Pediatric Nutrition
CONTINUING EDUCATION FOR CLINICIANS

Bioactive Components of Human Milk Including HMOs and Other Prebiotics
✦ Course Transcript ✦

Overview

Human breast milk is comprised of several bioactive components, including human milk oligosaccharides (HMOs), which are nonnutritive carbohydrates unique to human milk. In this course, **Lars Bode, PhD,** introduces HMOs and describes the structure, composition, and infant health benefits of these compounds. Topics include the prebiotic effects of HMOs, particularly related to the gastrointestinal and immune systems, but also growth and development and allergies. Also reviewed are the oligosaccharides found in infant formulas, with a focus on clinically relevant information that may help to guide counseling and decision making for parents.

Target Audience

This activity was developed for neonatologists, pediatricians, nurses, advanced practice clinicians, dietitians, and other healthcare providers with an interest in infants and children.

Learning Objectives

At the conclusion of this activity, participants should be better able to:

- Summarize ongoing research into the bioactive components of human milk
- Describe the evidence for use of prebiotics in infant feeding.

Faculty

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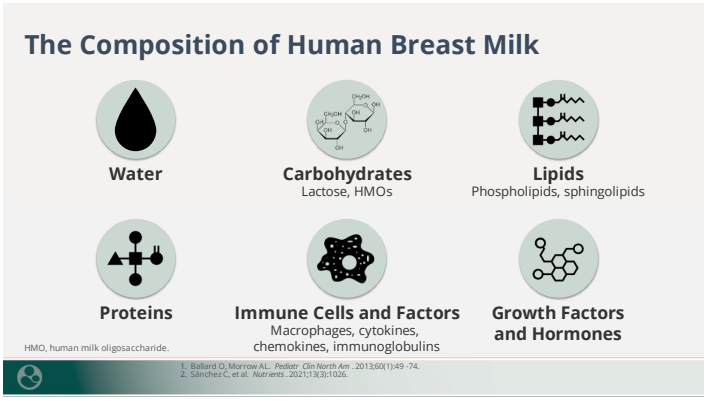
Editor's Note: This is a transcript of an online presentation broadcast on October 7, 2021. It has been edited and condensed for clarity.

HUMAN BREAST MILK COMPOSITION

Introduction to Human Milk Oligosaccharides

Lars Bode, PhD: We're going to talk about human breast milk composition with a specific focus on human milk oligosaccharides (HMOs). So, let's dive right in and talk about human milk in general.

What is in human milk? What makes it so powerful? If you look at the different components of human milk (Slide 1), of course there's water as the primary component, but there are many other components as well. We have carbohydrates, with lactose as the milk sugar, and then HMOs, or human milk oligosaccharides, that we'll be focusing on throughout this webinar.



We have lipids in human milk, and they come in a very complex system called the milk fat globule, surrounded by membrane, which has many bioactive components, and then the lipids themselves are tucked into this globule and are delivered in this very specific way. We have proteins, of course, in human milk, and some of these proteins have specific functions. For example, they participate in protein digestion, so they're proteases themselves that then chop the milk proteins into smaller fragments that have different activities. You can see the ladder of protein digestion and cleaving out active fragments throughout the intestinal tract, and then delivery of these active components at different times and spaces.

Human milk is not just a list of different ingredients. There are active, live components in human milk, including immune cells, epithelial cells, and bacteria, and not when you have pathogens, but really there are live, potentially good bacteria living in human milk. Together with immune cells come immune factors like cytokines, chemokines, immunoglobulins, and antibodies that come through human milk and potentially protect the infant from disease.

In addition, we have growth factors, hormones, and a whole myriad of different things in human milk that we're still trying to catalog and identify what they're really doing in human milk, not just for the infant but potentially also for the mother.

Now, let's talk about HMOs, the third most abundant component in human milk after lactose and lipids. Very often the amount of oligosaccharides exceeds the amount of total protein in human milk. These carbohydrates or oligosaccharides are nonnutritive, so they're not providing direct energy to the infant like lactose, the other human milk sugar. In fact, HMOs are indigestible; they reach the small intestine and eventually the colon virtually

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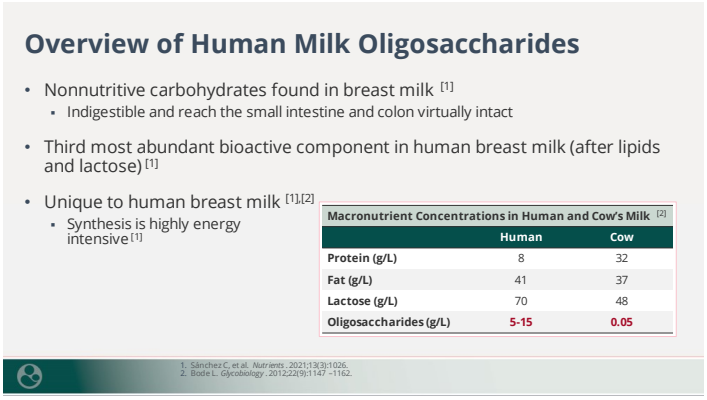
undigested and intact. Part of that is absorbed and refined in the systemic circulation (in the blood) and in the urine, so there are oligosaccharides flushing the baby, flushing the entire system, and they are not only present in the gut.

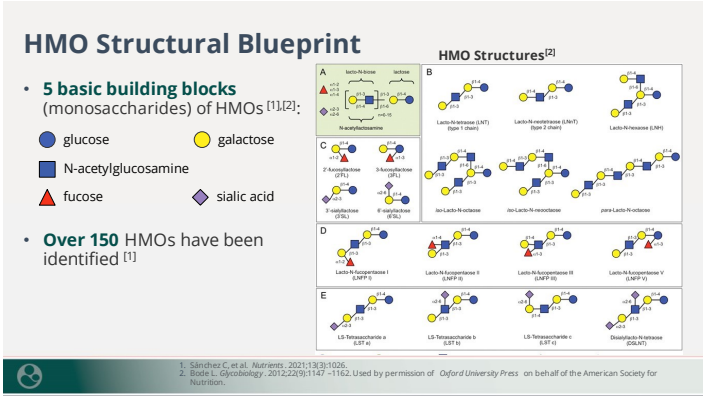


Slide 2 – Overview of Human Milk Oligosaccharides

[HMOs] are really unique to human milk (Slide 2). If you see the comparison here between human milk and cow's milk, there are about 100- to 1000-fold more oligosaccharides in human milk, and the structures are quite different as well.[1] We have about 150 to 200 different oligosaccharides in human milk.[2] Most of them are fucosylated, and we'll get into that in the next slide, whereas in cow's milk, there are hardly any fucose oligosaccharides—maybe only 40 structures or so compared to the 150 or 200 in human milk. So, just between human milk and bovine milk, there are huge differences in total amount and in composition of oligosaccharides.

Talking about composition, how do these oligosaccharides look? Oligosaccharides are complex sugars that are built with 5 basic building blocks or monosaccharides, and we give each of these monosaccharides here a little symbol to make it easier to talk about oligosaccharides (Slide 3). The 5 building blocks are glucose, galactose, N-acetylglucosamine, fucose, and sialic acid. So, depending on how we build these different building blocks together in different linkages, we get different oligosaccharides that can be fairly simple to extremely complex—everything from putting 3 to 30 of those building blocks together in different linkages. That really expands the chemical space of HMOs tremendously.

There are a few examples here on Slide 3. If you want to read more about that, we published a review paper in *Glycobiology* in 2012[1] that is still one of the most cited papers in this field and gives a nice overview of what these oligosaccharides are and what they potentially do.



Slide 3 – HMO Structural Blueprint

When we talk about HMOs today, many times we talk about 2'-fucosyllactose because that is currently one of the oligosaccharides that we find now in infant formula. If it says it contains HMOs, in the fine print it says it really contains 2'-fucosyllactose.

Now, what is this 2'-fucosyllactose or 2'-FL? It's one of the simple human milk oligosaccharides, a trisaccharide that consists of glucose, galactose, and fucose in this particular alpha1-2 linkage (Slide 4). It is the most abundant

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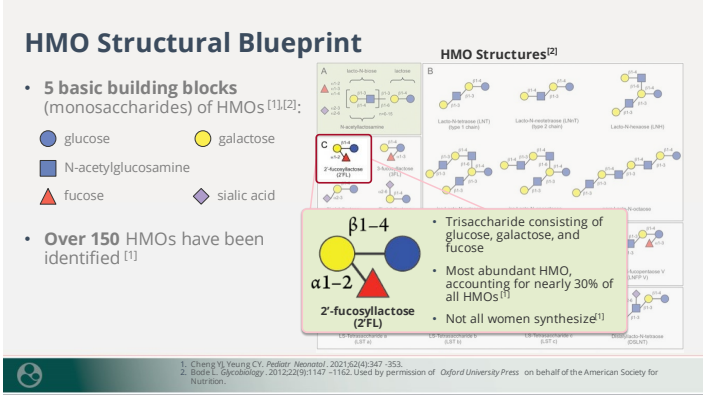
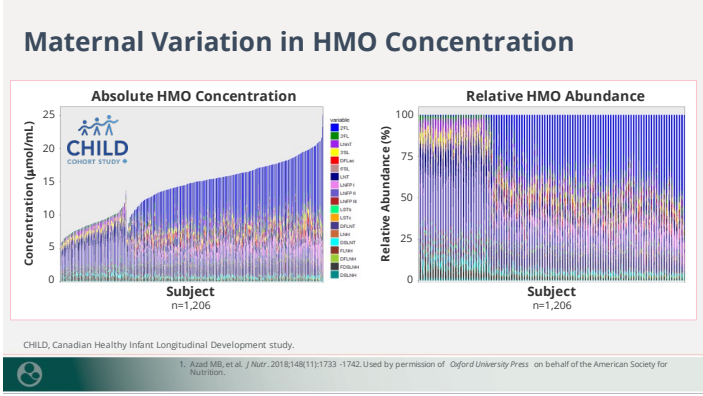
human milk oligosaccharide in the milk of many women, and we have to say that because there are some women who do not make any of this 2'-fucosyllactose, and we haven't really fully understood why that is. We call those that make a lot of 2'-fucosyllactose the "secretors," and the nonsecretors are those that make hardly any of it. Worldwide, approximately 20% of women do not make this particular oligosaccharide (ie, nonsecretors).[3] That is purely genetically determined, and we don't really know what this means for the quality of milk and for infant health. It is certainly not the case that all of those infants that get milk without this oligosaccharide are at a major disadvantage—that certainly is not the case.



Slide 4 – HMO Structural Blueprint

Okay, so here's a visualization of different oligosaccharide profiles from different women and you see very clearly different subjects here on the X-axis and then concentrations of different oligosaccharides in different colors on the Y-axis (Slide 5). Between different subjects, there is a massive variation. So, on the very left side here, you see very low concentrations overall; on the right side, much, much higher concentrations. The colors or patterns look very different too. If we take the same data and set it at 100% to get the relative abundance of different oligosaccharides [as shown on the right], there's this dramatic variation between different women, between different subjects, and you can already see there's some clustering going on here toward the left side of this graph. There's something missing there (the dark blue color on the top), and that's 2'-fucosyllactose, which I was talking about earlier [noting] that some women do not make this specific oligosaccharide.[4]



Slide 5 – Maternal Variation in HMO Concentration

That brings us to the genetic drivers of the maternal variation in human milk oligosaccharide secretion and production (Slide 6). Like I said, [human milk oligosaccharides are] genetically determined in the sense that there are genes that contribute to the fucosylation of oligosaccharides, namely the secretor gene, which facilitates the alpha1-2 fucosylation. Then there are the Lewis genes that participate to a certain extent in the alpha1-3, alpha1-4 fucosylation.[3]

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Genetic Drivers of Maternal Variation in HMO Production

- HMO composition in breast milk is determined in part by maternal genetics and the activity of genes in the Lewis antigen system
  - ***Se* (secretor) gene:** α1-2-fucoslyltransferase (FUT2)
  - ***Le* (Lewis) gene:** α1-3/4-fucosyltransferase (FUT3)
- FUT2 is responsible for synthesis of 2′-FL and other α1-2-fucosylated HMOs
  - Women who do not encode a functional FUT2 enzyme cannot synthesize α1-2-fucosylated HMOs

Maternal Genetic Background and Associated HMO Composition

Lewis Gene (FUT3)	Secretor Gene (FUT2)	Phenotype	Associated HMOs
+	+	Lewis positive secretor	All HMOs
+	-	Lewis positive nonsecretor	LNT, LNFP II, LNFP III, LNDFH II
-	+	Lewis negative secretor	2′-FL, 3FL, LNFP I, LNFP II
-	-	Lewis negative nonsecretor	3FL, LNT, LNFP III, LNFP V

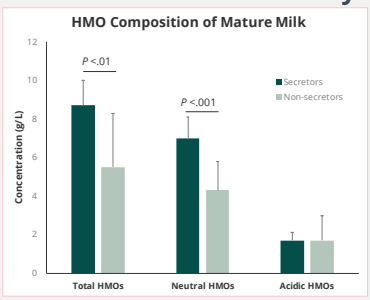
LNT, lacto-N-tetraose; LNFP, lacto-N-fucopentaose; LNDFH, lacto-N-difucohexaose; FL, fucosyllactose

1. Cheng YJ, Yeung CY. *Pediatr Neonatol*. 2021;62(4):347-353.

Slide 6 – Genetic Drivers of Maternal Variation in HMO Production

HMO Composition of Mature Breast Milk Varies by Secretor Status

- Study of 96 milk samples from 32 mothers with preterm and term infants found significant differences in HMO composition based on genotype
- Secretors had higher concentrations of **2′-FL** and **LNFP I**, and lower concentrations of **LNT**, **LNFP II**, and **LNDFH II**



LNFP, lacto-N-fucopentaose; LNT, lacto-N-tetraose; LNFDH, lacto-N-difucohexaose.

1. Kunz C, et al. *J Pediatr Gastroenterol Nutr*. 2017;64(5):789-798.

Slide 7 – HMO Composition of Mature Breast Milk Varies by Secretor Status

If we just bring it down to the secretor and the nonsecretor, those that make a lot of 2′-FL and those that do not make 2′-FL, single nucleotide polymorphisms really drive this variation and drive the near absence of 2′-fucosyllactose in some of these milk cells.

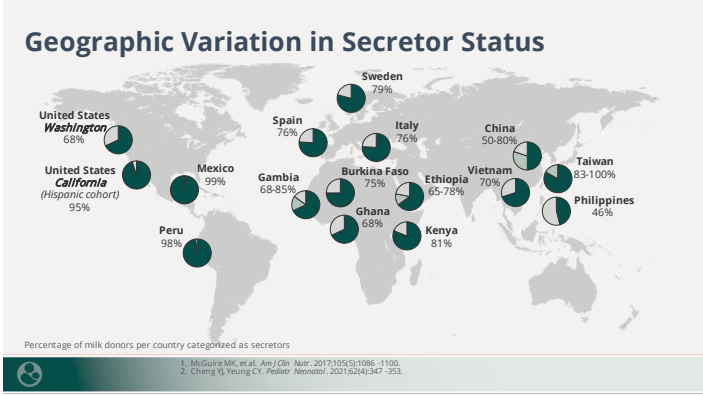
That has implications for oligosaccharides overall. It's not just 2′-FL that is affected. There are other alpha1-2 fucosylated oligosaccharides that are different, so it really changes the entire makeup of oligosaccharides. You can imagine 1 oligosaccharide going up or down in concentration changes the entire map of oligosaccharides because their synthesis is somewhat interconnected.

Here you see in a study from Clemens Kunz that was published in 2017, where they look at total concentration of oligosaccharides between secretors in the dark green and nonsecretors in the light green, and nonsecretors significantly make fewer overall oligosaccharides (Slide 7).[5] The levels of neutral oligosaccharides are also reduced. In fact, fucosylated oligosaccharides are reduced as well (not shown here on the graph). And at the same time, you see that the sialylated or acidic oligosaccharides are not affected as much between the 2 different groups.[5]

The bottom line here is that genetics drive the variation in human milk oligosaccharide composition between mothers, and that has a tremendous effect on 2′-fucosyllactose concentration but also affects all the other oligosaccharides because oligosaccharide synthesis is interconnected (ie, on a shared pathway). So if you reduce one oligosaccharide, something else will pop up or something else will also be reduced, depending on where it sits on the pathway.

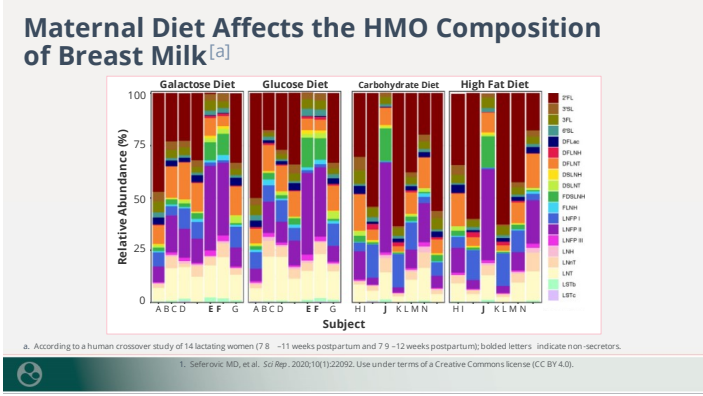
Remarkably, this variation in oligosaccharide composition based on the secretor status is different in different parts of the world (Slide 8). If we look at Latin America, South America, Peru, and Mexico, there are hardly any nonsecretors. Now, most women make a lot of 2′-fucosyllactose. In other parts of the world, such as the African continent, you find up to 30% or 35% of women do not make this specific oligosaccharide.[3,6] So, they're nonsecretors. And again, why this has developed in different areas of the world, what the benefits are, and what the disadvantages are of being a secretor or nonsecretor is not fully understood. It could very well be that there was an evolutionary or selective force that happened hundreds,

maybe thousands, of years ago or longer, and that selection pressure is no longer there. So, there's really no advantage or disadvantage. But, of course, that's very difficult to study. So, we haven't fully understood why there is such huge variation in oligosaccharide composition, but the bottom line is if we look at HMOs, there is no one average human milk oligosaccharide composition that is natural, healthy and normal. That does not exist. So, there is no normal HMO composition.



Slide 8 – Geographic Variation in Secretor Status

We know that genetics determine HMO composition, but other factors also have an effect. In a small pilot study last year where women were their own control subjects, we put women either on a high-galactose or high-glucose diet or switched them from high-carbohydrates to a high-fat diet (Slide 9).[7] It's difficult to see on this graph. Individual oligosaccharides in this graph here don't really seem to be affected much, but if you then group them together you can see [in Slide 10] that, for example, in the galactose/glucose crossover, that HMO-bound fucose goes down to a certain extent in the glucose group, and the HMO-bound sialic acid goes down in the group that is on a high-fat diet.[7] You see that, in addition to genetics, there are factors like diet and exercise—we've seen this in other publications—that play a role. So, we're now trying to understand what modifiable factors there are to change oligosaccharide composition.



Slide 9 – Maternal Diet Affects the HMO Composition of Breast Milk

Now, when I say that oligosaccharide composition is very different between different women, we have seen that oligosaccharide composition in a short period of time—over the course of a day, over the course of a week—is remarkably constant. So, it's not shown on Slide 11, but we have data that show that within a week or a couple of weeks, if you take a sample on Monday of the first week or on Friday of the second week, the oligosaccharide composition is almost identical.[8] However, it changes over the longer course of lactation. That's what's shown in Slide 11.

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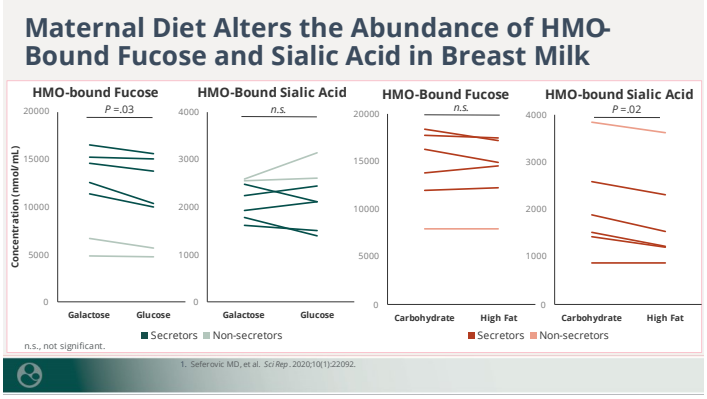
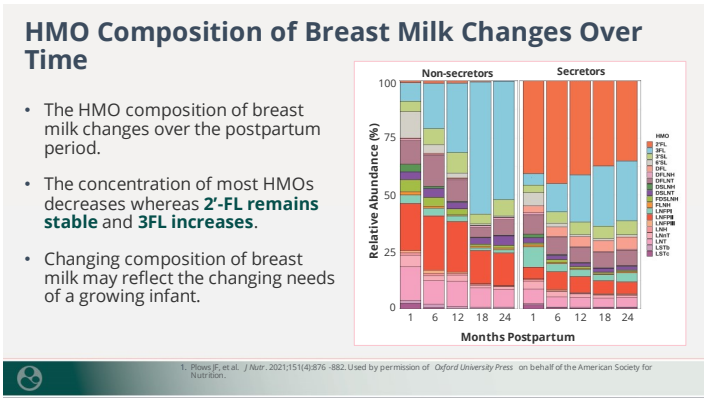


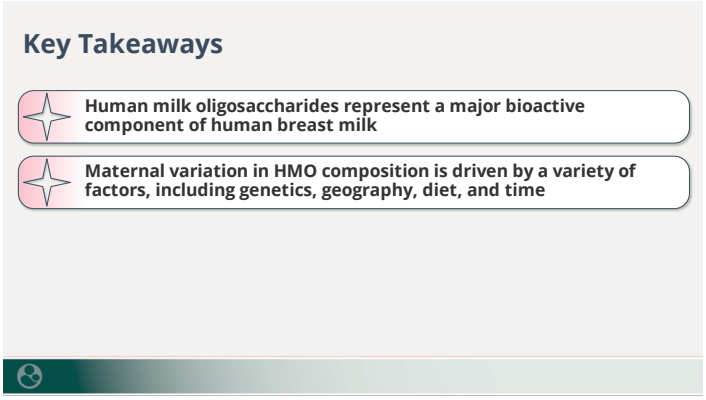
Slide 10 – Maternal Diet Alters the Abundance of HMO-Bound Fucose and Sialic Acid in Breast Milk

In Slide 11, we're looking at months postpartum, so from 1 month to 24 months or 2 years postpartum, there are dramatic changes. Most of the oligosaccharides decrease in concentration, and there are a few oligosaccharides, like 3-FL, that increase over the course of lactation. Again, why that is, if there is any physiological relevance to that, we haven't fully understood yet. How HMOs are synthesized and what drives the variation still remains to be elucidated.[9]



Slide 11 – HMO Composition of Breast Milk Changes Over Time

Key takeaways for this first part are that human milk oligosaccharides represent a major bioactive component of human breast milk—the third most abundant component in human milk after lactose and lipids—and the maternal variation in HMO composition is driven by a variety of factors, mainly by genetics. You can see these differences between secretors and nonsecretors, which we find is different in different parts of the world. But also diet plays a role; exercise plays a role. [Human milk oligosaccharide levels] change over a longer period of time, and there are still many questions to be answered.



Slide 12 – Key Takeaways

Effects of HMOs

Why are we so interested in what drives human milk oligosaccharide composition? What is the ideal target? Where do we want oligosaccharides to be? What is the ideal concentration? Does it even exist? And that triggers the next question, the next part of this presentation, what are the effects of HMOs? What do they actually do? Do we want to enrich for certain oligosaccharides because some oligosaccharides may do better or different things than other oligosaccharides? What's the target here? What do oligosaccharides actually do?

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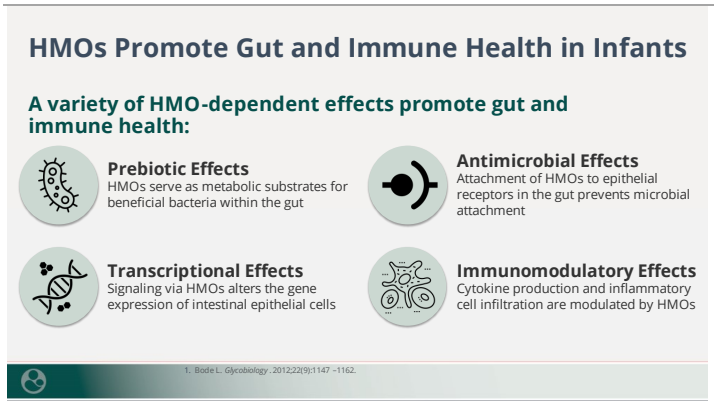

Slide 13 – HMOs Promote Gut and Immune Health in Infants

We do know that oligosaccharides promote gut and immune health in the infants (Slide 13), and potentially there are also effects for mom. I'm not going to go into that much in this presentation, but there might also be benefits for mom from making these oligosaccharides and contributing to breast health, either during breastfeeding or beyond.

When we talk about gut health and immune health, we see that HMOs have what we call prebiotics. Prebiotics serve as metabolic substrates for some potentially beneficial bacteria. They get eaten up by the bacteria, and that's the end of it—but that's not the full story. We also have seen that oligosaccharides have the opposite effect. They have antimicrobial effects, meaning that for certain undesirable bacteria, for certain pathogens, HMOs may actually stop the growth of these pathogens or kill them altogether. So, in addition to having prebiotic effects, they might have antimicrobial effects, and you can imagine that if they have antimicrobial effects, then you don't want these oligosaccharides to be eaten up by the good guys. You still want them, to a certain extent, available to actually hit the bad guys as well.

In addition to these effects directly on microbes (either good or bad guys), we also see that oligosaccharides have direct effects on the host, in part, independent of microbes. We see that if you expose epithelial cells, for example, to oligosaccharides, you see that it alters those pathways and the response of them as well. And the same is true for immune cells as well. We see that cytokine production is altered when we expose immune cells to oligosaccharides, and that's different with different oligosaccharides.

Let's go a little bit deeper into the prebiotic effects and how that then alters the health of the infant gut (Slide 14). Undigested HMOs go through the small intestine and are not digested by a baby's enzymes. They make it all the way into the gut, and that's where they serve, to a certain extent, as metabolic substrates for beneficial bacteria. That then forms the basis for improved gut health for the baby.

Certain *Bifidobacteria* strains can metabolize all of the oligosaccharides, so they have the machinery to really break down every single linkage and every single building block and make full use of these oligosaccharides as an energy source. But there have been other *Bifidobacteria* that utilize only some oligosaccharides, leaving others intact, and you can imagine that those intact, leftover oligosaccharides can have other effects as antimicrobials or through directly affecting epithelial cells and potentially immune cells.

It's not just *Bifidobacteria*. There are other strains, for example, *Bacteroides* or *Lactobacillus* that can also utilize human milk oligosaccharides—and some remarkably well in the sense that they can utilize a lot of the different oligosaccharides. Again, some

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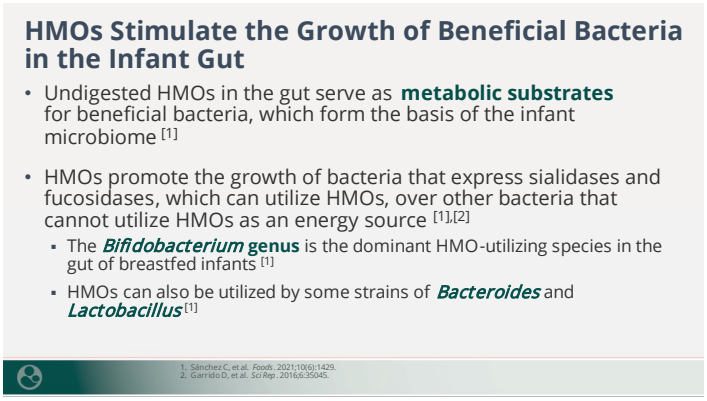
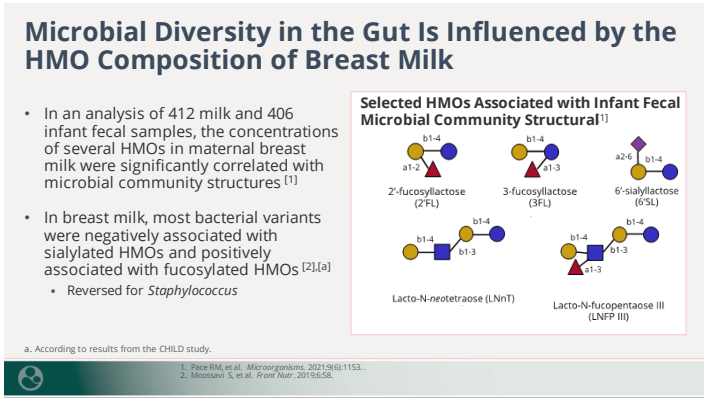
[bacterial species] only go after specific oligosaccharides and leave others intact. This really gives us a huge opportunity to develop specific probiotics or synbiotics where we design or select probiotics depending on what kind of oligosaccharides can be utilized so that the probiotics have their beneficial effect, but then, at the same time, leave some of the oligosaccharides intact so that those oligosaccharides now can have other impacts.



Slide 14 – HMOs Stimulate the Growth of Beneficial Bacteria in the Infant Gut

And the probiotic or prebiotic effects of oligosaccharides have been nicely documented in multiple different studies where we see that there are associations between different oligosaccharides and microbial communities in the infant gut and potentially also in the mammary gland. We know that human milk is not sterile. There is a niche for certain bacteria. There is a milk microbiome. It's not just pathogens that lead to mastitis. There are healthy microbes in human milk, and since they live together with the oligosaccharides already in human milk, you can image that those bacteria and the oligosaccharides already start interacting while they're sitting in the milk together.

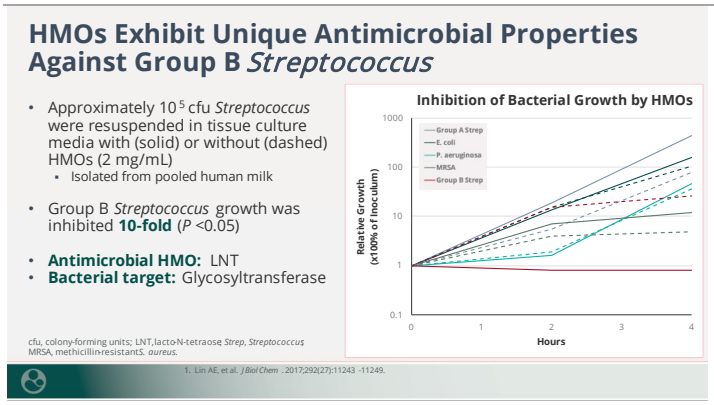
Lots of studies find associations between oligosaccharides in human milk and then either the microbial communities in the milk or in the infant gut (Slide 15).[10,11] And many of those studies are not 100% conclusive and many also look just at a very shallow level of either diversity measures or on a 16S high-level inventory of what kind of bacteria are there, but functional associations are still lacking.



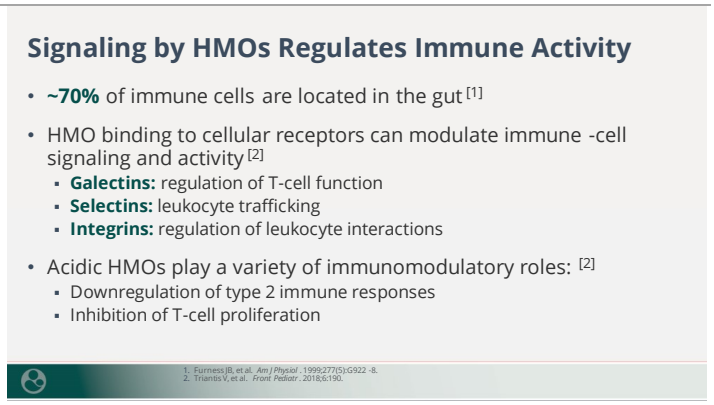
Slide 15 – Microbial Diversity in the Gut Is Influenced by the HMO Composition of Breast Milk

So that's the prebiotics effects. Let's move on to the antimicrobial effects (Slide 16). This is a study that we published in 2017, so almost 5 years ago, where the postdoc in the lab back then pulled out a few pathogens from the -80°C freezer and wanted to see what happens if I grow these pathogens in the presence or absence of different HMOs.[12] The different colors in the graph [represent] different pathogens. The dotted lines show the pathogen growth without oligosaccharides, and the solid line is the pathogen growth with oligosaccharides. The dark red line on the bottom here really stands out. That's group B *Streptococcus* in the presence of HMOs, and with the flat line, that group B *Streptococcus* simply does not grow in the presence of HMOs.

Bioactive Components of Human Milk Including HMOs and Other Prebiotics



Slide 16 – HMOs Exhibit Unique Antimicrobial Properties Against Group B Streptococcus



Slide 17 – Signaling by HMOs Regulates Immune Activity

We've seen this over and over again. Other groups have confirmed this in the meantime as well, that HMOs really inhibit the growth of group B *Streptococcus,* and we've since identified which oligosaccharides are most effective here. Lacto-N-tetraose (LNT) seems to play a role. We've also used the transposon mutant library to identify the target on group B *Streptococcus* which seems to be a glycosyltransferase.

Most importantly, and what I find quite impressive, is that the oligosaccharides in human milk synergize with commercially available antibiotics, meaning that you need less antibiotic to inhibit the growth of group B *Streptococcus* in the presence of HMOs. A true synergistic effect between antibiotics and HMOs, in times of the emerging antibiotic resistance crisis, could be a huge opportunity to use oligosaccharides as antimicrobials in addition to antibiotics.

We also see that oligosaccharides have a direct effect on immune cells, and this could be in the gut where we have the highest concentration of HMOs, but also the highest density of immune cells (Slide 17). Many, many immune cells are located in the gut and sense what's going on in the intestine and then transfer that information back to the entire system. There's a lot of sampling and immune stimulation going on in the gut, and that's exactly where we have human milk oligosaccharides present to interact with immune cells and immune-cell receptors.

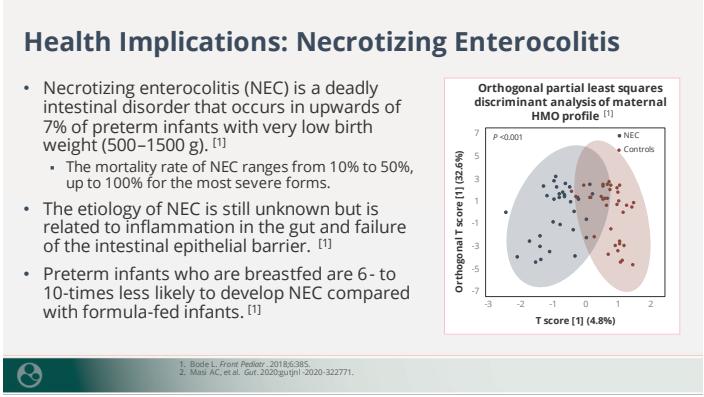
There are many different immune-cell receptors known to interact with glycans, (ie, sugars), and human milk oligosaccharides are, of course, sugars. For example, we know that there are galectins, selectins, integrins, and siglecs, as well, that interact with different oligosaccharides, and many of those target oligosaccharides look very similar to human milk oligosaccharides. And studies have shown that if you incubate immune cells with different oligosaccharides, you can change their cell response, and you can change immune responses that way.

For example, the acidic HMOs—those are the ones that contain sialic acid—seem to play a

role in immune-modulatory paths, [such as] down-regulation of type 2 immune responses that inhibit T-cell responses. And some of this has to be taken with a grain of salt because many of those oligosaccharide preparations also contain LPSs and other immune-modulatory components as a contamination. Only a few years ago, people realized that there were contaminations in the oligosaccharide preps and that might have contaminated some of the literature when it comes to oligosaccharides and immune responses.

Now, this is more like on a molecular or a cellular level. What does this really mean for infant health? What do we know about how different oligosaccharides impact infant health and not just changing some microbial communities or doing something in tissue culture where we see some receptors going up and down or interacting and immune-cell responses changed? What does this actually mean? Do we have any evidence that oligosaccharides do something positive for the infant?

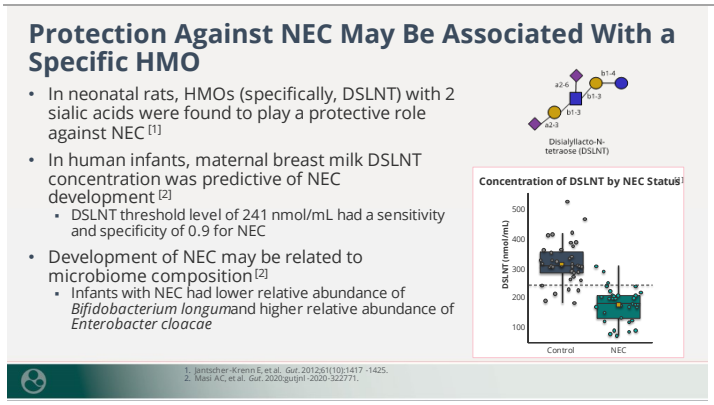
Probably the strongest evidence that we have is in the preterm infant space, particularly in necrotizing enterocolitis (NEC), which is still one of the most deadly diseases in preterm infants when it comes to the gut (Slide 18). About 5% of very low-birth-weight infants develop NEC with a fairly high mortality rate. And we still don't fully know how NEC develops in the first place. It might be multiple different routes to get to this symptom block that we call NEC. But inflammation plays a role; intestinal barrier function integrity breakdown plays a role. And that, eventually, leads to the devastating symptom block called NEC.



Slide 18 – Health Implications: Necrotizing Enterocolitis

What we do know though is that preterm infants who are breastfed or receive human milk are at 6- to 10-times lower risk of developing NEC compared with formula-fed infants (Slide 18).[13] So, that's a huge difference, of course, and we wanted to know: what is it in human milk that contributes to these beneficial effects when it comes to NEC? And there's multiple different ways of how we did that. First (and first meaning this was before 2008, 2009), we started to test HMOs in an animal model of NEC in rats and we found that oligosaccharides actually have an effect. Not only did they reduce pathology, but they also improved survival of the rat pups (of the rat babies) that we induced to develop NEC-like symptoms (Slide 19).[14] We then continued that research and found that it's not all oligosaccharides in human milk, but it's specifically 1 oligosaccharide called DSLNT, which stands for disialyllacto-N-tetraose, and the structure is shown here on the right.[14]

Bioactive Components of Human Milk Including HMOs and Other Prebiotics



Slide 19 – Protection Against NEC May Be Associated With a Specific HMO

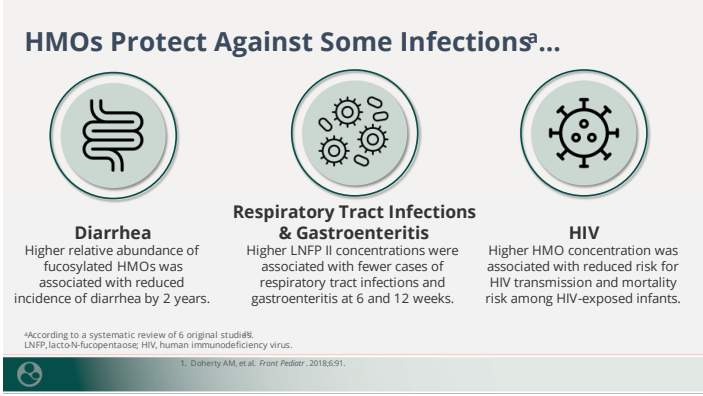
Now, you know, [those studies were done in] neonatal rats, and people argue that that's not a particularly good model to capture what NEC looks like in the preterm infant. So we, of course, needed to get one step further here and look at whether this even translates to the human preterm infant. So, there we developed multiple different cohort studies—first here in the US and Canada,[15] then in South Africa,[16] and most recently in the UK,[17] which is shown on Slide 19—where we recruited moms and their preterm infants, sampled milk about every second day and then looked at what oligosaccharides are in the milk and [asked whether] that somehow predicts which infant develops NEC.[15-17] To our surprise, in all 3 of these studies, both North America, South Africa and the UK, we found that there was indeed 1 oligosaccharide [that is] different that predicts very well whether the infant develops NEC or not, and that oligosaccharide was the same oligosaccharide that we found to be protective in the animal model, DSLNT.[15-17]

In other words, DSLNT protects from NEC-like symptoms in the rat model and, if it is in lower concentrations in human milk, then the infant is at higher risk to develop NEC. Those many data sets really fit nicely together where we have an animal model and then human association studies to see that a specific oligosaccharide plays a role in protecting an infant from a disease (ie, necrotizing enterocolitis).

Of course, we still need that intervention study to say, well, what happens if we give DSLNT now to infants, can we protect them from NEC? I showed you associations; I showed you that this works in rats, but does the intervention actually work in the human preterm infant? And that's something we're working toward right now and we're trying to source DSLNT and make it available for a larger clinical study to really test and give the ultimate proof that an intervention with this oligosaccharide has an impact on health.

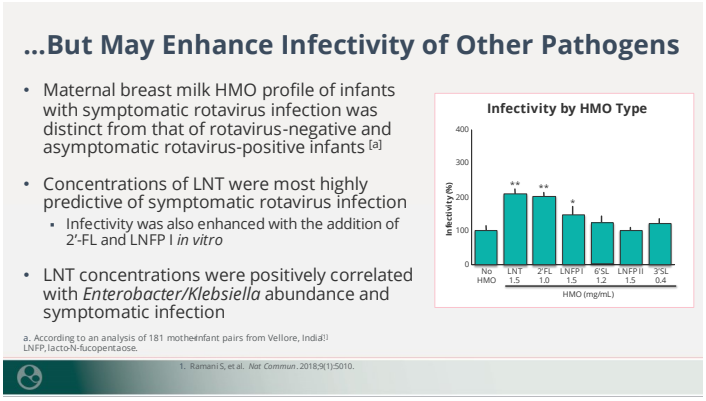
In Slide 20, we also see that HMOs protect against some infections, diarrheal disease for example (a huge problem still): about 2,200 infants and children under the age of 5 years are lost every day in the world to diarrheal disease. And we see that HMOs have some impact on some of those pathogens that drive diarrheal disease as well. The same is true not only for diarrheal disease, but also for infectious agents that affect the respiratory tract and, not just bacteria, but also for viruses. We've done some work here in the HIV space where we've seen that, yes, higher concentrations of total oligosaccharides are associated with reduced risk for HIV transmission and mortality among HIV-exposed infants. At the same time, if we go a little bit deeper, we see that [with] individual oligosaccharides, if they are at higher concentrations, your risk of transmission

actually goes up. So, it's not that simple to say all oligosaccharides do the same thing, and if they're higher in concentration, then we have a reduced risk. Overall, that's correct, but individual oligosaccharides may have different effects on different outcomes, since there are different structures. I think that's key to keep in mind: not all oligosaccharides do the same thing. Really, structure determines their function and determines their impact on health.



Slide 20 – HMOs Protect Against Some Infections...

And that's nicely illustrated here in a paper that we published a couple of years ago in *Nature Communications*.[18] [Sasirekha Ramani at Baylor and our lab found that individual oligosaccharides would actually increase infectivity of a very specific strain of] rotavirus that is found in India and mostly affects early neonates in that particular area. This is very counterintuitive; we would think that all oligosaccharides reduce pathogen burden, but in this case, for this strain, we found that there are individual oligosaccharides that increase infectivity. Not only do we see this in the lab, but if we take this out to the field and do association studies where we recruit moms and infants, analyze the milk, and then associate that with rotavirus infection risk and severity, you see the same oligosaccharides stand out as increasing the risk, increasing symptoms.[18]

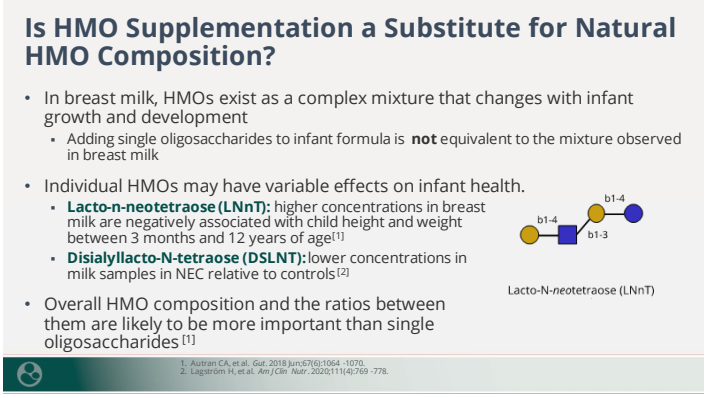


Slide 21 – ...But May Enhance Infectivity of Other Pathogens

That was a surprise to us, but fortunately every challenge comes with an opportunity, and we think that these oligosaccharides might actually help increase the efficacy of the rotavirus vaccines. So, if there is a pathogen that thinks it can get ahead by abusing and exploiting different oligosaccharides, I think we have a very good handle using those same oligosaccharides now against the pathogen by using that as a booster to vaccination. It's really constantly a battle back and forth, you know, how different pathogens try to get ahead and how science keeps catching up and gets ahead of the curve.

Oligosaccharides are becoming available now in larger quantities—tons really, megatons—of individual oligosaccharides and at very low cost. I can tell you when I did my PhD in the early 2000s, we did some calculations if we wanted to add 1 of those oligosaccharides to infant formula, what would the box cost in the supermarket, and it was absolutely prohibitive. The box would cost multiple millions of dollars if we had done that 15 or 20 years ago. It was completely unrealistic. But now, with all the advances in oligosaccharide synthesis, we are

at a stage where you can walk through the supermarket and you see first infant formula products that indeed say contains HMOs and, again, fine print is it contains 1 or 2, or maybe a couple more, oligosaccharides, not the entire repertoire.



Slide 22 – Is HMO Supplementation a Substitute for Natural HMO Composition?

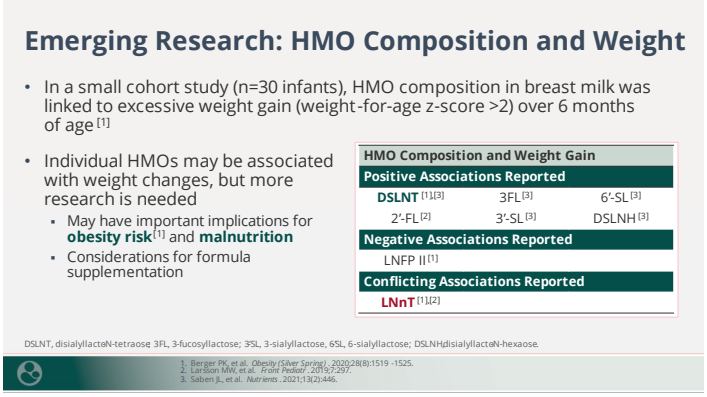
Does it matter? So, in human milk, we have a mixture of, like I said, 150 or 200 different oligosaccharides. Is that the same as adding 1, 2, or 5 different oligosaccharides that we can now synthesize? And, in some cases, the answer might be yes. In some cases, individual oligosaccharides may be sufficient to improve a certain health condition, like NEC, which I showed you earlier. In many cases, that's not the case. In many cases, we rely on that entire mixture of different oligosaccharides and you can imagine if it comes to shaping microbial communities, for example, you don't just want 1 oligosaccharide that is now able to feed certain bacterial strain or maybe a few bacterial strains, but all the other bacteria. Like if a bacterial strain can't deal with this 1 oligosaccharide, it needs that other oligosaccharide also to thrive, and that might give you some imbalances in microbial community composition (Slide 22).

So, yes, it's not the same to add 1 oligosaccharide or 2, compared to having 150 or 200 different ones. In some cases, it might work. In many other cases, it's not sufficient. However, of course, it's the right way forward. Just to give you an example, in Slide 23, we looked at associations between oligosaccharides and food sensitization.[19] There was not a single oligosaccharide that was associated with food sensitization in this particular study and the child cohort. However, if we did a pattern analysis, where we take all the oligosaccharides together and see are there any patterns associated with food sensitization, that gave us a fairly good estimate. So, if we look at all the oligosaccharides together, we might get some ideas of how they're linked to certain disease outcomes, highlighting that individual oligosaccharides are often not sufficient to drive a specific outcome.

Slide 23 – Emerging Research: HMO Composition Is Associated With Food Sensitivity Risk

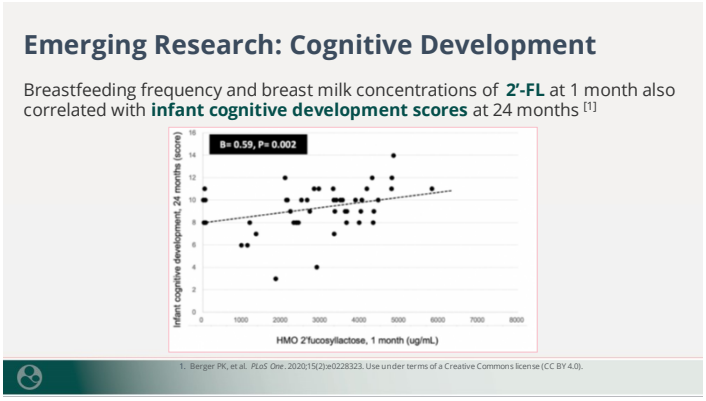
There are data on oligosaccharides and body composition (Slide 24).[20-22] Many different studies show that there are associations between 2'-FL and height and weight, for example.[21] The opposite seems to be true for LNnT, where the associations are negative.[20,21] What this really means in some cases, of

course, [is that] growth and weight can be a disadvantage when we talk about obesity risk. If we talk about malnutrition, it might have completely different implications. So, again, different oligosaccharides have different effects. Their ratio plays a role, and the context is very important too. If we talk about HMOs and health in developed countries with high energy burdens, it's very different than talking about oligosaccharides in low- and middle-income countries where malnutrition is prevalent and where we have a lot of pathogens that we are exposed to. So again, different oligosaccharides doing different things. One size does not fit all.



Slide 24 – Emerging Research: HMO Composition and Weight

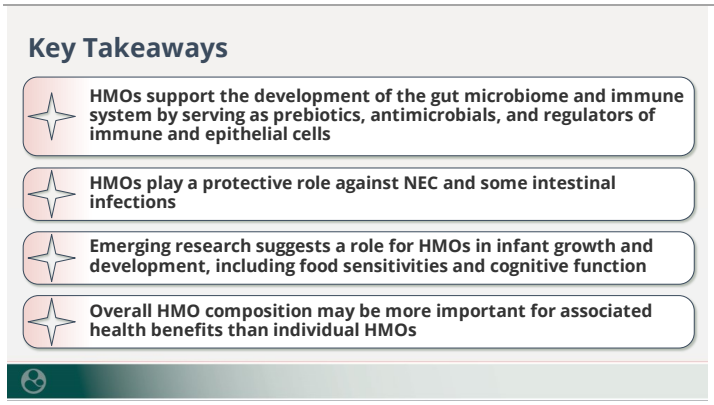
In Slide 25, we show very interesting data when it comes to infant cognitive development. Can we get our infants smarter by adding oligosaccharides or by breastfeeding longer or shorter or differently? And here is data that, where we've shown that 2'-FL concentration measured in mom's milk at 1 month of age is associated with infant cognitive development at 24 months, so 2 years of age.[23] And other studies have shown similar data on 2'-FL or 6'-SL where the exposure early on, so at 1 month, predicts cognitive outcomes, at 6 months or 2 years or beyond that, meaning that whatever happens in the first few months of life somehow determines what happens later on.



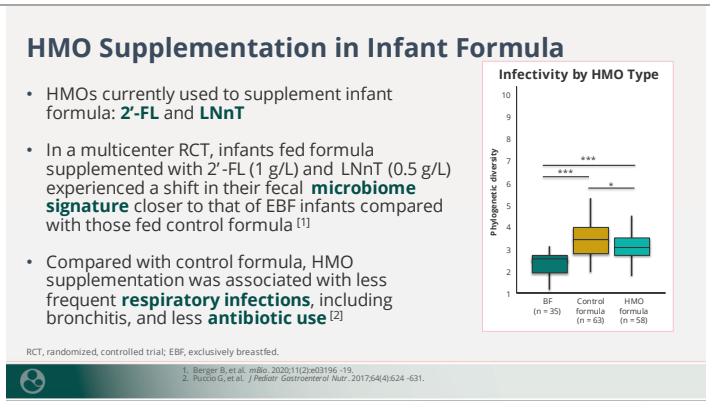
Slide 25 – Emerging Research: Cognitive Development

So, what are the key takeaway for this section (Slide 26)? HMOs support the development of the gut microbiome and immune system by serving as prebiotics, but also serving as antimicrobials and, independent of microbes, serving as regulators of the immune and epithelial cells. We have probably the best data currently on HMOs, one specific HMO, in the protection against NEC, but there are other intestinal infectious diseases and respiratory infections where oligosaccharides may play a role as well. When it comes to infant growth and development, food sensitivities, and cognitive functions, either individual oligosaccharides are associated or we need that entire mix—the entire blend—of oligosaccharides. So, overall, HMO composition may be more important than individual oligosaccharides; although, for some outcomes, individual oligosaccharides may be sufficient.

Bioactive Components of Human Milk Including HMOs and Other Prebiotics



Slide 26 – Key Takeaways

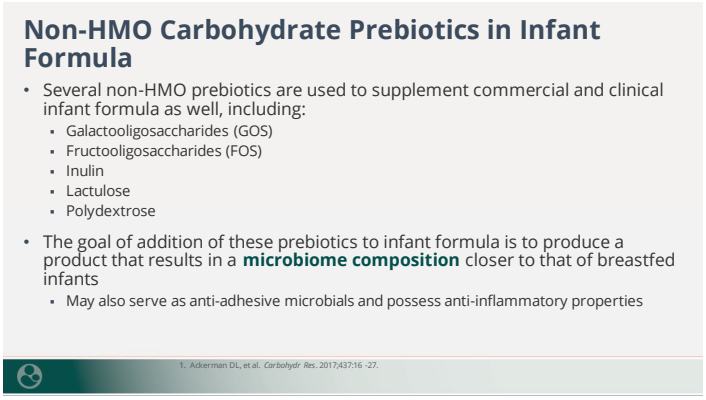


Slide 27 – HMO Supplementation in Infant Formula

Oligosaccharides in Infant Formula

Now let's talk about oligosaccharides that we have in infant formula. I mentioned that specific oligosaccharides, like 2'-FL, LNnT, and others are available and are currently added already to some of the infant formula products, and I'll go through this a little bit faster now due to the limited time we have. In Slide 27, I am showing you that if you add something like 2'-FL and LNnT in certain concentrations to infant formula, the diversity of the microbiome in the infant is more close to [that of infants exclusively receiving] human milk than it is [compared with those infants receiving] the infant formula without the oligosaccharides; although, it's still far away from [exclusively breastfed infants].[24] And there was some secondary analysis here also for less frequent respiratory infections and less use of antibiotics.

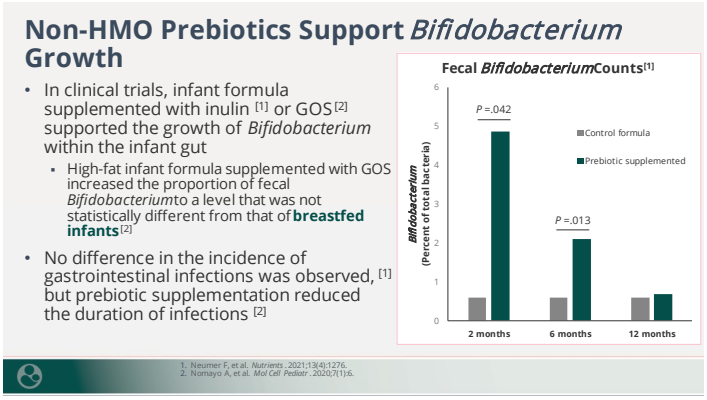
Then, in addition to HMOs like 2'-FL and LNnT and others, previously infant formula companies have also used nonhuman milk oligosaccharides as prebiotics (Slide 28). These are your galactooligosaccharides, fructooligosaccharides, inulin, lactulose, polydextrose, all carbohydrates, and non-HMO carbohydrates, that we do not find in human milk but that may have some similar effects when it comes to some of the benefits of human milk, mainly when it comes to the prebiotic effects of oligosaccharides.



Slide 28 – Non-HMO Carbohydrate Prebiotics in Infant Formula

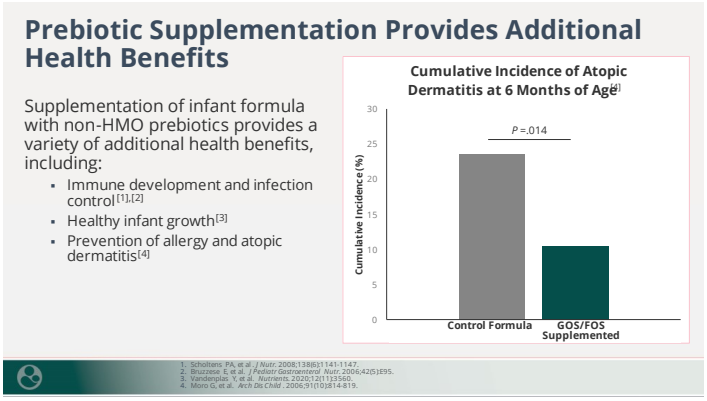
As shown in Slide 29, [non-HMO carbohydrates] do change the microbial community to a certain extent, but really if you look a little bit deeper, if you look at functional ability of those microbial communities, it's far away from [those

produced by] human milk. So, those nonhuman milk carbohydrates certainly do not have the same functional effects as HMOs; although, you can see here that there is some prebiotic effects that change *Bifidobacterium* relative abundance and so on and so forth.[25,26]



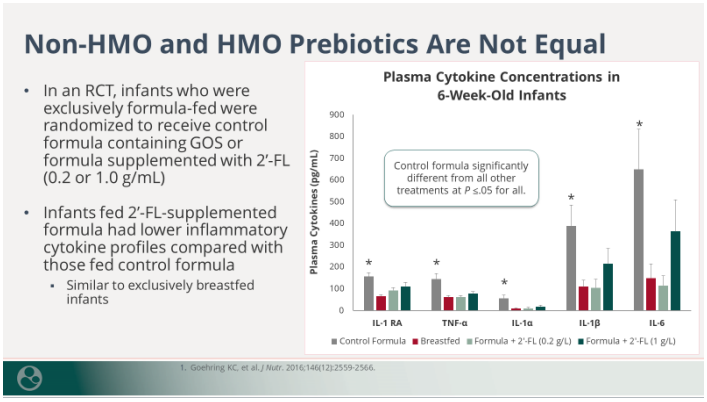
Slide 29 – Non-HMO Prebiotics Support Bifidobacterium Growth

In addition to the prebiotic effect of some of those non-HMO prebiotics, there are reports that GOS/FOS, for example, change the incidence of atopic dermatitis (ie, improves that),[27] and there are a few other effects where it is claimed that these nonhuman milk oligosaccharides are somewhat beneficial beyond just serving as limited prebiotics (Slide 30).[28-30]



Slide 30 – Prebiotic Supplementation Provides Additional Health Benefits

But they are clearly not the same. So, if you look at this study in Slide 31, where you compare a formula that contains only galactooligosaccharides with a formula that contains 2'-FL in different concentrations, the formula with just galactooligosaccharides is very different than the formula with HMOs and certainly very different from the breast-fed effect.[31]

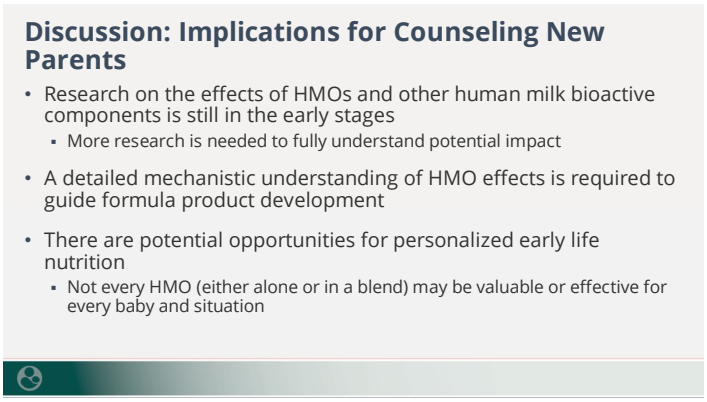


Slide 31 – Non-HMO and HMO Prebiotics Are Not Equal

Now, what does this all mean for parents, for healthcare providers? What kind of guidance can we give at this point? One piece of advice I would always give is that it's very early on; we're in the very early stages of research and discovery. Although there are a few products already out in the market that contain human milk oligosaccharides, please keep in mind that the research on the effects of human milk oligosaccharides and other human milk bioactives is in the very early stages. And we need a lot more research to fully understand what is going on with these oligosaccharides. But I strongly believe that if we have a detailed, deep mechanistic understanding of the effects of different oligosaccharides, it will really give us great opportunities to improve infant health, and also really help people of all ages where either the effects in the early stages have long-

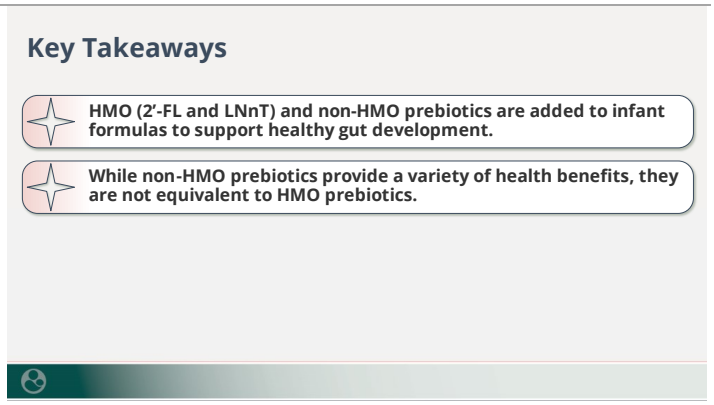
lasting impacts, or if we can identify effects of oligosaccharides for other age groups as well (Slide 32).

Lots of opportunities for personalized, early-life nutrition, but keep in mind that not every oligosaccharide is the same. There is no one-size-fits-all solution to this, and we probably need more than just 1 oligosaccharide to cover all these different opportunities that are in front of us.



Slide 32 – Discussion: Implications for Counseling New Parents

Takeaways from the third part are that HMOs, like 2'-FL and LNnT, and non-HMO prebiotics are currently already added to infant formulas with the claims to support a healthy gut development. And while some of those nonprebiotics provide a variety of health benefits, they're not the same as human milk oligosaccharides. Most of these non-HMO prebiotics are not present in human milk. Keep in mind that we are currently feeding our babies potentially structures that we know are not in human milk and are not really designed to be fed to them.



Slide 33 – Key Takeaways

Audience Question & Answer

Editor's Note: This is a transcript of audience questions together with presenter responses from the October 7, 2021, audio webcast.

Given that maternal diet can influence HMO composition, do you have any recommendations for foods a parent should eat to optimize HMOs?

Lars Bode, PhD: Yeah, that's a great question. We get asked that quite a bit, and the truth is we don't, and that is for 2 different reasons. One, we don't have enough data to say that if you eat this, then this will affect your oligosaccharide composition in this and this way. We have some early data from both animal and human studies (I showed a couple of those) where we see that if we change diet to a certain extent, it changes the oligosaccharide composition.[7] We actually have a paper in revision that shows that vegan and vegetarian diet has no effect on oligosaccharide composition, so that might help people that are on those diets.

But the other truth to the story is that what does it mean to optimize oligosaccharide composition? I think we don't even know at this point what an optimal HMO composition looks like and it might be different for different

people. There might be an optimized HMO composition for infants that are at a high risk because they're exposed to certain pathogens in low- or middle-income countries, and we know that some of those oligosaccharides go after those pathogens. But that might not be the optimized HMO composition for an infant that receives human milk and is in the neonatal intensive care unit because it is at risk of NEC. So, what matters here is we don't really have a target that we would call the optimized HMO composition, and we don't have enough information to say that this and this specific diet will get it to that target. So, still lots of research to be done there.

How do you end up finding out which infant has the protective microbiome to protect against NEC?

Yeah, great question. So, the whole story about microbiome and NEC is still out for debate. We see that there is an association with a certain microbiome that leads up to NEC. So, there are some associations between what we call a favorable microbiome—a healthy microbiome maybe—and a microbiome that now leads to NEC. But you could also turn those around and say whatever leads to NEC changes the intestinal environment, and that's why the microbiome's different. So, we still don't know exactly the causalities here, and that is currently under active study as well, to see how would a microbiome look that is potentially protective against NEC or is potentially the other way around. When it comes to HMOs and NEC, many people always think that this specific HMO now leads to improving microbial community composition and that protects from NEC, but we really don't have any evidence for that. It seems more that this oligosaccharide has a direct effect on host cells independent of microbes and protects that way from NEC, which may also change the microbial community. So, we don't really know what comes first there and what the effect chain is. Again, lots of research there as well.

Does the composition of HMOs change according to maternal and infant health status?

Yeah, very interesting, and potentially yes. So, we have some preliminary data that looks at, especially on the infant health status, where Donna Geddes' group in Paris has done some very nice studies to show that milk immune factors, for example, are different depending on infant health.[32] So, if an infant has an ear infection or something, all of a sudden, mom's milk composition changes as well, and we've looked at that for oligosaccharides as well and it might be the case that that is actually also true for oligosaccharides. But, from the maternal side, there are several studies that looked at the maternal health and oligosaccharide composition. We see, for example, gestational diabetes, has an impact on HMO composition.[33] We see that women with arthritis, for example, (not published yet), but women with arthritis also seem to have slightly different oligosaccharide composition. We've seen that women that are HIV-infected make different oligosaccharides.[16] And again, we don't know exactly what comes first there, but certainly we know more about maternal health and oligosaccharide composition than we know about infant health and oligosaccharide composition.

Formula companies have been adding human-identical milk oligosaccharides to infant formulas in varying amounts. I appreciate you addressing the importance of HMOs and breast milk functioning together. Do you see any harm in adding human-identical milk oligosaccharides to infant formula without knowing appropriate dosing and combinations?

Yeah, so, always safety needs to come first, of course, but maybe let's unpack this question a little bit. When the question I hear says human-identical milk oligosaccharides, that's a term in the field. So, when we take a look at a given oligosaccharide, like 2'-FL, it is indeed structurally identical to the 2'-FL that we have in human milk. So, if you took both out of infant formula and human milk, and just look at 2'-FL, they are structurally identical—and the same is true for other oligosaccharides. You can't tell the difference. It's just 1 is made in mom's mammary glands and the other one is made either in the lab or in bioreactors or wherever. But structurally, they are indeed human-milk identical. Now, what's not identical is that in human milk, we have 150 or 200 different compounds, and there are many other bioactive components in human milk, not just 1 or 2 different oligosaccharides. So, do we see that there is any interaction between different oligosaccharides so that 1 oligosaccharide isn't sufficient? Yes, we do, and in some instances, we see that individual oligosaccharides work. DSLNT for NEC, as a prime example, but in many other cases we see that we need to have them somehow act together. Do we see there's any harm in adding these HMOs to infant formula without knowing exactly what the dosing is? Fortunately, the concentrations currently added to infant formula are at the fairly low end, so most infant formula containing 0.2, 0.8, or 1 g/L of 2'-FL is still in the lower range of what we have in human milk. To my knowledge, there is currently no data that shows that there is any harm using these specific concentrations. Do we want to increase that concentration and see what happens? Probably not in infants. I think there needs to be a lot of research to evaluate the safety of higher doses, but at this point I don't think we have any evidence that there is any harm in adding the oligosaccharides in these low concentrations.

Is it possible that oligosaccharides in infant formula require a protective shell in order to get to the gut and be effective?

Hmm, I'm not sure what is meant by protective shell, necessarily, but the beauty of HMOs is that they are not destroyed in the passage along the gastrointestinal tract. And, you know, they are in human milk, in solution, and I'm assuming that they are in infant formula in solution, so we know that they're not degraded by the low pH in the gut or by enzymes from the brush-border membrane or pancreas. So, I don't think we need any protective shell to get them into the lower parts of the gut to be effective. Do we need to protect them potentially from bacteria that can chew them up readily? Maybe, but maybe just don't use bacteria that chews them all up right away. So, certainly interesting.

This question is on 2'-FL and cognition. Is there an optimal concentration for 2'-FL for better cognitive outcomes?

As far as I see in the data, it seems to be continuous and not like there's a threshold

concentration where you say that you have to have at least this much of 2'-FL to reach a certain cognitive outcome. I'm not sure that we can say that at this time.

Is there a difference in HMO composition and benefit between expressed breast milk vs donor breast milk?

We have not seen this for oligosaccharides. There are a few studies out there that look specifically for the milk microbiome and that being different between, you know, fresh milk directly fed on the breast and then pumped milk fed from the bottle afterward, which makes sense. But we don't see that with oligosaccharides.

Abbreviations

DSLNT	disialyllacto-N-tetraose	**LNnT**	lacto-N-neotetraose
FL	fucosyllactose	**LNT**	lacto-N-tetraose
FOS	fructooligosaccharides	**LPS**	lipopolysaccharide
GOS	galactooligosaccharides	**NEC**	necrotizing enterocolitis
HMO	human milk oligosaccharides		

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