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Summary of the joint National Institutes of Health and the Food and Drug Administration workshop titled “exploring the science surrounding the safe use of bioactive ingredients in infant formula: Considerations for an assessment framework”

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The US government and many professional societies recommend that human milk be the sole source of nutrition for infants up to 6 months-of-age.^{1,2} However, only about 25% of infants in the US meet this goal signaling an urgent need for improved multi-level support for breastfeeding among societies, communities, and families.² In addition, when breastfeeding is not possible, access to a safe and effective source of nutrition—provided by infant formula (IF) is critical. The search for bioactive ingredients found in human milk (HM) that could be added to formula has been intense among academic institutions, governmental research institutes, and industry.¹ The National Institutes of Child Health and Human Development developed a strategic plan (<https://www.nichd.nih.gov/about/org/strategicplan>) that recognizes the importance of optimal nutrition across the lifespan. One aspirational goal is to optimize infant survival and health by optimizing formulas to mimic the composition of HM. more closely.

This is a summary of a workshop sponsored and organized by the US National Institutes of Health and the Food and Drug Administration (FDA)*. The workshop focused on the state of the science regarding what is known about HM constituents that are thought to have bioactivity in infants and to think broadly about how to evaluate the safety of constituents that are already included or that could be included in the future as ingredients for use in IF. The workshop included 14 invited speakers with broad expertise in basic research related to lactation, HM composition, bioactivity of HM components and clinical neonatology and pediatric medicine.

The panel discussed issues related to study design and evaluation of safety and bioactivity, including: (1) which animal models and in vitro systems seem most promising, (2) statistical considerations for studies in animals, (3) whether bioactive ingredients can be separated into those about which enough is known and those for which more research is needed, (4) whether more thought should be given to maternal secretory status for HM composition or bioactive ingredients, (5) beyond human milk oligosaccharide (HMO) composition, whether the levels or doses of bioactive ingredients in IFs matter whether the sources of bioactive ingredients matter (eg, isolated from bovine milk, recombinant, or synthetic). In addition, the panel discussed the potential to create a structure to address how to integrate HM and IF research with big data analysis and the importance of including benefit and efficacy as part of safety evaluations, whether there are certain types of functionalities or modes of action for which there is a reasonable certainty that they will not lead to adverse events and the importance of assessing both imminent and developmental safety. Lastly, the panel identified the need to implement strategies to recruit clinician-researchers into HM and IF research.

IF and HM both provide basic nutrition, but HM contains numerous additional biologically-active components, such as enzymes, immune and stem cells, immunologically-active molecules, and living microbes.^{1,3} The dynamic changes in HM composition over different

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stages of lactation can be recapitulated in IF; however, changes during a single feeding⁴ or with circadian rhythms⁵ will likely never be able to be duplicated. Grant funding and regulatory agencies, as well as the scientific community and industry, need to have a better understanding of the current scientific knowledge and gaps that need to be addressed as opportunities to enhance the composition of IF that will safely provide benefits to the infants.¹

This workshop focused on the state of the science regarding what is known about HM constituents that are thought to potentially have desirable bioactivity in infants, and to think broadly about how to evaluate the safety of constituents that are already included or that could be included in the future as ingredients for use in IF. For this workshop, 3 “guidelines” were articulated as follows.

- A focus on IF intended for use in healthy, term infants up to 12 months of age.
- The definition of bioactive ingredients is “non-human derived ingredients that may mimic components typically present in HM and are not traditionally considered essential nutrients but are thought to have physiological activity along with clinical relevance.
- A focus on integrating science within the context of the regulatory framework

Five scientific sessions were presented: (1) normal infant development outcomes; (2) HM as a reference for bioactive ingredients; (3) consideration of interactions between bioactive ingredients during processing or via the matrix; (4) functional equivalence of bioactive ingredients from different sources; and (5) consideration of interactions between and among bioactive ingredients. In this paper, we do not address variations in endpoints within the normal range. This was done in another recent review where safety of bioactive ingredients was reviewed.⁵

Session 1: Normal Infant Developmental Outcomes

Milk and the Developing Intestinal Microbiome

Three critical areas are changing due to advances in science and technology, as follows:

- The notion of the dyad of the mother and infant is moving toward that of a triad,^{3,6} where the microbial ecosystems of the mother and infant play interactive roles.
- Growth curves provide guidance for nutritional practice, but nutritional interventions undertaken after growth faltering may be too late in many situations to make meaningful nutritional interventions. We are evolving toward more individualized paradigms driven by a better understanding of the intestinal microbiome, multiomics and artificial intelligence that will likely provide information allowing us to be pre-emptive for providing precision nutrition for the infant.
- The idea of HM as a “conditionally perfect” model is a static convention that does not define the type of HM. Does HM refer to colostrum, transitional

milk, or mature milk? HM is a biological system with interacting components that include a microbial ecosystem that affects and are affected by interactions with the mother, child, and the environment.¹ The maternal–infant interactions associated with breastfeeding appear to be extremely important and very difficult to mimic with formula feeding.

Over the first 1000 days' postconception, there is an ongoing interaction between nutrients and the microbiota with the production of metabolites that are epigenetically active. Microbes thought to be commensals and potentially health promoting are present in HM⁶ exhibits maternal-specific microbial patterns.⁷ Microbes and their components, as well as microbial metabolites, play a role in the intestinal mucosal immune system.⁸ This is particularly the case during the first 6 months of life, when the imprinting of immune cells, the setting of immunologic memory and tolerance, and the development of key immune cells (eg, dendritic cells) occur, which will affect the infant for the entire lifespan. Some other HM bioactives that are closely related to the microbes that affect mucosal immunity, include lactoferrin (LF), immunoglobulins, and HMO.⁹

Findings from the Canadian Healthy Infant Longitudinal Development Cohort Study and other cohorts have shown that formula-feeding significantly alters the gut microbiota^{10–12}—even when exposure is limited to just a few feedings in the neonatal period.¹³ The Environmental Determinants of Diabetes in the Young study assessed breastfeeding and multiple other factors (eg, maternal body mass index, birth mode, geographical location), and found breastfeeding to be the most impactful factor in shaping the infant microbiome, particularly from 3 to 6 months-of-age.¹⁴ The study, which followed the babies to 40 months of age, indicated that the impact of HM wanes over time, which is not surprising because babies are often no longer breastfeeding in later infancy and encounter more varied food and other exposures. However, it is important to note that microbiome “recovery” after an early perturbation (such as formula-feeding) does not necessarily mean there are no long-term health impacts.

Transient perturbations during critical periods of early development can have a permanent impact on immune development and metabolism. For example, in a study where mice were briefly given low-dose penicillin in early life to perturb their microbiota, the microbiome recovered after treatment, but there were permanent effects on the metabolism and body composition of the mice. In adulthood, they were more likely to become obese and exhibit metabolic derangement.¹⁵ Another rodent study demonstrated that early life antibiotic exposure induced different antibiotic-induced disruptions of the enteric nervous system, microbiota and mucosal serotonin biosynthesis in male and female mice,¹⁶ thus necessitating consideration of sex as a biological variable. Additionally, even if certain bacteria are present, their function can change depending on the nutritional substrates available. For example, HMO utilization genes expressed in certain infant gastrointestinal (GI) microbes during breast-feeding disappear after weaning.¹⁷

HM itself has a complex microbiome^{18,19} that is not fully understood. Unanswered questions about HM and infant gut microbiomes include the following:

- Do HMOs shape the milk and/or infant microbiome?

- Do HM microbes colonize the infant GI tract, mouth, and/or nose?
- Do HM microbes interact with the intestinal epithelium and/or resident microbiome? If so, what is the nature of this interaction?
- Is there a core HM microbiome common to all women, or is it completely personalized?

In addition, research is needed to determine whether HM components alone or combined with other HM components influence the infant microbiome structure or function. Components of interest include, but are not limited to, immunoglobulin A (IgA),²⁰ leptin,²¹ milk extracellular vesicles,²² and fatty acids.²³

Some considerations related to IF and the microbiome are as follows.

- IF is lacking many HM bioactive components and contains many non-HM components that are likely relevant to the infant microbiome and subsequent development.
- When measuring the microbiome, one must choose meaningful types of measures from the many options. For example, a simplified “diversity score” may not provide sufficient information; amplicon sequencing may not be sufficient to identify strains and confirm their viability and activity.
- IF and bottle feeding may affect microbiomes beyond just the GI, including oral and nasal.^{24–26}
- Microbiome-targeted additives might be an important area, but new approaches are needed to move beyond studying “traditional” probiotics or prebiotics, many of which have been applied from adult enteral applications. An ecological approach to understand which types of bacterial communities develop in a healthy vs unhealthy infant GI tract over time and determining their function using a full array of multiomics would be needed. Looking toward HM for inspiration about which microbes (as probiotics) or HMOs (as prebiotics) to include in IF, based on their presence and biological activity, could be a useful approach.

Milk and Immune Development

The term infant is born with a functionally immature and naïve immune system, necessitating development of systemic and mucosal immunity undergo developmental maturation in the first year of life.^{27,28} This occurs under the influence of environmental exposures, novel antigens and the gut microbiota²⁹ and is necessary for short- and long-term health outcomes, including resistance to infection and the development of atopic diseases, including atopic dermatitis, food allergy, wheezing illnesses or allergic rhinitis. Lifestyle factors (eg, prolonged breastfeeding, farming lifestyle)³⁰ affect immune system development. HM modulates immune development by providing immunoprotective proteins and by modulating the gut microbiome development.²⁹ HM components such as food antigens, IgA, cytokines, microbiome, and HMOs affect the mucosal barrier and immune microenvironment, providing microbiome feeding or seeding, and providing initial antigen

and epitope repertoire.³¹ Given the potential life-long implications of immune dysregulation, a recent workshop specifically made recommendations for assessing the safety of bioactives in IF on immune outcomes.³²

Several clinical outcomes in terms of immune system development in the first year of life are important:

- Protection against infections (eg, gastroenteritis, pneumonia, ear, and skin infections)
- Oral tolerance: failure to develop this leads to food allergy, celiac, or early onset inflammatory bowel disease
- Other chronic inflammatory conditions (eg, allergic diseases, type 1 diabetes, autoimmune diseases)

The following outcomes measured at 3, 6, 9, 12, and 24 months are likely to be the most useful to evaluating the effects of new potential bioactive ingredients for use in IF on the immune system: complete blood count and differential (monocytes, neutrophils, lymphocytes, eosinophils, basophils), (1) T helper 1, T helper 2, T-regulatory, T helper 17, and B cells, (2) Lymphocyte responses after stimulation, (3) Total immunoglobulin (IgA, IgG, IgM, IgE), (4) Vaccine responses (tetanus, diphtheria, mumps, measles, rubella, pneumococcal), and (5) Outcomes specific to the bioactives (eg, food specific IgE, IgG).

Neurological System

Brain growth and development are more rapid in the first 1000 days than at any other point in the lifespan.³³ Given the dynamic nature of early brain development, timing is a key concept with respect to the effect of the infant's nutritional milieu on the developing brain. Overall, the adequacy of IF fortification strategies depend on their timing relative to the peak periods of growth and development, known as sensitive periods, with the potential to benefit cognitive functioning well into childhood and adulthood. In epidemiologic and interventional studies, greater breastfeeding is associated with improved cognitive outcomes later in life.^{34,35} For example, in Project Viva, a longer duration of breastfeeding and greater breastfeeding exclusivity were both associated with better cognitive outcomes at both preschool and school age.³⁶

Bioactives in HM represent a possible mechanism through which breastfeeding may benefit the developing infant brain, although currently very little is known about how specific HM bioactives, or the combination of bioactives within the matrix of HM, act on neurobiological processes during human development. Bioactives added to IF might influence brain development through similar mechanisms. In animal studies, milk bioactives, such as microRNAs and milk fat globule membrane (MFGM), are ingested, survive digestion in an active form, are absorbed across the GI tract and into the circulation, cross the blood-brain barrier, and accumulate in specific brain regions and structures.^{37,38} Human clinical trials support the potential for MFGM to support neurocognitive development in the first year, but benefits may diminish over time.³⁹ Bioactives that may act indirectly include the HMOs, which are not absorbed in large amounts, but substantially impact the gastrointestinal (GI) microbiome composition of the infant, which in turn may influence the brain through diverse

pathways including the vagus nerve, hypothalamic-pituitary-adrenal axis, immune system, and neuroactive metabolites, such as short chain fatty acids and sialic acid.⁴⁰

Key concepts about ideal measurements of infant neurologic outcomes as they relate to infant brain development include the following.

- During infancy, look for physiologically significant bio-indicators that show the net effect or response within the brain. Examples are visual attention (looking measures) and quantitative electroencephalogram based markers such as event-related potentials, which allow inferences about attention and memory systems, as well as brain magnetic resonance imaging to measure aspects of infant brain structure such as myelin.^{41,42}
- Consider the timing of the developmental processes in the brain—specifically, myelination and the refining of connections between neurons and brain regions that develop in the first postnatal year—which overlap with timing of exposure to IF bioactives.
- Counterbalance limitations of measures during infancy with longer follow-up into childhood using a range of parent reports and other resources, such as the National Institutes of Health Toolbox in older children.
- Additional considerations are provided through examination of the COGNIS (A Neurocognitive and Immunological Study of New Formula for Health Infants) study as a useful exemplar.⁴³ This Spanish randomized controlled trial tested an experimental formula supplemented with several bioactives (MFGM, synbiotics, probiotics, long chain polyunsaturated fatty acids, gangliosides, nucleotides, sialic acid) as compared with an unsupplemented control formula. There was also a breastfed reference group. Few differences were seen between formula groups in the first year of life, whereas visual function was better in breastfed as compared with formula-fed infants. Subsequent follow-up at 18 months and 2.5 years revealed better parent-reported behavioral outcomes in children who had received the experimental as compared with control formula.⁴³ At 4 years-of-age, language outcomes somewhat favored the experimental formula; children in the breastfed reference group had substantially better outcomes.⁴³ Strengths of this study included physiologically- and developmentally informed outcome measures in infancy as well as follow-up through school age when higher-level domains such as language could be assessed. Notably, the breastfed reference group performed better than either infant formula group across most domains.

Session 2: Human Milk Composition as a Reference for Bioactive Ingredients

IF is designed to provide biological outcomes as similar as possible to those provided by HM. Therefore, it is logical that HM composition be utilized as a reference for IF composition. However, HM composition varies widely by time postpartum, time of day, time within a feed, maternal factors (eg, genetics, diet, obesity), breastfeeding patterns, and

environmental exposures).^{44,45} Indeed, carefully standardized global studies have shown that there is no single “normal” HM composition. For example, the INSPIRE (Evolutionary and Socioculture Aspects of Human Milk Composition) study, which used standardized collection and analysis methods to assess HM composition, found wide variations in immune factors, immune specificity, HMOs, protein, lactose, and microbiomes in milk produced by healthy women 1–3 months postpartum.^{46–48} Variation in HMO profiles has been, at least in part, driven by evolutionary genetic selection.⁴⁹ Nonetheless, despite the dynamic nature and variability of HM bioactive constituents, it is the natural choice for use as a standard reference for IF composition.

There remain a multitude of unanswered, yet critically important, questions related to what is (and is not) known about HM composition and its use for IF formulation. For example, are there adaptive consequences to variation in HM composition? In other words, is there really a one-size-fits-all HM composition, or has HM composition been customized to a particular environment and set of customs to maximize infant health and wellbeing in that environment and culture? For example, it is unclear whether infants with different α (1,2)-fucosyltransferase (FUT2) genotypes should consume milk with different HMO profiles. Also, there is considerable variability in the microbiome profiles of HM produced around the world. Are these various microbiomes customized to various environmental microbial ecologies? If so, then adding the same constellation of microbes to IF designed to be fed to infants exposed to different environmental milieus might be unwise.

Additional considerations related to IF and the use of HM as a reference include:

- The science describing the biological significance of observed changes or differences in levels of various HM constituents over a lactation or among women, respectively, is still in its discovery phase. Some differences in HM composition may have been driven by evolutionary pressures and other environmental conditions that have customized milk for optimal infant survival and wellbeing.
- Our understanding of potential health risks for infants fed HM in which the activity of some bioactive ingredients has been abolished or reduced is not settled. However, many infants fed pumped/frozen HM or banked HM that has also been heat-treated have been experiencing this phenomenon with no known negative outcomes. It is time that we examine this experimentally.

Bioactives added to IF are approved under FDA’s “Generally Recognized As Safe” process. However, there are limitations and shortcomings to this process.⁵⁰ Major modifications to IF composition require growth monitoring studies, which are challenging to conduct and exclude small-for-gestational-age and late preterm infants who are often fed standard IF. These infants make up about 30% of newborns globally. There is also a concern about the standards being used. For example, breastfed and formula-fed babies have different growth patterns; which pattern should be used? Finally, there is the question of which bioactives might require a more thorough biochemical assessment.

More research is needed about assessing the cost/benefit ratio of adding bioactives, meaningful clinical outcomes related to infection or allergy prevention and management,

and how to connect common infant symptoms (eg, colic) to specific components of HM or IF. There should be caution about aiming for IF to be “closest to HM.” This does not account for possible risk: benefit exposures and the changes that occur in HM over time. There is also a need for equity in providing the best IF to everyone, regardless of cost. In an analysis by Abrams,⁵¹ it was suggested that from a safety perspective, the addition of a component should be studied throughout childhood, not just in infancy and early childhood. Postmarketing surveillance of bioactive ingredients is also needed. Adding single ingredients to IF and making changes as knowledge evolves is reasonable, so long as there is evidence to believe that the ingredients are beneficial (<https://nap.nationalacademies.org/catalog/10935/infant-formula-evaluating-the-safety-of-new-ingredients>).

Other considerations related to IF and the use of HM as a reference for IF include:

- It is clinical outcomes, not just matching the composition of HM that matters for safety and efficacy of IF, and as we move forward identify these and advertise health benefits, not just the comparison with how “close” an IF is to HM. Further, comparisons with HM should not be used as advertising to avoid confusing families.
- There are equity issues in IF bioactive access—women infants children IF formulations are different, and often contain less bioactive ingredients than standard IF available to consumers purchasing their formula not from the women infants children program
- Evaluations of bioactives should include mixed-fed children (consuming HM and IF) to provide a more realistic representation of effects in children, as many are mixed-fed (https://www.cdc.gov/breastfeeding/data/nis_data/results.html)
- Long-term research is needed on bioactives as related to development outcomes and disease implications. Research funding for long-term studies and outcomes beyond those needed for FDA approval are needed and ideally are provided independent of corporate sponsorship
- Pipeline of researchers, especially pediatricians, who have a career interest in this field is limited in part due to funding limitations.⁵²

Session 3: Interactions Between Bioactive Ingredients during Processing or via the Matrix

Most studies evaluating the addition of bioactives to IF test a single ingredient at a time. However, there is evidence that bioactives interact during processing or in the infant. HMOs are stable during pasteurization and other typical process operations, allowing the inclusion of HMOs in IF. In contrast, immunologically-active HM proteins interact with each other and with lactose during heating,^{53,54} which reduces the bioactivity of a bioactive protein, likely due to aggregation, as was shown for LF.⁵⁵ Heating on confirmation and functionality of bioactive proteins, but may also be modulated through the effect on digestion. For example, casein is important because aggregated bioactive whey proteins attach to the casein micelle upon heating, thereby changing both the digestion of these milk proteins

and the immune system response.⁵⁶ Protein degradation begins at between 70 °C and 75 °C, suggesting that regular pasteurization may already lower the level of milk proteins. Immunoglobulins are slightly more stable and require slightly more intense heat treatment to be affected. This effect of heating on functionality may depend on the composition of the product or ingredient that is heated. For example, LF behaves differently when heated alone (very little aggregation) than when it is in a milk or IF matrix (large amounts of aggregated protein), due to differences in the interactions among whey proteins.⁵⁶

Potential alternatives for heating include non-thermal techniques, such as UV light, high pressure, and ultrasonication.⁵⁷ These may be used alone or in combinations of either multiple non-thermal or lower-heat treatments.^{58,59} Studies have shown that non-thermal technologies can retain the functionality of the bioactive proteins in milk,⁵⁷ but more work is needed on a larger scale to determine their safety profiles.⁵⁹

In considering safety assessments for bioactive milk constituents introduced into IF, functionality maybe a key consideration. The situation in which some subgroups of infants would benefit from certain additives could be examined from the perspective of genetic risk. For example, infants with a family history of wheezing have a genetic predisposition and benefit more from breastfeeding, perhaps due to certain HM components.⁶⁰ It may be more feasible to explore this with infants who have received antibiotics or been delivered by cesarean, because these traits are more easily identified. This knowledge may be implemented through making personalized IF depending on the specific infant predisposition, as currently there is one basic one-size-fits-all formulation.

Several questions need to be better addressed. These include:

- What role do preclinical trials have in assessing bioactives?
- What are the effects of a breastfeeding mother being on a vegetarian or vegan diet, or the use of non-milk protein sources in IF, considering the interest in plant based IF?
- Is it possible to evaluate the safety of IF without understanding the functionality of the countless constituents of HM?
- Is it acceptable to assume that an HM constituent's functionality will be similar when added to the IF matrix?
- Is it preferable to use the lowest level of HM constituents measured in healthy infants as the safety target rather than the upper limit?
- Is the intake of a bioactive more important than its concentration in HM? Those values are often unavailable, as the volume of HM ingested is rarely measured.
- Are researchers focusing on only a few HM components and ignoring or overlooking essential ones?

There is a need to balance innovation in the IF industry with the regulation of specific proteins or components manufacturers want to add to IF. Would it be more efficient to do studies comparing IF with and without a specific component of a manufacturer's interest

to speed up the process? In the past, companies have added ingredients to their IF, such as nucleotides or galactooligosaccharides/fructooligosaccharides mixtures, without proper data that they provide the same benefits as provided by HM compounds. Considerable research on ingredients focuses more on potential functionality while ignoring safety, but the product is still marketed as one that contains something like a compound found in HM. One clear fact is that safety must be evaluated. An example of a potential safety issue was shown for short-chain galactooligosaccharides generating anaphylactic reactions in specific populations,^{61,62} which also shows that adding components not present in HM may have unanticipated risks.

Although long-term safety studies are ideal, determining long-term safety outcomes in humans in general, and in infants, is very difficult, because it may take up to 50 years to see the effects. In the future, government-industry partnership for funding research will be essential, as both companies and government do not have the resources to study all possible endpoints by themselves.

Session 4: Functional Equivalence of Bioactive Ingredients from Different Sources

Example of Docosahexaenoic Acid (DHA) Supplementation to IF

The scientific focus in the late 1970s and early 1980s was on linoleic and α -linoleic acid, the parent fatty acids for the omega-6 and omega-3 polyunsaturated fatty acids. Much work has been done in the last 40 years on the conversion of these 18-carbon fatty acids to the long-chain metabolites DHA and arachidonic acid (ARA) and to understanding their physiologic function.

There is very little DHA in the brains of preterm infants. DHA is present at 22 weeks gestation and then accumulates dramatically over the first 2 years of an infant's life, suggesting that it is important.⁶³ A study in nonhuman primates showed that a reduction in brain DHA was associated with lower cortical visual acuity,⁶⁴ suggesting that lower DHA in the brains of preterm infants⁶⁵ might be functional. This led to subsequent studies of visual acuity in preterm infants fed IF with and without DHA.^{66,67} By 2002, numerous studies had correlated DHA supplementation to IF with better visual and cognitive function.⁶⁸ That same year, just as IF containing DHA and ARA was starting to be produced, the DHA Intake and Measurement of Neural Development trial began in term infants to determine the optimal dose of DHA (ie, 0.32%, 0.64%, or 0.96%) in IF, while keeping the concentration of ARA constant (0.64%). Some of the children were followed to 6 years-of-age with cognitive testing; a subset of the cohort was followed to 9 years-of-age with evidence of brain structure-function effects of postnatal DHA and ARA supplementation. The study showed that positive effects on cognition to school age^{69,70} and benefits for brain development were still present at 9 years of age.⁷¹

The studies of DHA and ARA differed from some biologicals that are now being considered for IF in several ways. First, a membrane biomarker that was low in infants who were fed IF compared with infants fed HM was the first indication that DHA and ARA might

be important nutrients. Second, there was already evidence that brain DHA and ARA accumulation were incomplete at birth, and nonhuman primate studies had shown that low brain DHA accumulation meant a permanent reduction in cortical visual acuity. Third, a possible functional deficit existed, and there was a testable hypothesis: that adding DHA and ARA to IF could improve visual acuity. Finally, the focus was on brain function; DHA was the first biological entity added to IF for a purpose other than providing macronutrients or essential vitamins or minerals.

Example of HMOs Supplementation to IF

HMOs have also been an area of intense interest due to their high concentration in HM and their resistance to digestion in the upper GI tract intact. There are many potential structures, however the typical number for each mother is approximately 100.^{72,73} The HMOs can be phenotyped into 2 groups (using the abundances of α (1, 2)-fucosylated structures.⁷⁴ Secretors have an activated FUT2 gene that encodes for a FUT2. Non-secretors produce little-to no α (1, 2)-fucosylated structures but will produce other fucosylated structures with different linkages. The expression of the FUT2 gene varies geographically,⁷⁵ thus, some infants consume HM with no α (1, 2)-fucosylated structures. 2'-Fucosyllactose is commonly the most abundant α (1, 2)-fucosylated structure. HMOs also include many antigens and receptors, which can be quantitated by specific Lewis antigens and by which fractions of the compounds contain fucose, sialic acid, both, and neither.

HMOs are found in virtually every bodily fluid of the mother and infant, including in HM and blood and in the infant's blood, urine, and feces.^{76,77} HMOs are not only protective to the infant, but also to the mother that produces them. Several studies have shown that HMOs bind to pathogens.⁷⁸ They may also function in immunomodulation⁷⁹⁻⁸¹ and as prebiotics. The most well characterized is the symbiotic relationship between HMO and *Bifidobacterium longum* subspecies *infantis* (*B. infantis*). When HMOs affiliated with these bacteria were purified, the enzymes found in the *B. infantis*, included sialidases and fucosidases.⁸² *B. infantis* interacts with the very specific linkages of these HMOs.⁸³ In addition, other bacterial inhabitants of the neonatal gut, including *Lactobacillus* (Bai), *Bacteroides*, and *Parabacteroides* species can utilize HMOs.

To date, 7 synthetic HMOs (lacto-N-neotetrose, lacto-N-tetrose, 3'-sialyllactose, 6'-sialyllactose, 2'-fucosyllactose, 3-fucosyllactose, difucosyllactose/lactodifucotetraose) have received Generally Recognized As Safe status in the US and have positive opinions from the European Food Safety Authority regarding their safety. One⁸⁴, 2⁸⁵ or up to 5^{86,87} HMOs are currently being added to some commercial IF. Although the clinical trials and lack of adverse events reported from long-term market availability of formulas containing 1 or 2 HMO do not raise concerns, many questions still need to be address related to HMOs, particularly as more complex HMO are considered for addition to IF. For example, can all structurally different individual HMOs be classified as functionally equivalent, and can human and non-human homologs be classified as functionally equivalent?

Considerations Related to HMOs in IF:

- Structurally different individual HMOs cannot always be classified as functionally equivalent, as it depends on the function of interest.
- Human and non-human homologs cannot always be classified as functionally equivalent, again, depending on the function of interest.

Proteins and Peptides

The FDA allows different sources of protein in IF (bovine milk and soy protein). From a safety and basic nutrition perspective, IF provides adequate, safe protein that is essential to the growth of formula-fed infants. However, IF lacks bioactive milk proteins due to the array of IF processing methods (various heat treatments, which can alter milk protein structure and functionality) and various degrees of enzymatic hydrolysis that reduce functional activities. Infants fed HM have numerous biological benefits over those fed formula (including reduced risk of infection, decreased risk of necrotizing enterocolitis in preterm infants^{88–90} and potentially decreased allergy.⁹¹ These differences in outcomes could relate to the difference in proteins provided in IF.

Proteomics has revealed that HM contains hundreds to thousands of unique proteins.⁹² Many of these have at least partially known functions (eg, isolated lymphoid follicles, immunoglobulins, lipases like bile salt-stimulated lipase that can assist with lipid digestion), but many, have yet undiscovered functions in the neonate. Moreover, the partial digestion of many milk proteins releases an array of peptides. Infant gastric and intestinal digestion releases a large array of peptides highly homologous with known bioactive peptides with antimicrobial, calcium-binding, antihypertensive, immunomodulatory, and opioid activity.^{93–98} This complexity produces regulatory challenges.

The question is how should these novel formula additions be evaluated? Beyond safety, each novel protein proposed for use in IF should be supported by demonstrated structure, function, and digestive behavior like that of the HM protein counterpart, at least by in vitro assays of interactions with relevant receptors and target cells (eg, specific microbes, immune cells, or gut cells). Recombinant proteins should be shown to match as closely as possible to native structures in HM, as shown by determination of mass, amino acid sequence and post-translational modifications (eg, glycosylation, phosphorylation) using techniques, like liquid chromatography mass spectrometry. However, identical structure may not be necessary, if functionality is sufficiently similar. For example, bovine milk LF differs somewhat in sequence and glycosylation from HM LF, but still possesses many of the same functions, and thus could serve as an adequate alternative to HM LF.

Milk proteins are exposed to an array of proteases in the stomach and gut, resulting in the partial or complete digestion of many milk proteins. It is critical to determine which HM proteins survive, so that the potential relevance of their function can be assessed. Novel milk proteins should match the extent of survival of HM proteins and proteins. Optimally, this can be achieved by in vivo human testing in infants with sample collection from the stomach and intestine as well as stool, followed by mass spectrometry, enzyme-linked immunosorbent assay, and functional testing. When it is not possible to obtain approval for direct feeding of a protein, *ex vivo* incubation in infant digestive samples can provide an approximation of in

vivo digestion. A lower form of evidence could be simulated GI digestion to mimic that of infants. Other strategies for digestion assessment could include feeding to piglets or primate models, yet the extent to which protein digestion in the models matches that of humans is not clear, particularly on an individual protein basis. For proteins or peptides suggested to have biological actions that would require systemic absorption, blood sampling and analysis would be needed.

Considerations Related to Proteins in IF:

- Novel formula proteins should be evaluated for structural, functional, and digestive similarity to HM proteins.
- Optimal digestive studies would include in vivo infant feeding and digesta analysis (gastric, intestinal, stool)
- Absorption studies are needed for proteins with potential systematic actions which would require infant feeding studies with blood sampling
- Studies should assess the allergenic potential of recombinant HM proteins or analogs isolated from bovine milk.

Session 5: Consideration of Interactions Between Bioactive Ingredients and Other Components

For HMO-prebiotic interactions, it is believed that different microbes utilize different HMOs. Introducing a blend of HMOs in the absence or presence of other prebiotics could lead to a different microbial structure and function than a single HMO will. What happens if the introduction of an individual HMO shifts microbial communities in a direction very different from where they would be if they had been fed the complex mixture of HMOs in HM?

Other aspects to consider are that HM nutrients and bioactives exist in discrete compartments, which may influence their functions. Analogues to these components are available in recombinant or synthesized forms or can be isolated from the milk of other species. How these components interact within the matrix of HM or IF is not understood well, which limits the ability to anticipate the biological actions of bioactive components added to IF. Additionally, we do not fully understand interactions between bioactive ingredients in IF—some evidence of interactions is found for osteopontin-ILF,^{99,100} whereas studies with HMO show that components can have distinct, but complementary actions.^{101,102}

Lastly, HM bioactives can have direct or indirect effects on infant outcomes. The intestinal, neural, immune, and microbial systems all interact.^{103,104} Thus, studies that examine a single outcome cannot fully explain the complexity of the system and the other effects underlying different mechanisms. Considerations related to ingredient interactions in IF:

- We need mechanistic, preclinical studies that investigate multiple outcomes within the same study to fully understand how ingredients interact and the effects, if any, on multiple systems

- The microbiome, immune system, and cognition are especially relevant outcomes for studies of the effects of any interactions between IF ingredients.
- Understanding the mechanism(s) of effect of any bioactive ingredient, and their interactions, would allow for better prediction of effect on outcomes. However, in vivo data is needed to understand any developmental outcomes, or unanticipated outcomes, because of bioactive ingredients.

A Clinician's Perspective

Infant formulas need to be understood from both the nutritional and bioactive standpoint. From a clinician's perspective, the FDA defines IF as "food which purports to be or is represented for special dietary use solely as a food for infants by reason of its simulation of human milk or its suitability as a complete or partial substitute for human milk." (<https://www.fda.gov/regulatory-information/search-fda-guidance-documents/guidance-industry-frequently-asked-questions-about-fdas-regulation-infant-formula>) Infants, and particularly preterm infants, have undeveloped immune systems and are vulnerable to the effects of bioactives added to IF. IF was first developed as a source of nutrition to address the immediate need to support growth and development. This paradigm shifted with the realization that HM is a complex biologic fluid that provides more than nutrition, varies from mother to mother, and changes over time.^{105,106}

Clinicians consider IF to be inferior to HM not only because IF does not mimic clinical outcomes of HM (eg, reduced mortality, immune and infection protection, production of a healthy gut microbiome, improved neurodevelopment in preterm infants) but also because IF may contain additives that cause harm.^{107,108} The GI reflux disease algorithm is an example of how mistakes can occur in the realm of infant feeding.¹⁰⁹ A common approach to this disease had been to thicken the feeds with a starch- or gum-based thickener (eg, rice cereal, xanthan gum) or alginates. Some literature supported the use of these thickeners, but there was also some evidence of untoward effects, including slower gastric emptying, altered caloric density, and mineral absorption.^{110–112} Xanthan gum, considered a good thickener because it is not digested by HM, was widely used until reports of NEC and death associated with specific xanthan gum use resulted in an FDA warning prohibiting its use in preterm infants.^{113,114} (<https://wayback.archive-it.org/7993/20170722060115/https://www.fda.gov/ForConsumers/ConsumerUpdates/ucm256250.htm>) Studies then illustrated the unintentional consequences of thickened feeding, including viscosity-related harmful changes to the gut, increased osmolality, and reduced mineral absorption.^{115,116} (https://www.infantjournal.co.uk/pdf/inf_063_str.pdf). In retrospect, more studies to better understand the chemistry of thickening with HM and IF before broad use could have forewarned adverse events.

Clinicians are looking for outcomes that include efficacy and safety, short- and long-term effects, and severe adverse or irreversible effects. They want advances in IF to be reasonably priced and to continue without overregulation that would stifle innovation, but with enough regulation to prevent a flood of new bioactives with unanticipated interactions. It would be beneficial to have a standardized definition of safety and efficacy.

Discussion and Summary

Several topics were discussed in this workshop that have implications for setting regulatory guidelines for bioactives in IF:

- Statistical considerations for studies in animals
- Strategies to recruit clinician-researchers into HM and IF research
- Whether bioactive ingredients can be separated into those about which enough is known and those for which more research is needed
- Whether more thought should be given to maternal secretor status for HM composition or bioactive ingredients, beyond HMOs.¹¹⁷
- Whether the levels or doses of bioactive ingredients in IFs matter Whether the sources of bioactive ingredients matter (eg, isolated from bovine milk, recombinant, synthetic)
- The potential to create a structure to address how to integrate HM and IF research with big data analysis
- The importance of including benefit and efficacy as part of safety evaluations, including whether a reasonable certainty that certain types of functionalities or modes of action will not lead to adverse events
- The importance of assessing both imminent and developmental safety
- Which animal models and in vitro systems seem most promising.

In terms of study design for milk bioactives, early studies can be conducted using in vitro or preclinical animal models, whereas infant clinical safety trials will be required prior to FDA regulatory approval. Using tissue cultures and organoids allows for testing under controlled conditions and for testing different ingredients on different cell types. If single cells are used, only effects on that cell type will be detected. This approach is good for understanding mechanisms. Preclinical animal models allow for controlling genetics and the environment and for testing an ingredient's safety and efficacy. This approach is good for understanding mechanisms. Piglets are considered one of the best models of infant immune, GI and brain development.¹¹⁸

The rigorous design of human studies aiming to assess effects of milk bioactives requires careful attention to epidemiologic principles. The best study design for establishing causality would be a randomized controlled trial comparing infants on IF with and without bioactive additives. Infant formula studies have often included a breastfed reference group, but because this reference group is non-randomized, between-group differences can be confounded by the shared determinants of feeding type and infant outcomes.⁹⁹ An equivalence design would be useful in assessing safety but demonstrating superiority of an IF with an added bioactive ingredient would provide stronger evidence in support of a change in clinical practice.

Efficacy trials should be powered to detect a plausible, clinically meaningful difference, taking expected attrition into account. These trials must consider sex as a biological variable

and allow for comparison with unsupplemented formula. For example, in selecting measures to identify effects of bioactives in IF on neurodevelopment, investigators should seek bio-indicators, which detect the response to a particular nutrient within a biological system, such as the brain.¹⁰⁰ To improve precision and capture important information about timing and longer-term impact of effects, outcomes should be assessed repeatedly over the course of an intervention, at the end of the intervention, and later in childhood. Use of invasive procedures is limited in infants due to ethical constraints, but non-invasive approaches are available to study brain development through imaging,^{101,102} intestinal transcriptome,¹¹⁹ and hostmicrobe interactions^{120,121} using exfoliated epithelial cells or genetic signatures using saliva or buccal swabs. This summary should provide a strong framework for future work in this area. ■

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Glossary

HM	human milk
IF	infant formula
FDA	Food and Drug Administration

HMO	human milk oligosaccharides
LF	lactoferrin
Ig	immunoglobulin
MFGM	milk fat globule membrane
DHA	docosahexaenoic acid
ARA	arachidonic Acid
GI	gastrointestinal

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