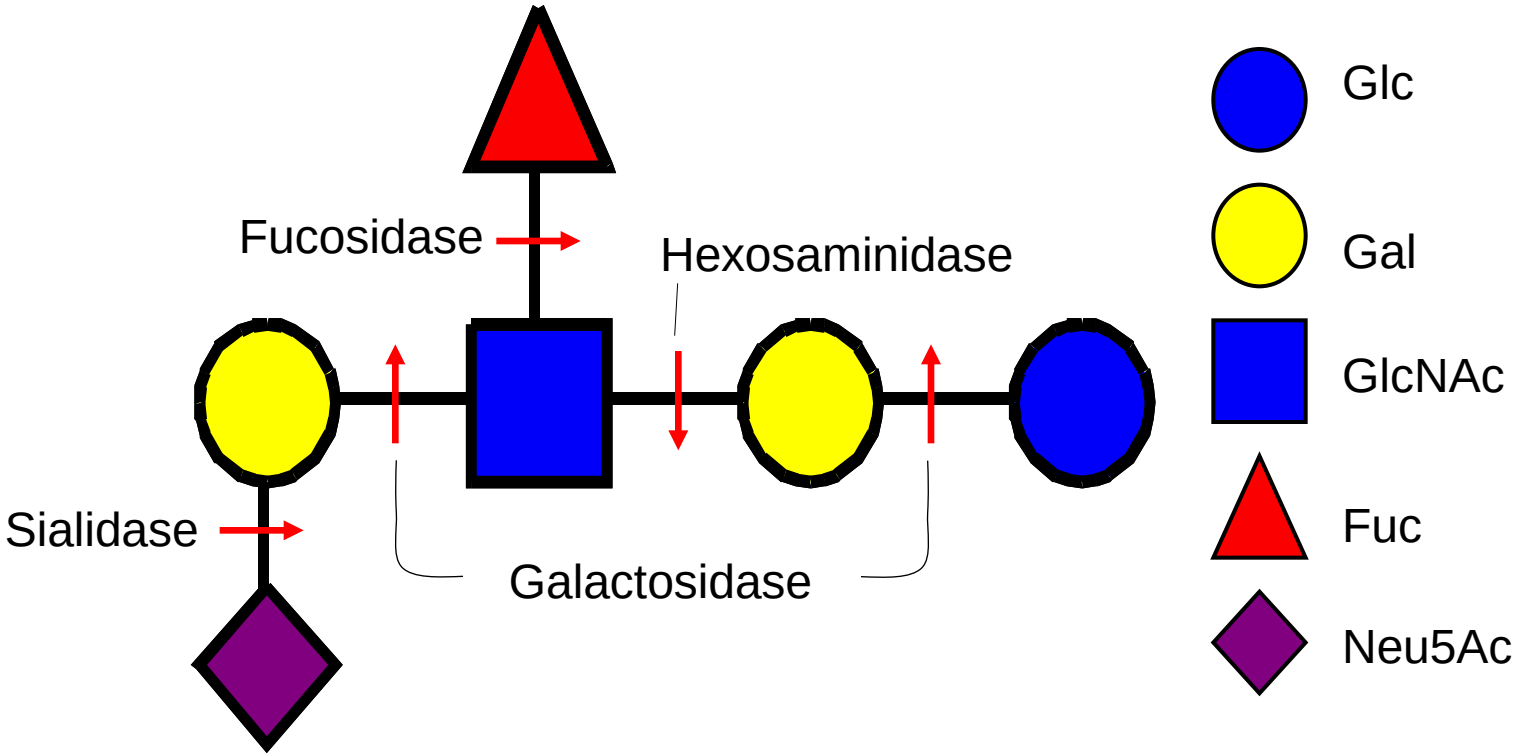


Infant derived strain

PNAS 2008

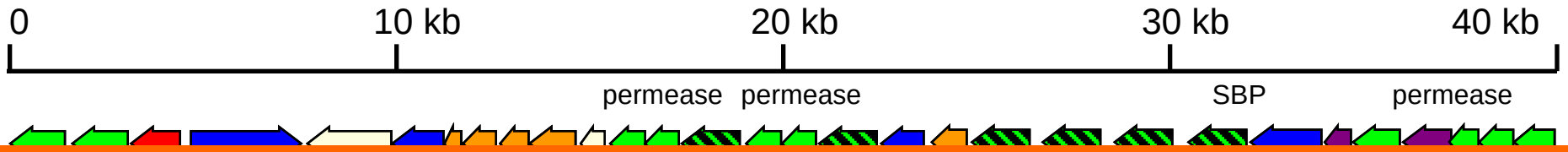
What's Needed to Deconstruct HMOs?

- Transport systems for oligo & monosaccharides
- Glycosyl hydrolases



B. infantis HMO cluster

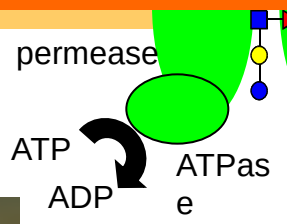
All 4 glycosyl hydrolases **Array of oligosaccharide transporters**

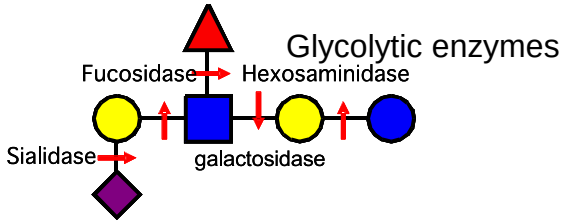


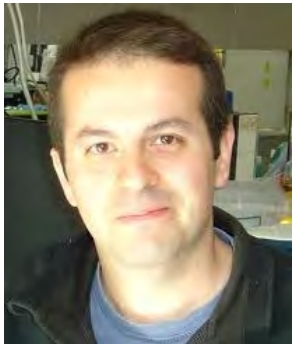
Genes unique to milk-associated bifidobacteria are uniquely expressed during growth on milk sugars

(PLoS One 2013, unpublished)

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

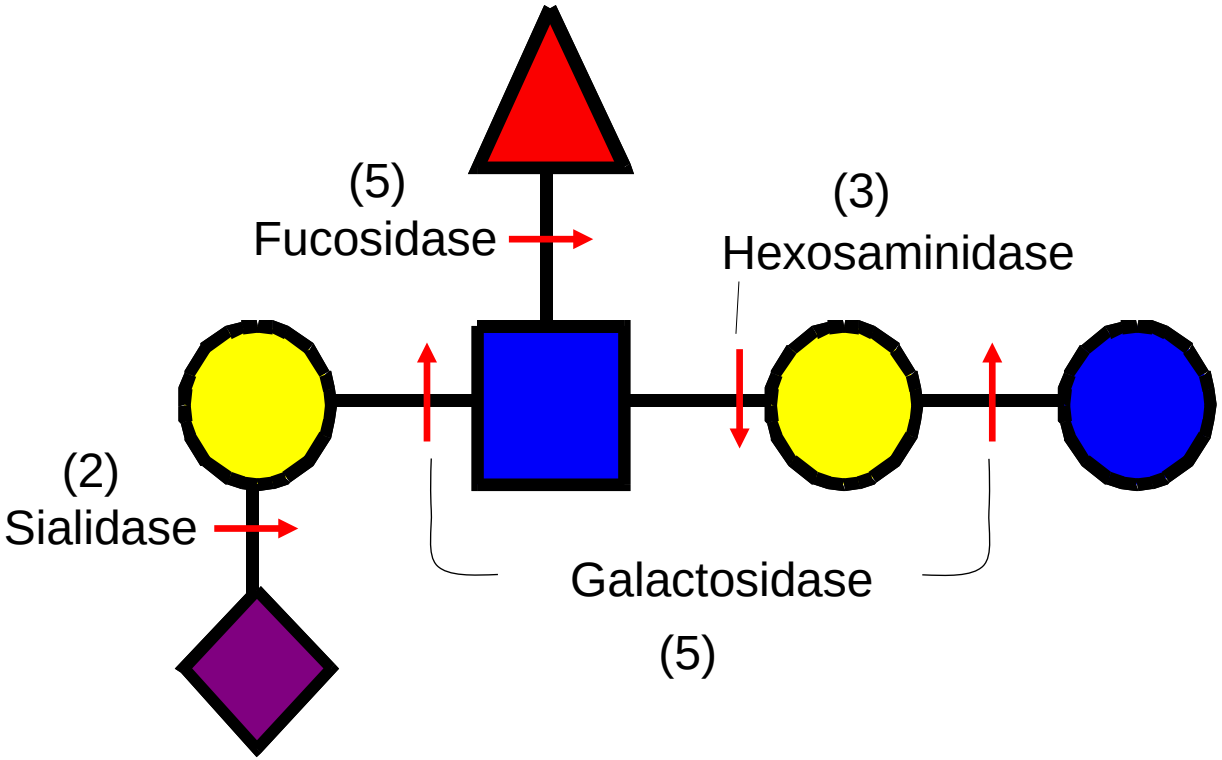


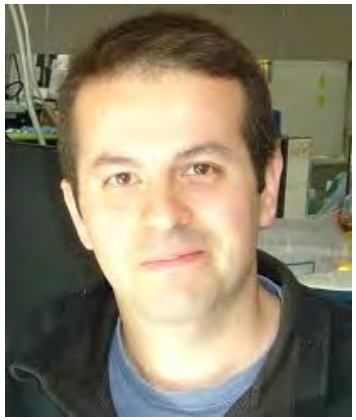




Sela PNAS 2008

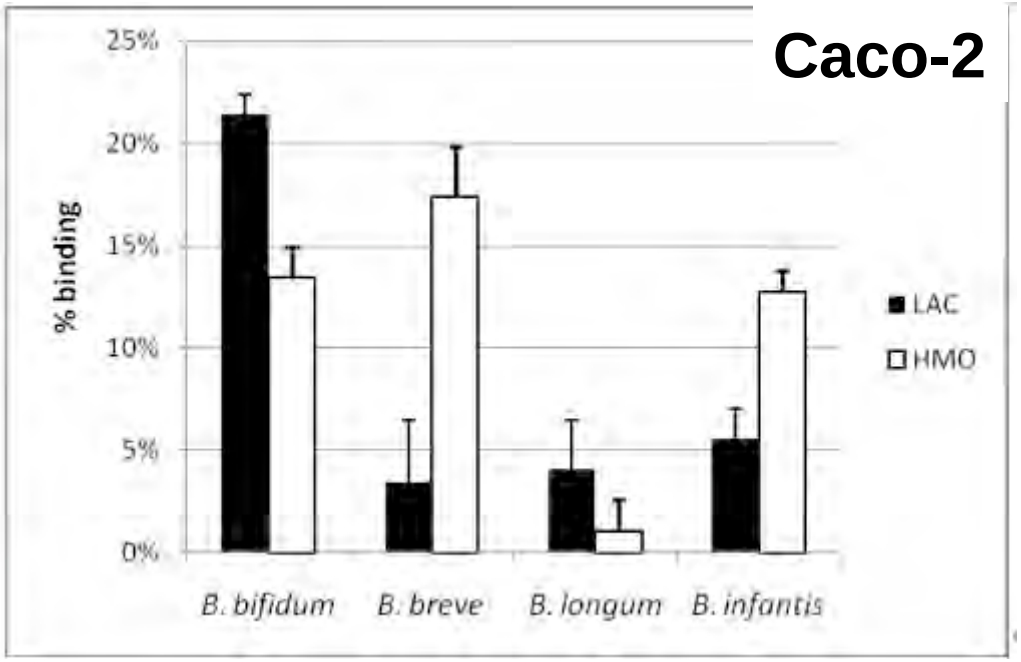
Characterization of the glycosidases and transporters from *B. infantis*?





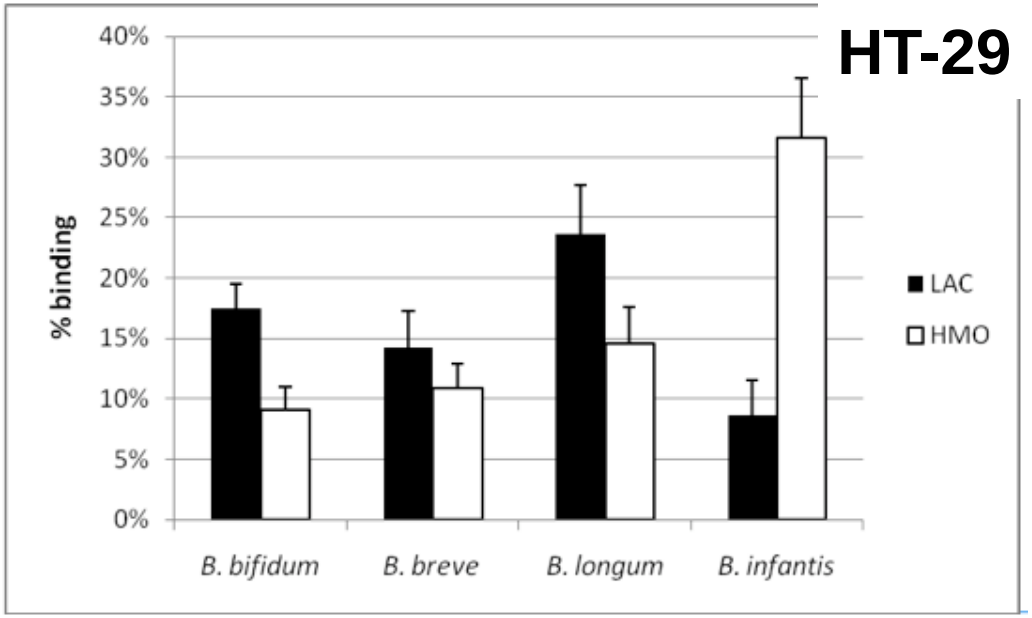
Sialidases - ----------	Sela et al JBC 2011
Fucosidases - ----------	Sela et al AEM 2012
Hexosaminidases - --------	Garrido et al Anaerobe 2012
Galactosidases - ----------	Garrido et al Food Micro 2012
Surface Binding Proteins	Garrido et al PLoS One 2011

Growth on milk oligosaccharides helps some bifidobacteria bind intestinal cells

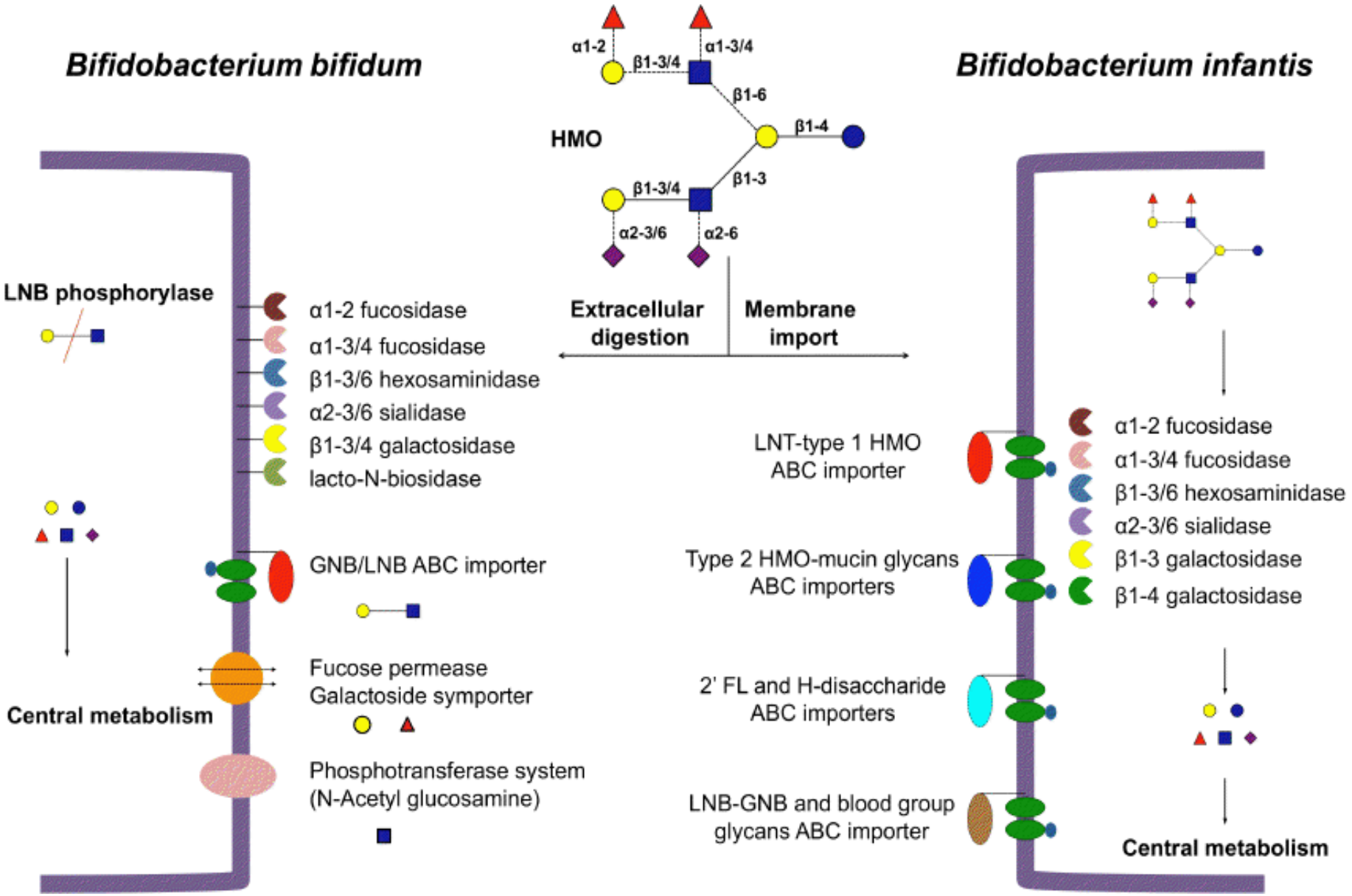


HMO vs Lac grown cells:

- Induce TJ proteins
- Induce anti-inflammatory cytokines (IL-10)

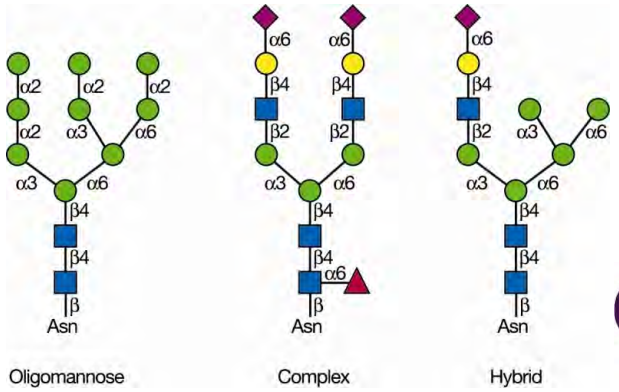


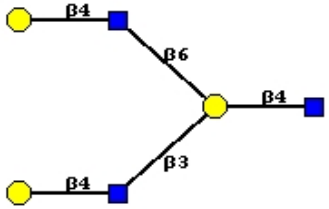
Chichlowski et al JPGN 2012

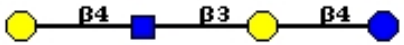


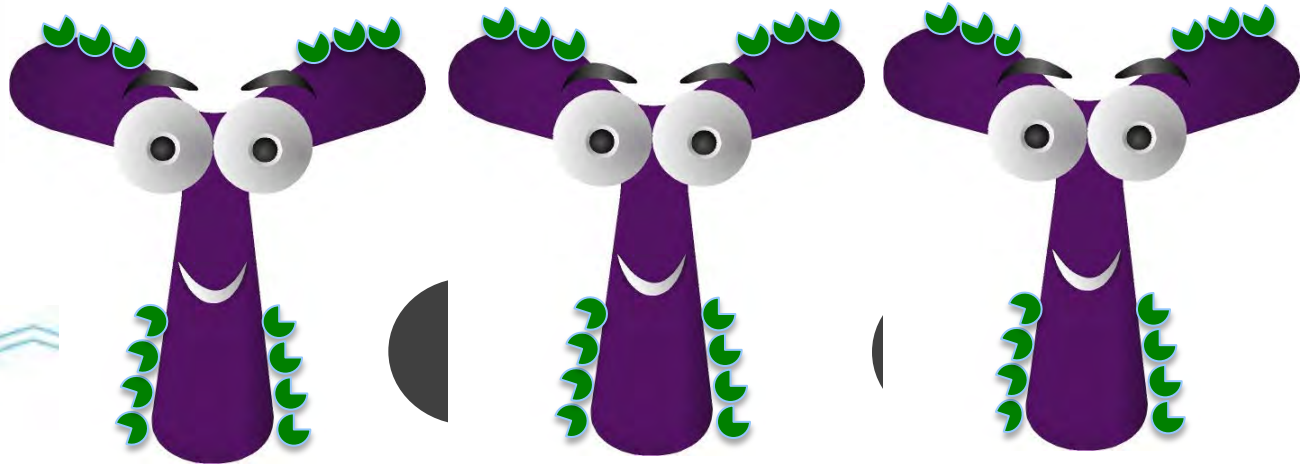
Garrido, et al. Advances in Nutrition (2012)

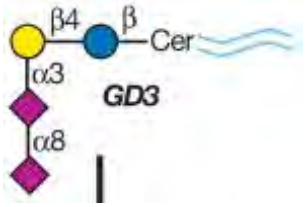
Model for bifidobacterial enrichment in the infant GIT

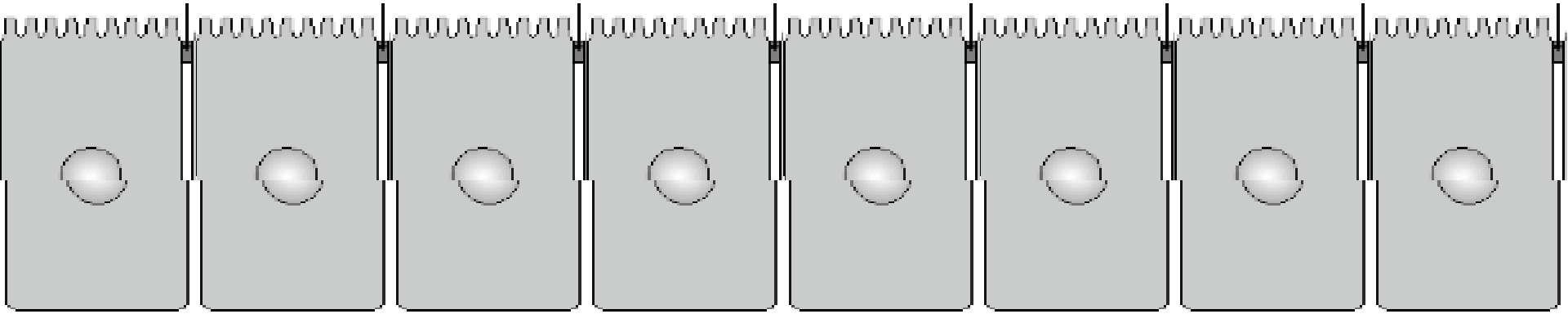




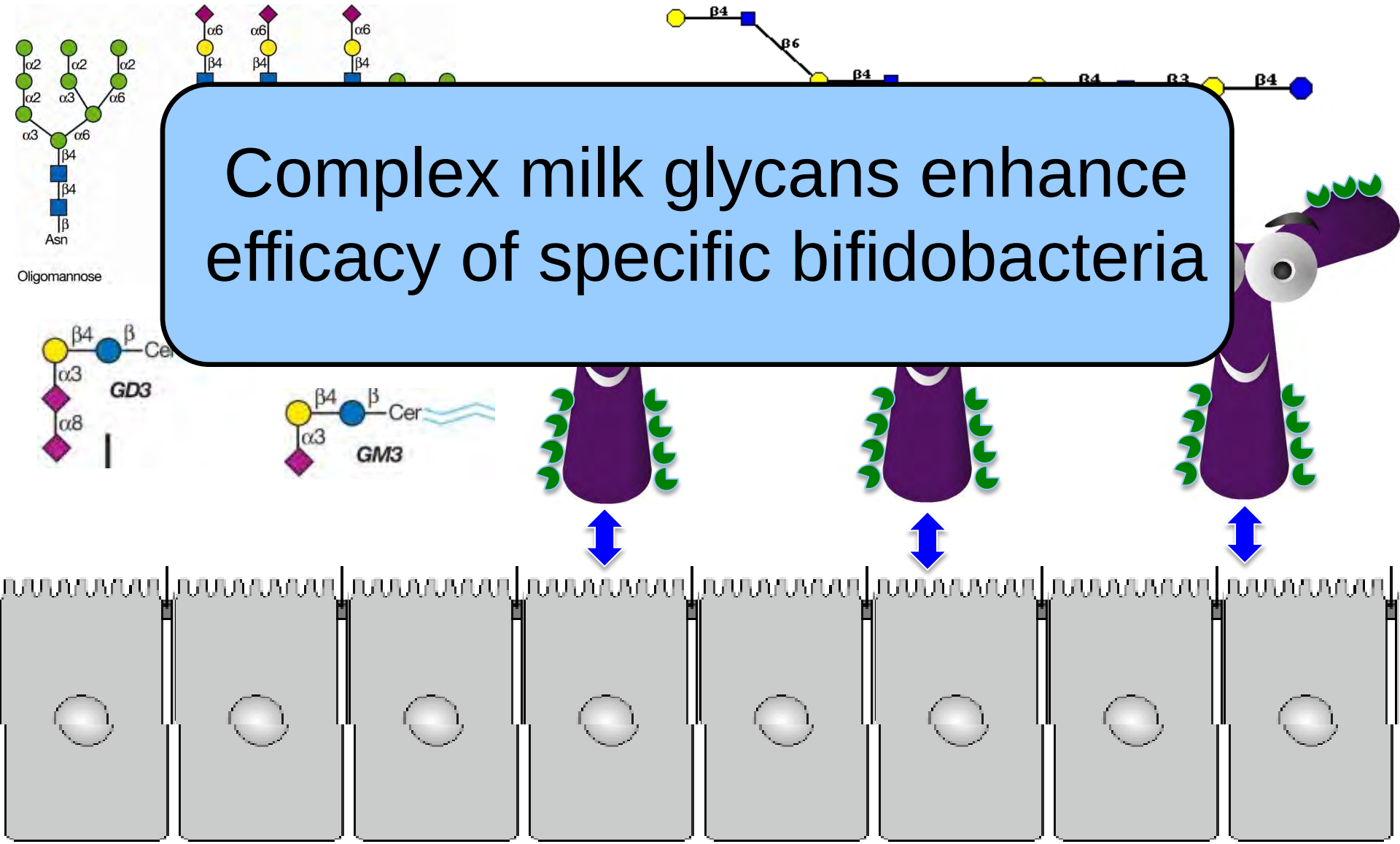






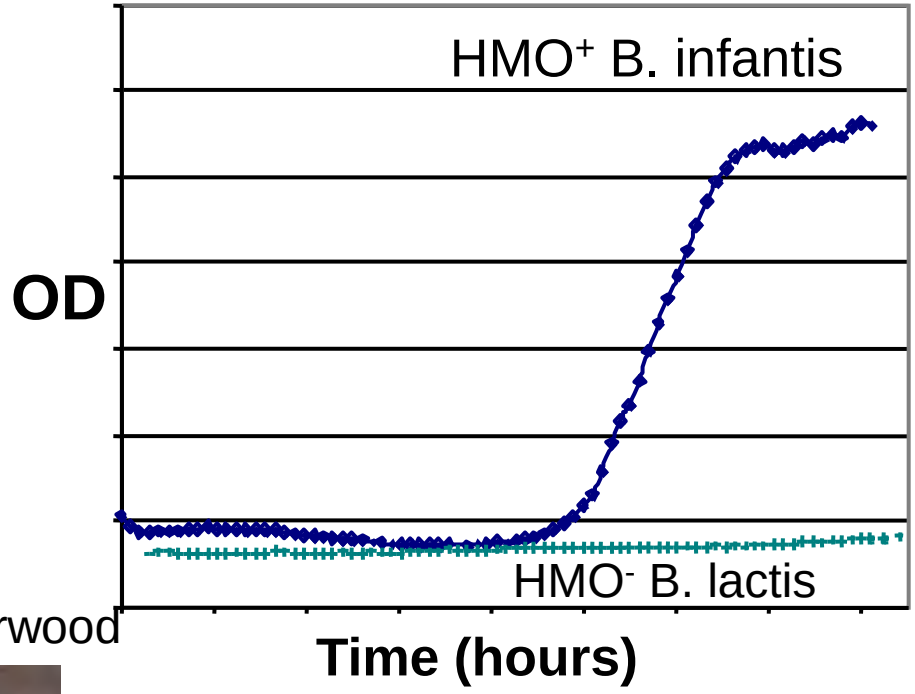


Model for bifidobacteria enrichment in the infant GIT

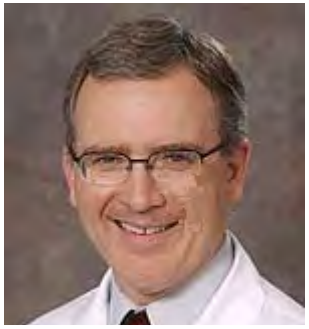


Can this knowledge be translated?

Will synbiotic feeding HMO$^+$ *B. infantis* with HMOs help establish bifidobacteria?



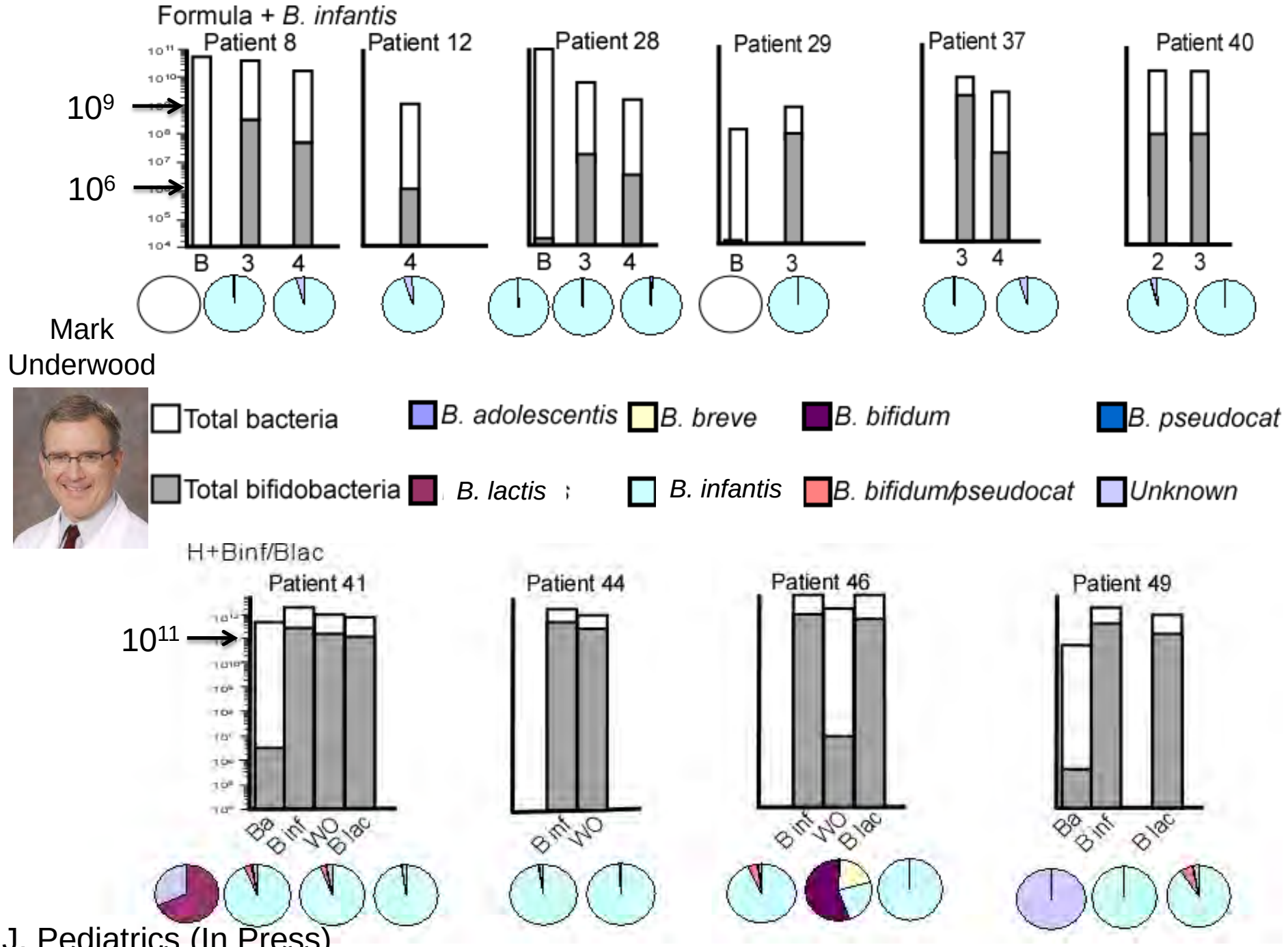
Mark Underwood



UCD Med School
Neonatology

Prolacta™ BIOSCIENCE
Advancing the Science of Human Milk™

National Institutes of Health
Eunice Kennedy Shriver NICHD
National Institute of Child Health & Human Development


Formula + B. infantis
Patient 8
Patient 12
Patient 28
Patient 29
Patient 37
Patient 40
10^9
10^6
B 3 4
4
B 3 4
B 3
3 4
2 3
Mark Underwood
Total bacteria
B. adolescentis
B. breve
B. bifidum
B. pseudocat
Total bifidobacteria
B. lactis
B. infantis
B. bifidum/pseudocat
Unknown
H+Binf/Blac
Patient 41
Patient 44
Patient 46
Patient 49
10^11
Ba
B inf
WO
B lac
J. Pediatrics (In Press)

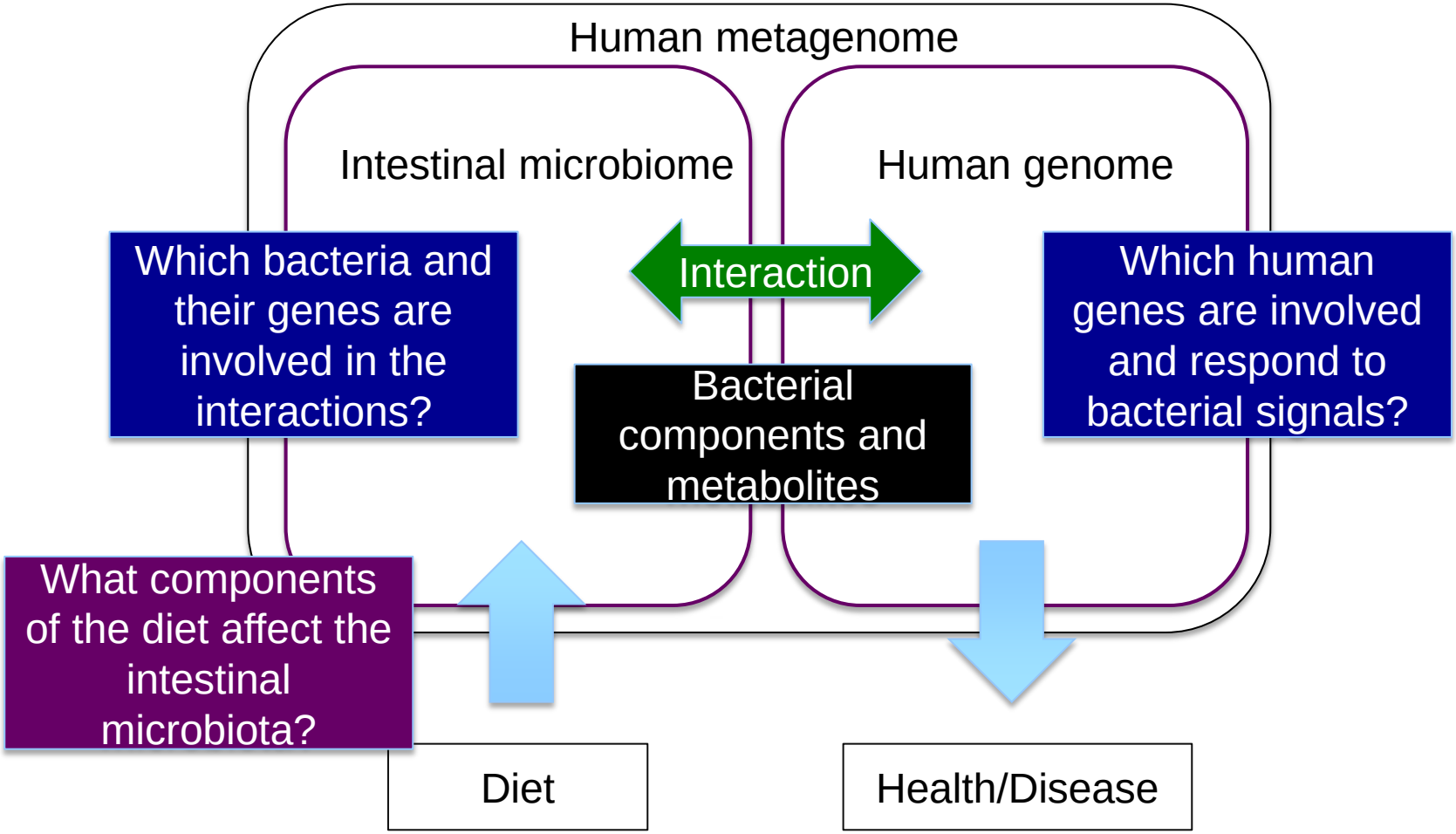
Take home points….

- 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

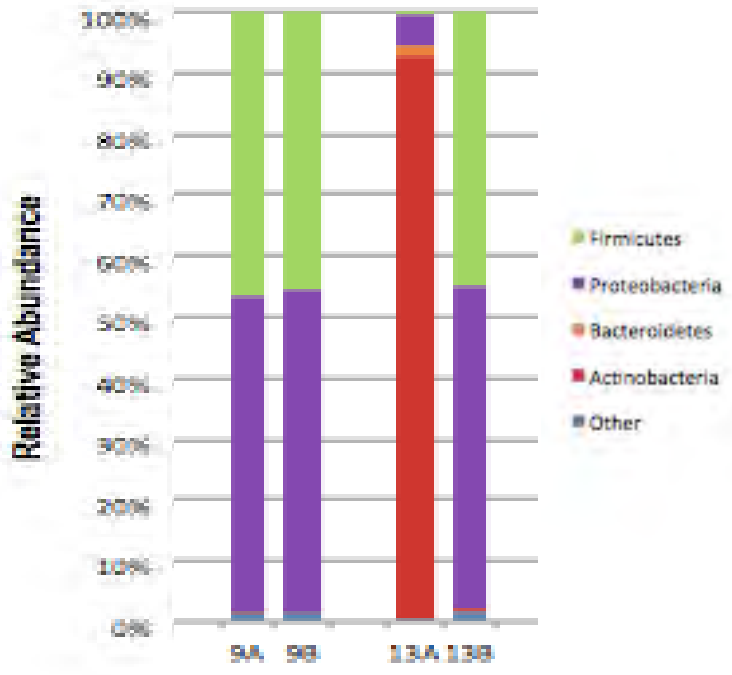


Gaps/Needs/Challenges

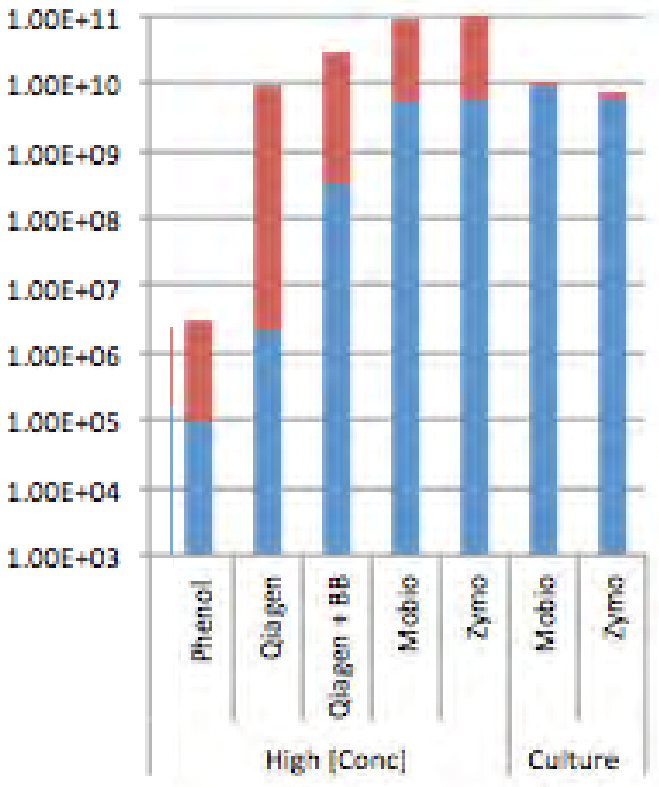
- Mechanistic research needed (systems biology + strain level examination)
- Interdisciplinary teams (thanks Vince)
- Better (supported) animal models (thanks Gary)
- Continued tool development…
  - Metabolomic/Metagenomic
  - Genetic tools!!
  - Glycomics
- Ability to stratify clinical populations

Needs/Challenges

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

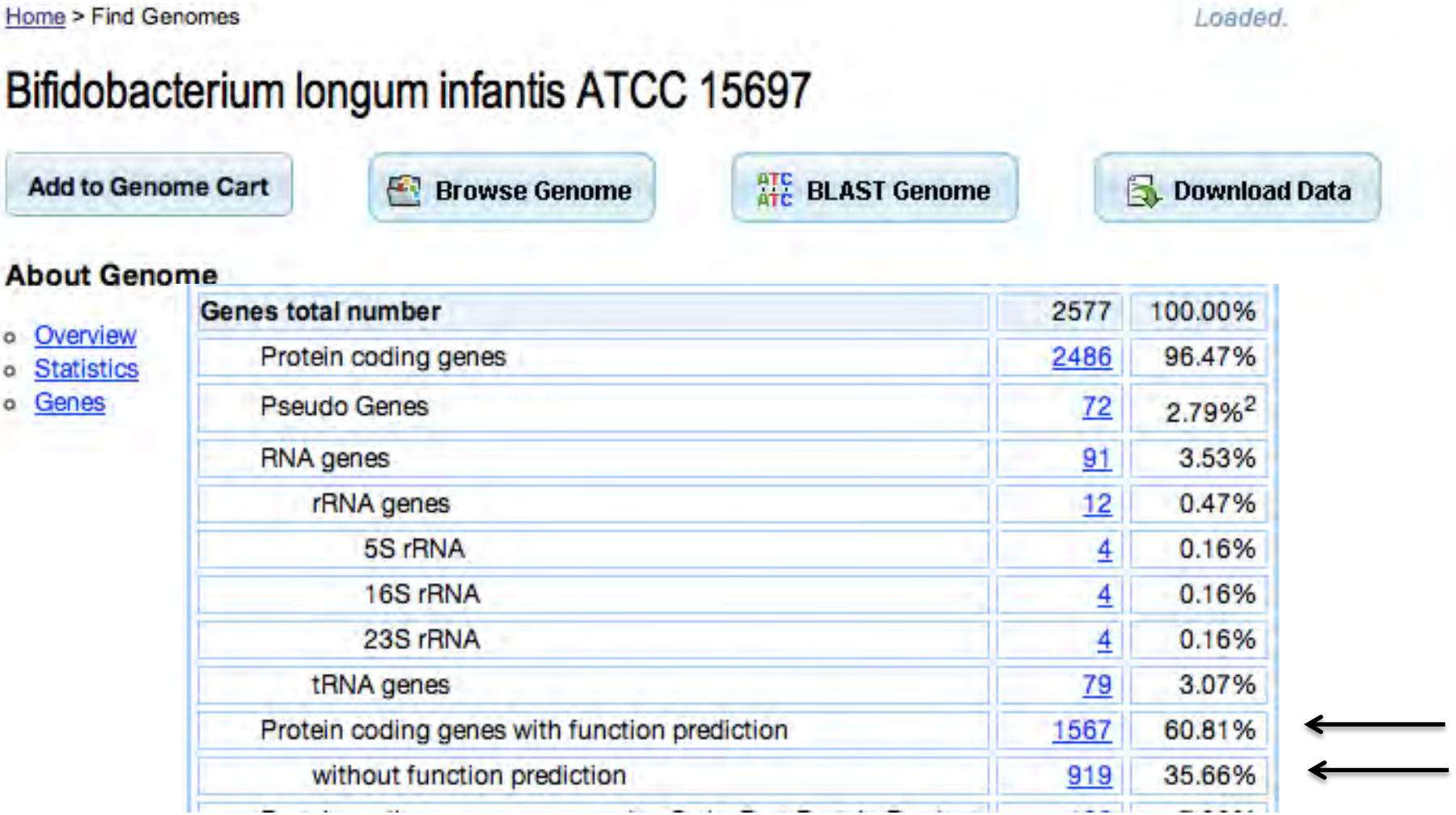


8F forward primer misses bifidobacteria (need to spike in a 8Fbif primer)



Lack of bead beating lowers bifidobacteria

Lack of accurate annotation/function will impede or misdirect microbiome work ….



Home > Find Genomes

Loaded.

Bifidobacterium longum infantis ATCC 15697

Add to Genome Cart | Browse Genome | BLAST Genome | Download Data

About Genome

- Overview
- Statistics
- Genes

Genes total number	2577	100.00%
Protein coding genes	2486	96.47%
Pseudo Genes	72	2.79%[2]
RNA genes	91	3.53%
rRNA genes	12	0.47%
5S rRNA	4	0.16%
16S rRNA	4	0.16%
23S rRNA	4	0.16%
tRNA genes	79	3.07%
Protein coding genes with function prediction	1567	60.81%
without function prediction	919	35.66%

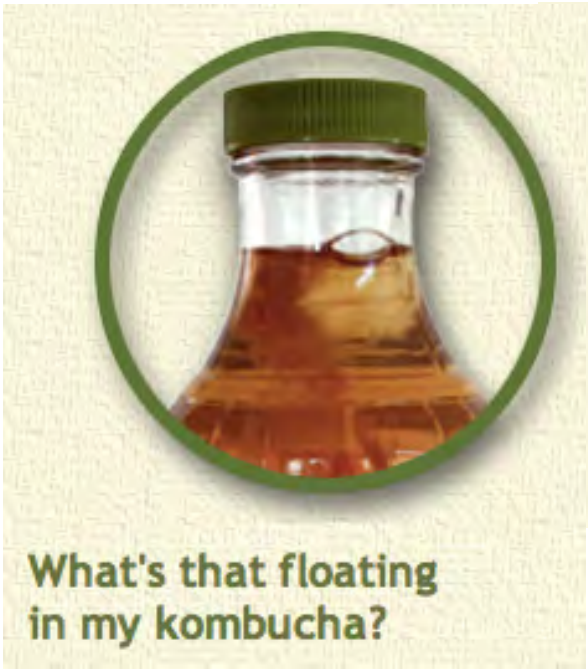
Media/Public/Physician Confusion on Probiotics

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

Genus	Species
Lactobacillus	*acidophilus* *brevis* *delbrueckii*[a] *fermentum* *gasseri* *johnsonii* *paracasei* *plantarum* *reuteri* *rhamnosus* *salivarius*
Bifidobacterium	*adolescentis* *animalis*[b] *bifidum* *breve* *infantis* *longum*
Streptococcus	*thermophilus* *salivarius*
Enterococcus	*faecium*
Escherichia	*coli*
Bacillus	*coagulans*[c] *clausii*
Saccharomyces	*cerevisiae*[d]

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

<u>But the public perception is not at the strain level</u>



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.

Are you delivering the probiotic species and strain you think you are?

Product	Microorganisms listed on the product label	T-RFLP	Species-specific PCR	Additional microbe T-RFLP patterns detected
1	*Lb acidophilus*	+	+	*Lb brevis*, *Lb plantarum*[*], *Lb jonhsonii*[†], *Lb amylolyticus*, *Lactobacillus* sp[‡]
	B bifidum	−	+	
	L helveticus	+	+	
	S thermophilus	+	+	
2	*Lb acidophilus*	+	+	*Lb brevis*, *Lb plantarum*[*], *Lb amylolyticus*, *Lactobacillus* sp[‡]
	B bifidum	+	+	
	Lb helveticus	+	+	
	Lb rhamnosus	+	+	
5	*Lb acidophilus*	+	+	*Lb bifermentus*, *Lactobacillus* sp[‡], *L lactis*, *Lb rhamnosus*[§]
	B bifidum	−	+	
	Lb helveticus	+	+	
6	*Lb acidophilus*	+	+	*Lb bifermentus*, *Lb sanfranciscensis*, *Lactobacillus* sp[‡], *Lb plantarum*[*], *Lb fructivorans*
	B bifidum	−	+	
	Lb helveticus	+	+	
7	*Lb acidophilus*	+	+	*Lb plantarum*[*]
	B bifidum	−	+	
	B longum	+	+	
8	*Lb acidophilus*	+	+	*Lb bifermentus*, *Lactobacillus* sp[‡], *Lb helveticus*, *Lb plantarum*[*] *Lb rhamnosus*[§]
9	*B longum*	+	+	
	B bifidum	+	+	
10	*Lb acidophilus*	+	+	*L lactis*, *Lb rhamnosus*, *Lb plantarum*[*], *Lb jonhsonii*[†]
11	*Lb acidophilus*	+	+	*Lb bifermentus*, *Lactobacillus* sp[‡], *Lb helveticus*, *Lb plantarum*[*], *Lb rhamnosus*[§]
12	*Lb acidophilus*	−	+	*Lb plantarum*[*], *Lb sanfranciscensis*, *Lb helveticus*, *Lactobacillus* sp[‡]
13	*Lb casei*	+	+	*Lb casei*, *Lb rhamnosus*[§]
14	*Lb acidophilus*	+	+	*Lb rhamnosus*[§], *Lb plantarum*[*], *Lactobacillus* sp[‡], *B animalis*, *Lb amylolyticus*, *Lactobacillus* sp[¶]
	B longum	+	+	
	B bifidum	+	+	

Marcobal et al. JPGN 2008

Understanding Responder Non-Responder Issues

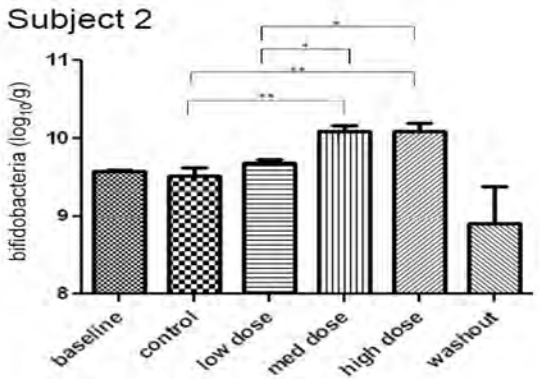
OPEN ACCESS Freely available online

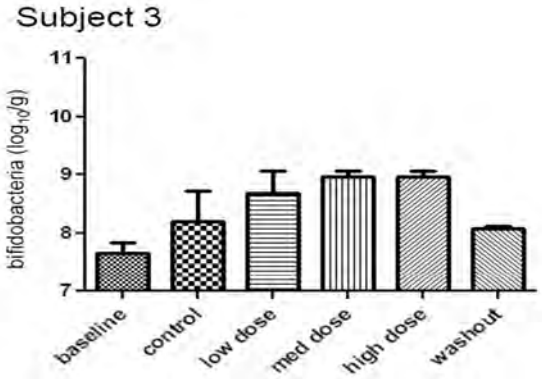
PLoS one

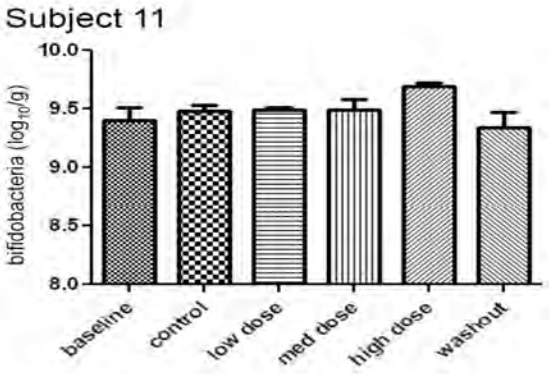
Barcoded Pyrosequencing Reveals That Consumption of Galactooligosaccharides Results in a Highly Specific Bifidogenic Response in Humans

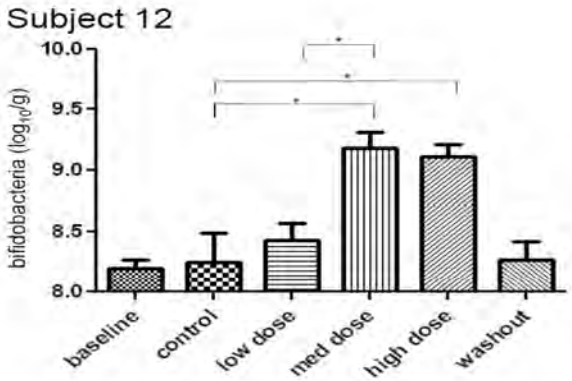
Lauren M. G. Davis[1], Inés Martínez[1], Jens Walter[1], Caitlin Goin[2], Robert W. Hutkins[1]*

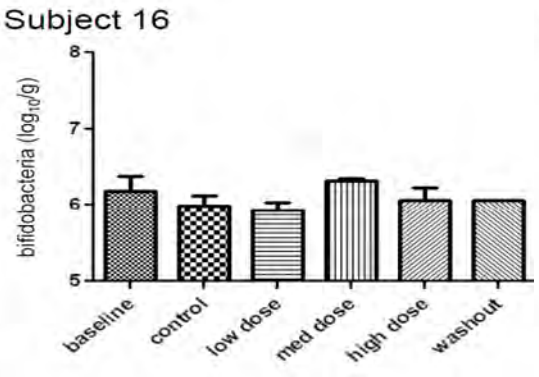
1 Department of Food Science and Technology, University of Nebraska, Lincoln, Nebraska, United States of America, 2 School of Biological Sciences, University of Nebraska, Lincoln, Nebraska, United States of America

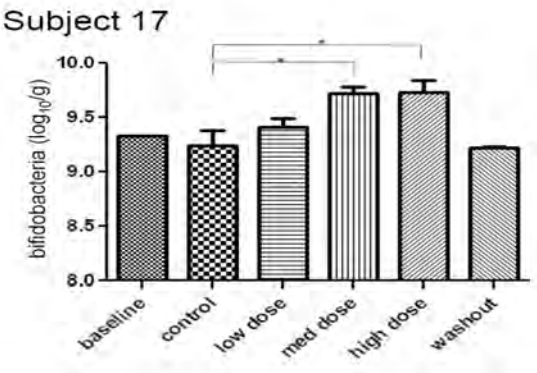




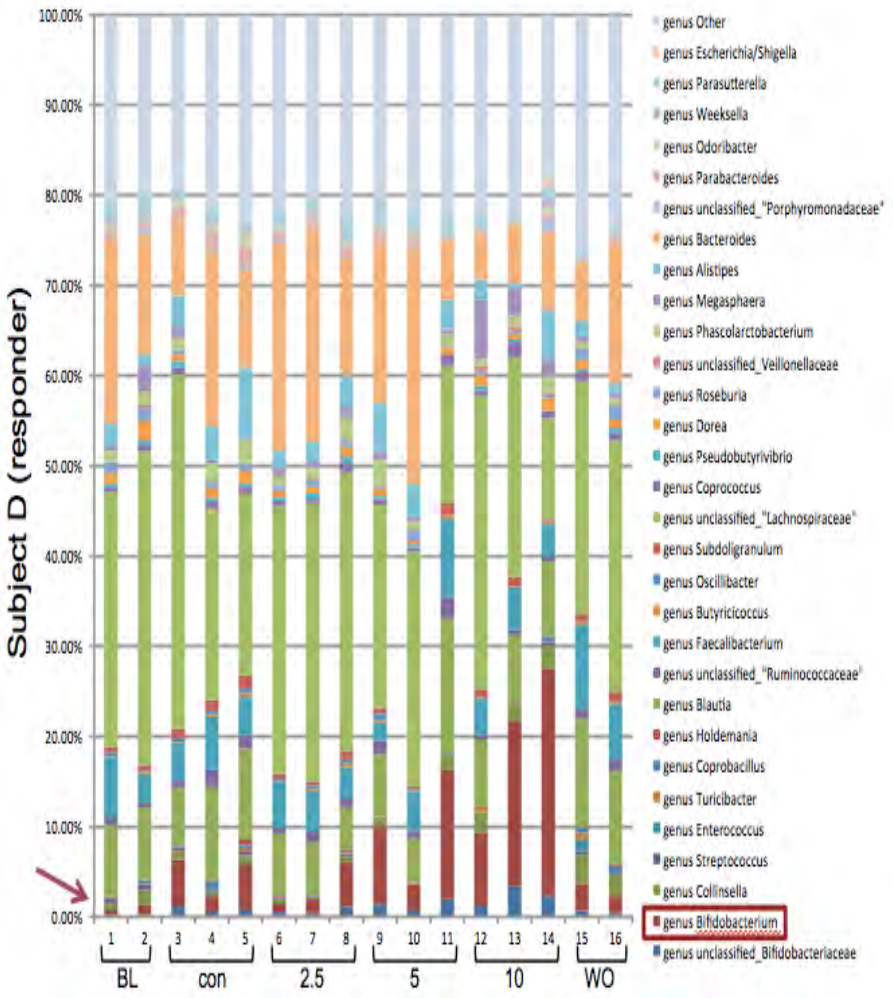


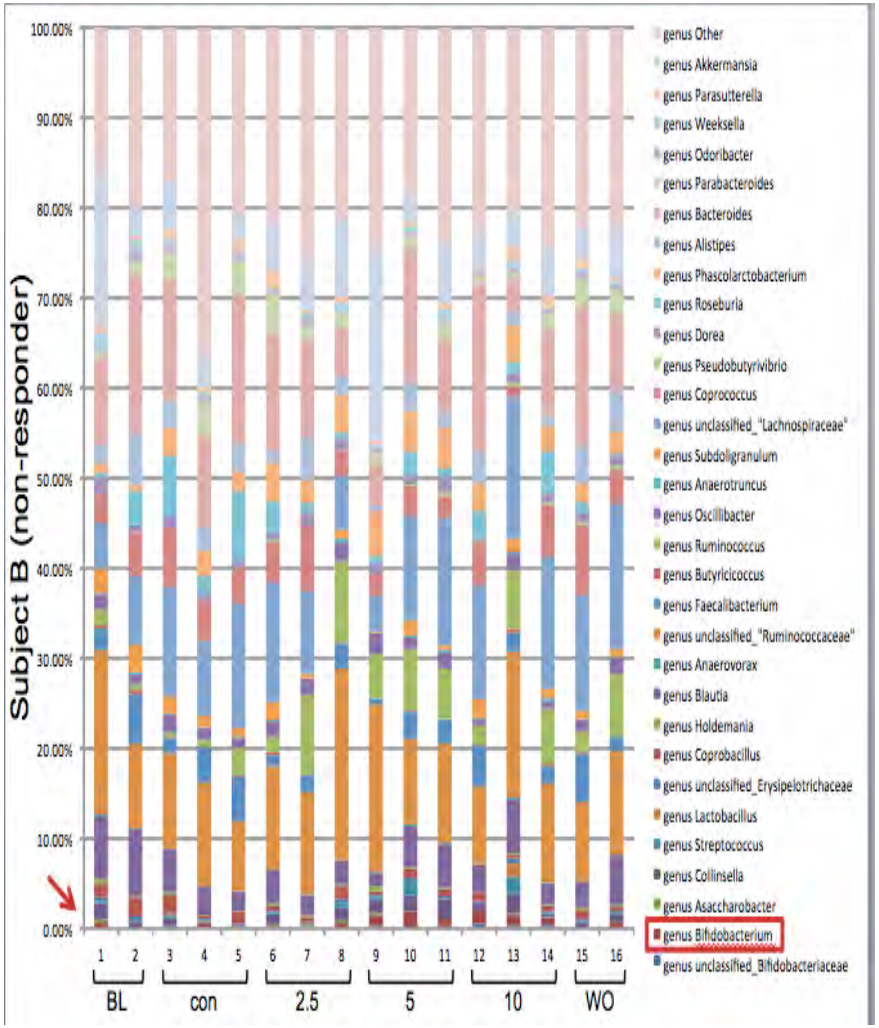






Understanding Responder Non-Responder Issues





Davis et al PLoS One 2011

PIs: **Carlito Lebrilla, J. Bruce German**, Xi Chen, Mark Underwood, Chuck Bevins, Helen Raybould

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the UC Discovery Grant

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Conflict of Interest Statement

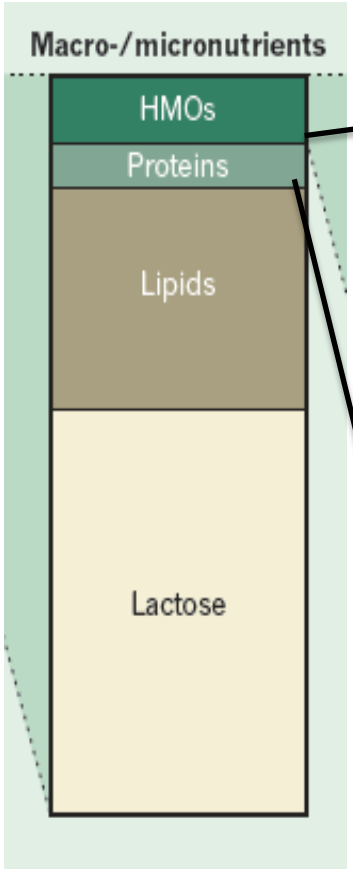
Co-Founder – Evolve Biosystems Inc.

Co-Founder – MicroTrek Inc.

What's that floating in my kombucha?

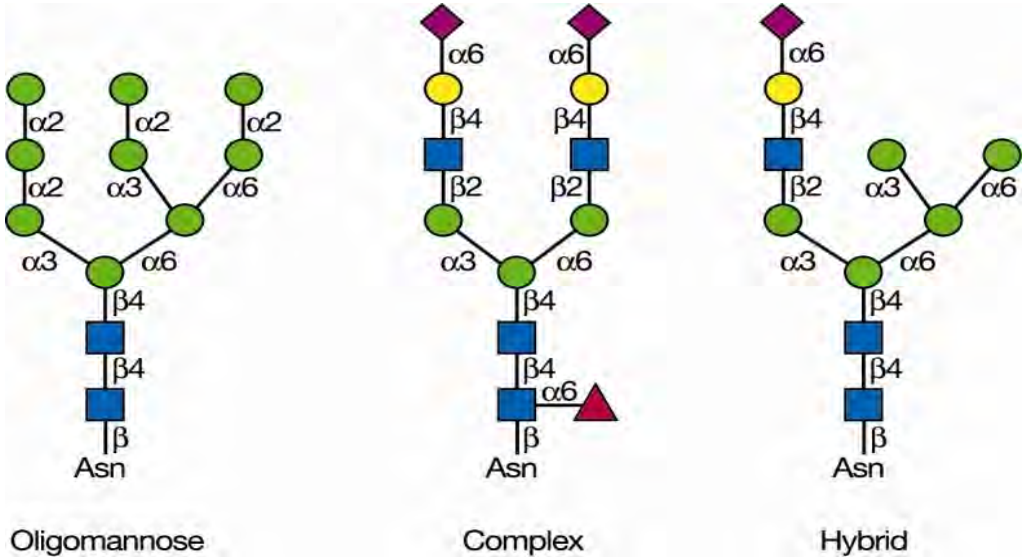
Our kombucha is raw meaning that it has never been pasteurized nor heat treated. As a living product, new cultures will continue to form even once bottled. Sometimes they are clear, like egg whites, and other times they are brownish in color. These cultures are harmless and indicate that the beverage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

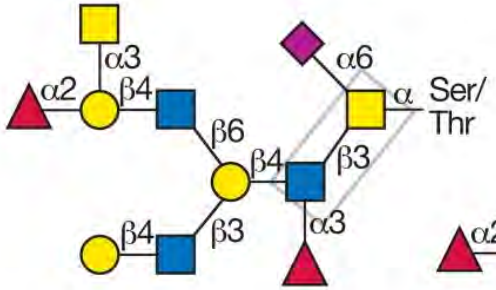
What about bifidobacterial growth on milk glycoproteins?

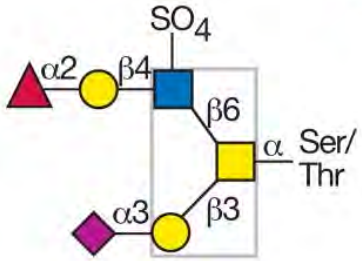


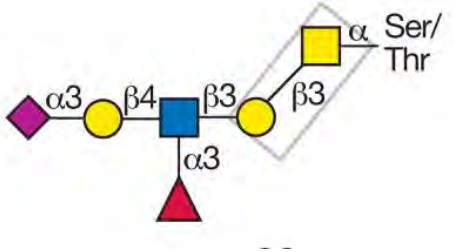
N-linked exp.
Lactoferrin
Immunoglobulins

O-linked exp.
Caseins (K)



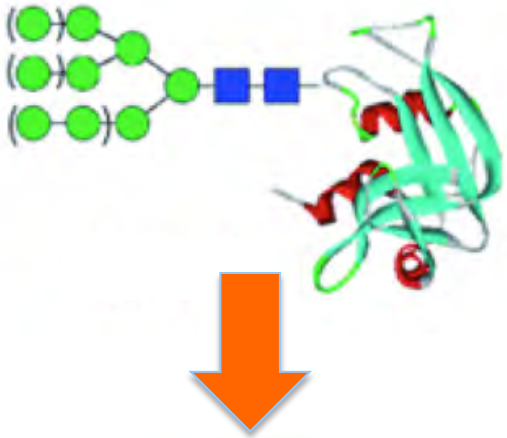


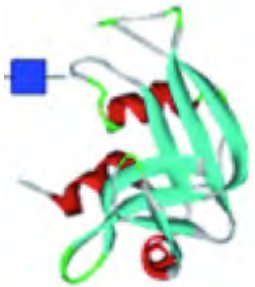


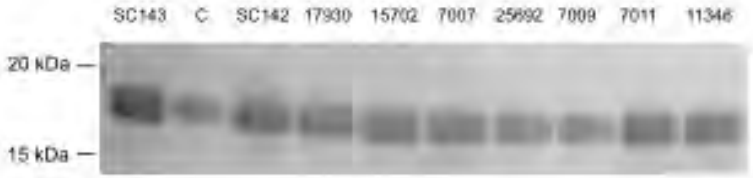


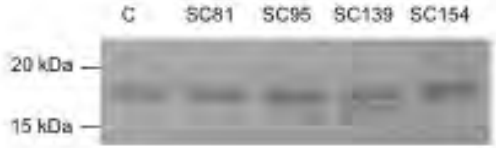
What about bifidobacterial growth on milk glycoproteins?

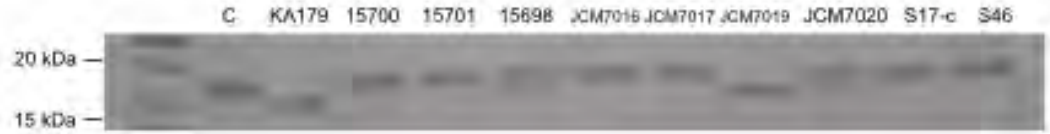
RNaseB as proxy
N-linked glycan





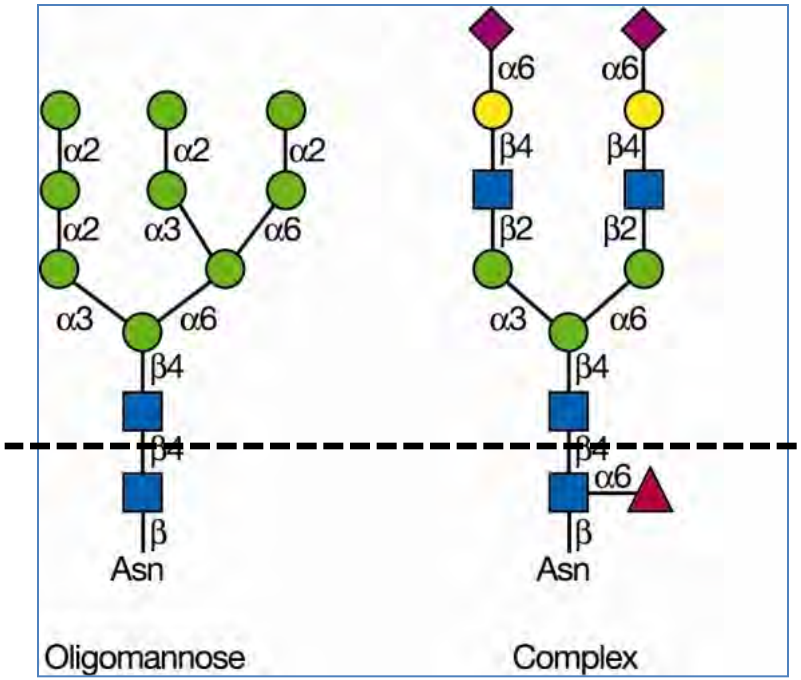


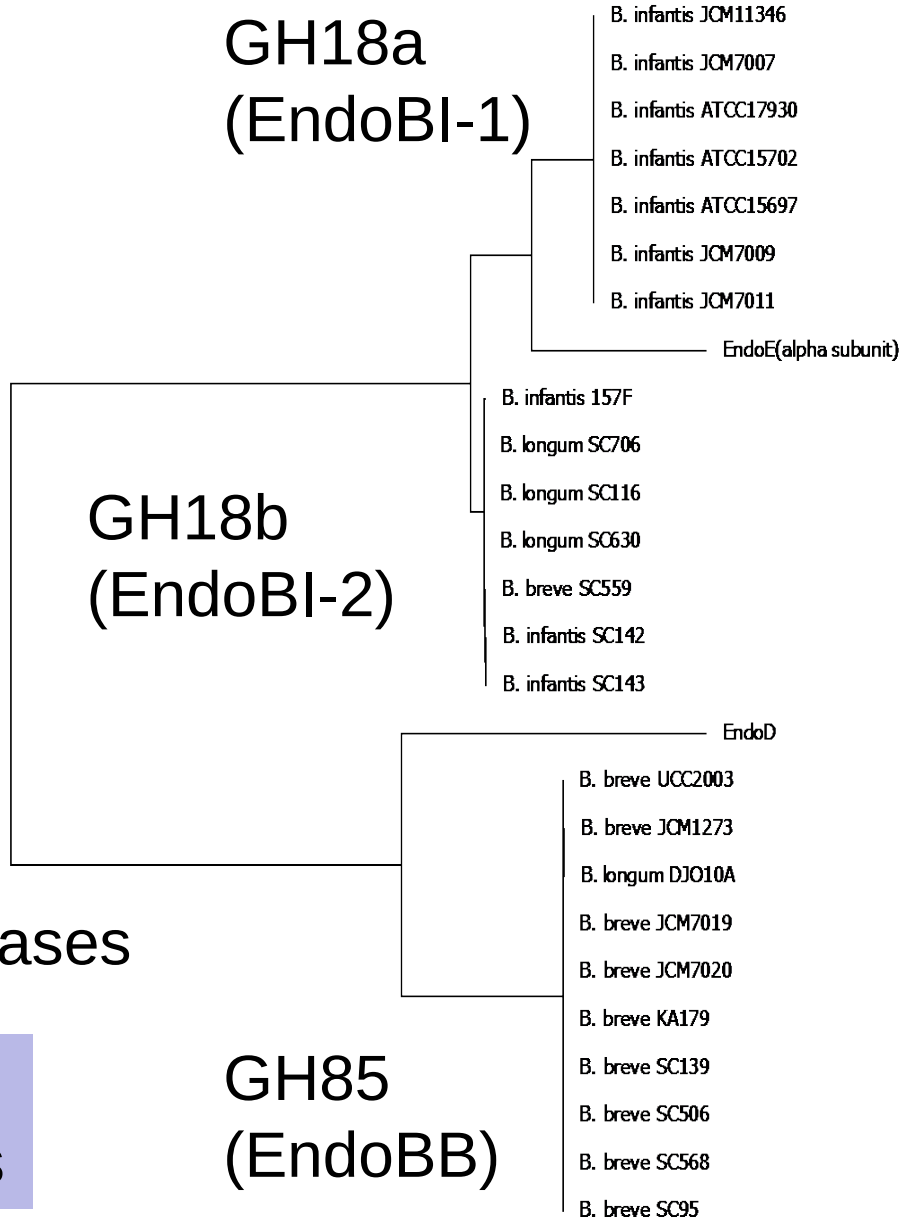




Garrido et al Molecular Cellular Proteomics 2012

Endoglycosidase genes in bifidobacteria



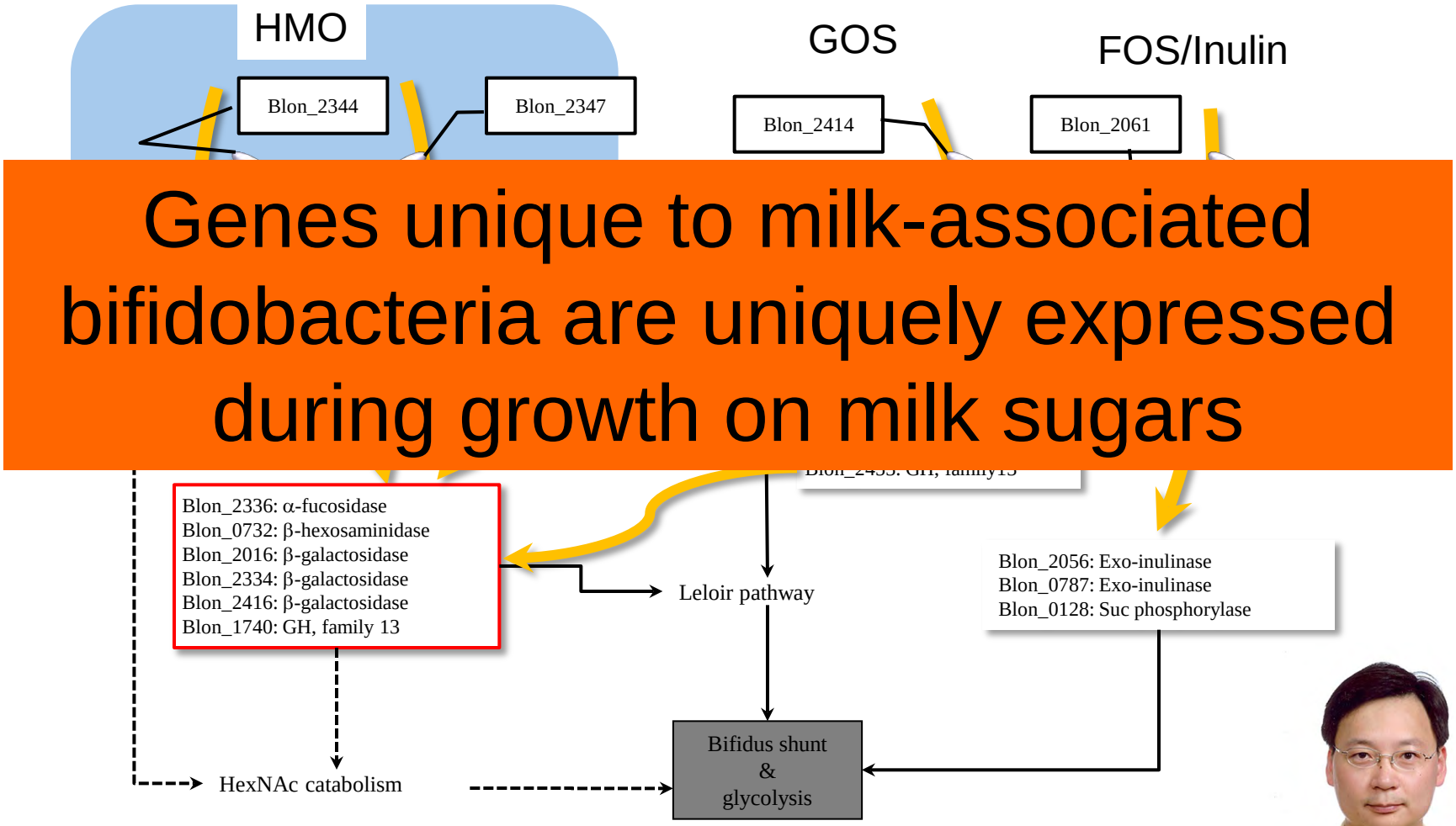


Endo-**beta**-N-acetylglucosaminidases

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

Garrido et al Molecular Cellular Proteomics 2012

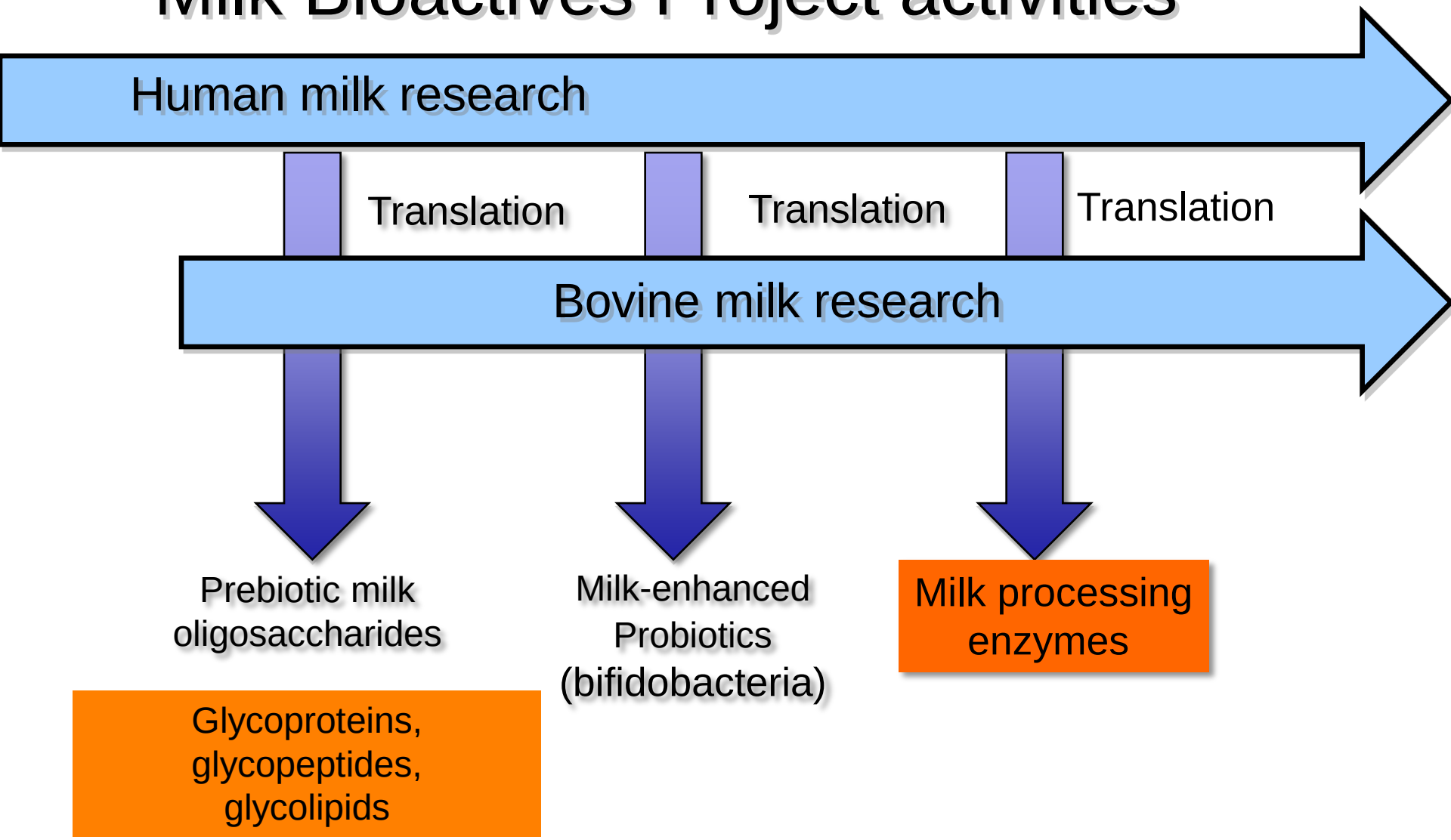
Whole cell proteomics of B. infantis grown on different prebiotic sugars



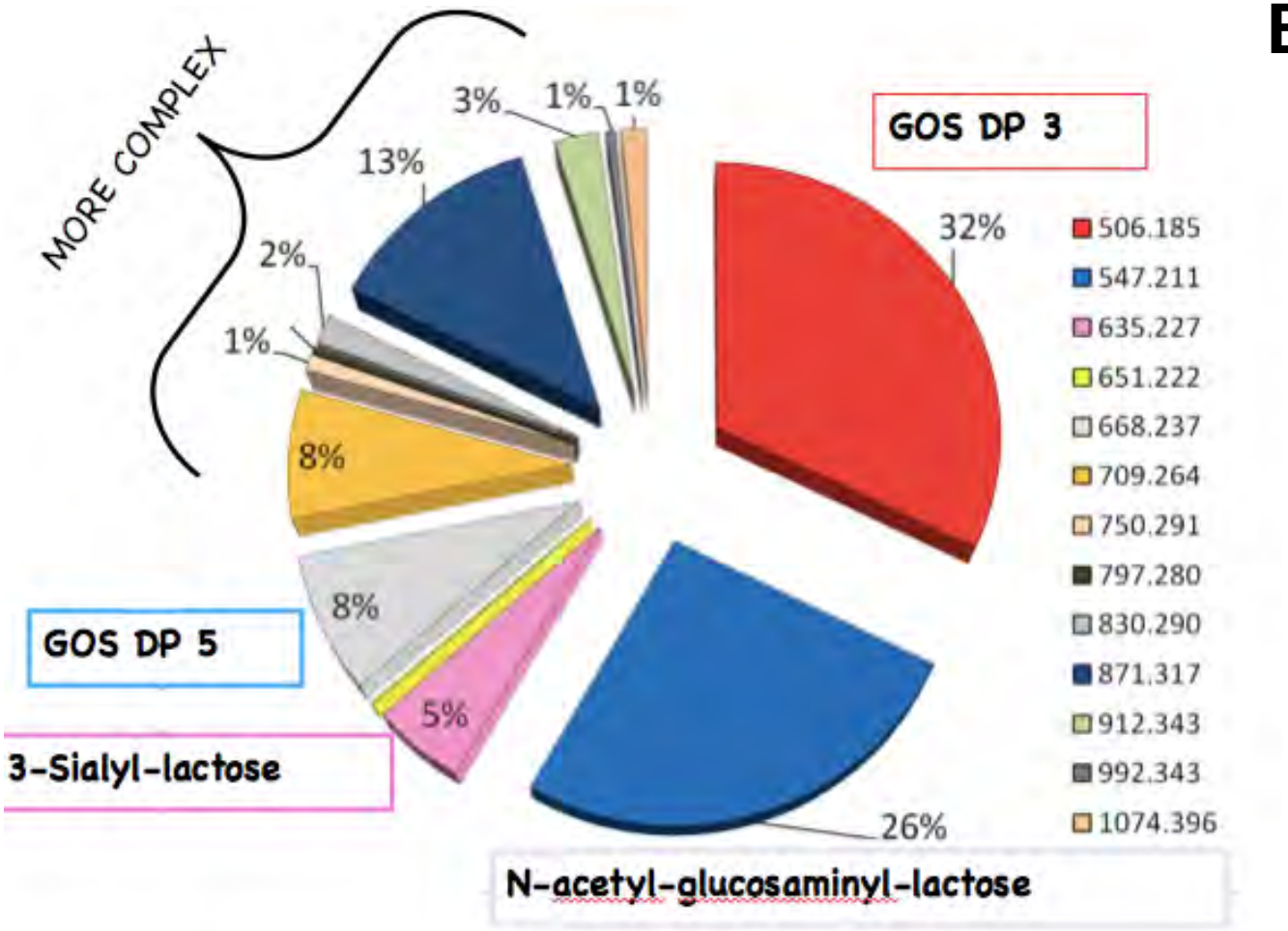
PLoS One 2013

Jae Han Kim

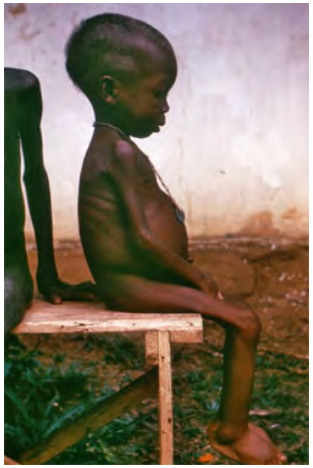
Milk Bioactives Project activities



Whey permeate oligosaccharides



BMMI Project



Jeff Gordon Wash U

BILL & MELINDA GATES *foundation*

HILMAR CHEESE COMPANY

Nutrition Facts

Serving Size 1 cup (228g)
Servings Per Container 2

Amount Per Serving

Calories 250	Calories from Fat 110
	% Daily Value*
Total Fat 12g	18%
Saturated Fat 3g	15%
Trans Fat 3g	
Cholesterol 30mg	10%
Sodium 470mg	20%
Total Carbohydrate 31g	10%
Dietary Fiber 0g	0%
Sugars 5g	
Protein 5g	
Vitamin A	4%
Vitamin C	2%
Calcium	20%
Iron	4%

* Percent Daily Values are based on a 2,000 calorie diet. Your Daily Values may be higher or lower depending on your calorie needs.

	Calories:	2,000	2,500
Total Fat	Less than	65g	80g
Sat Fat	Less than	20g	25g
Cholesterol	Less than	300mg	300mg
Sodium	Less than	2,400mg	2,400mg
Total Carbohydrate		300g	375g
Dietary Fiber		25g	30g

"In the 20th century we determined what food is…

Bruce German
UCD Food Science

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