ABSTRACTS

Oral Presentations

Many Human Milk Extracellular Vesicle Proteins are Lost in Human Digestion, Do the Survivors Convey Beneficial Effects?

Sarah Andres, Assistant Professor, Oregon Health & Science University

Extracellular vesicles (EVs) are lipid-membrane-encased nanoparticles that carry biological cargo (including proteins) from mom to baby via human milk. Although studies in animal models demonstrate the beneficial effects of human milk EVs on intestinal barrier function, we do not know if these cargoes survive in vivo human digestion. Addressing this knowledge gap will remove critical barriers to using human milk EVs and associated proteins as additives to neonatal nutrition or therapeutics for necrotizing enterocolitis. 1. Test if human milk EVs promote the survival of cargo proteins to the human intestine; 2. Delineate molecular mechanisms of EV-cargo mediated gut barrier protection. EVs were isolated from 3 paired human milk and neonatal intestinal content (digesta) samples using density-gradient ultracentrifugation. Digesta were collected after gastric feeding from naso- or orojejunal sampling tubes. EVs were characterized by nanoparticle tracking analysis, western blot, and electron microscopy. EV protein cargo was profiled by a C18-UPLC paired with Orbitrap mass spectrometry. Effects of candidate proteins on human intestine were examined using neonatal enteroids derived from patient intestinal stem cells. All studies are part of OHSU IRB-approved protocols. Only 4.68% +/- 0.02 (p=0.0012 vs undigested milk) of EV proteins from human milk survive to the human intestine, but nearly half 48.96% +/- 0.02 (p=0.007 vs undigested milk) of the protein diversity is preserved. Protein cargoes within the surviving EVs are involved in extracellular matrix interactions, membrane trafficking, metabolism, and cell survival. These EVs are taken up by intestinal cells where they exert their effects. We demonstrate that the majority of EV protein cargo is lost between the mouth and the intestine, but that nearly half of the protein cargo diversity is preserved. These findings indicate the importance of examining cargo that survives to the intestine when investigating potential milk-mediated mechanisms of disease prevention.

Co-authors: Brian Scottoline1, Bum Jin Kim2, David Dallas2, Madeline Kuhn1, Randall Armstrong1, Yang Zhang1
Affiliations: Oregon Health & Science University1, Oregon State University2

High Temperature Processing and Subsequent Storage of Infant Formulas Induces Protein Modifications, Gut Dysfunction and Inflammation in Preterm Pigs

Stine Brandt Bering, Associate Professor, Head of Section, University of Copenhagen

Processing of infant formula (IF) or protein ingredients using high temperature and subsequent storage may induce chemical protein modifications (e.g., Maillard reaction products, MRPs) and thereby reduce bioactivity. Ultra-high temperature (UHT)-treated IF is increasingly being used for newborn preterm infants when human milk is unavailable. We hypothesized that this negatively affects development in preterm infants fed high-temperature treated IFs. Using preterm pigs as a model for sensitive newborn infants, we investigated the effects of a model IFs subjected to indirect UHT treatment with or without subsequent storage (SUHT) compared to pasteurized IF (PAST) (Experiment 1). Likewise, the effects of a gentle processed serum protein concentrate (SPC) with or without extra heat treatment (HT-SPC) and storage (SHT-SPC) was compared to a whey protein concentrate (WPC) (Experiment 2). MRPs and other protein modifications were determined in the IFs and protein ingredients, and clinical outcomes and gut and immune maturation markers were investigated after 5 days of feeding. For both UHT-treated liquid IF and SPC-based IF, the heat-treatment and subsequent storage increased protein modifications and aggregates and reduced antibacterial activity. Piglets fed these formulas showed increased diarrhea and intestinal inflammation (necrotizing enterocolitis) and

reduced gut morphology and function. Furthermore, feeding the stored UHT formula resulted in gut accumulation of MRPs and protein-cross-links as well as upregulation of genes involved in acute inflammatory responses and cell turnover. Protein modifications, including MRP formation, mainly occurred in heat-treated IFs and protein ingredients during storage, particularly the liquid IFs. This was associated with MRP accumulation in the gut and impaired gut maturation with increased inflammation in preterm pigs in the first days of life. Processing and storage conditions are important for the quality of IFs and might affect gut and immune development in newborn infants, particularly those that are very diet-sensitive or immature.

Invited Presentation: Human Immune System Imprinting by Environmental Factors Early in Life
Petter Brodin, Chair and Professor, Imperial College London

Establishing immune-microbe mutualism after birth is a daunting task. Diseases like asthma, allergies, autoimmunity, obesity, and neurodevelopmental disorders have been linked to perturbed immune-microbe relationships early in life. Prior studies mostly involved human cord blood or mice, but neither of these can account for postnatal exposures in humans. In recent years, we have performed longitudinal systems-level immunomonitoring in children while simultaneously quantifying colonizing stool microbes. We find numerous associations between microbes and their metabolites imprinting on developing immune cells. Here I will present our most recent results related to the development of human immune systems early in life.

Student Award Presentation: Infant Feeding Practices and Parental Perceptions During the 2022 United States Infant Formula Shortage Crisis
Karina Cernioglo, Medical Student, University of California Davis

In May of 2022, parents living in the United States experienced a dramatic infant formula shortage largely caused by supply chain issues and the recent recall of several infant formula products over contamination concerns. An anonymous, electronic, cross-sectional survey was designed to understand infant feeding practices, parental experience and perceived support during the crisis. Ninety-nine parents that lived in the U.S. and fulfilled the study criteria completed the survey. Sixty-six percent of respondents were female, and 75% of respondents were recipients of the Special Supplemental Nutrition Program for Women Infant Children (WIC). Parental mean age was 30.0 years and the mean infant age was 26.8 weeks. In response to the infant formula shortage crisis, parents changed their infant feeding practices, of which several were unsafe. Seventy-nine percent of parents fed their infants U.S. infant formula brands and 39% of parents fed their infants imported infant formula brands before the shortage which were reduced during the shortage to 27% and 11%, respectively. Use of donor milk significantly increased from 2% of parents before to 28% of parents during the crisis. Use of breast milk from community sharing significantly increased from 5% of parents before to 26% of parents during the crisis. Use of watered-down infant formula had significantly increased from 2% of parents before to 29% of parents during the crisis. The resources that parents reported as most helpful in navigating the crisis differed by parental sex and WIC recipient status and included other parents, friends, and family; lactation consultants; healthcare providers; and WIC. Our study found that feeding practices in response to the infant formula shortage may pose health risks to infants. These data suggest the need for policy changes within regulatory and the healthcare system to provide families with clinical prenatal and postnatal lactation support, access to donor milk, and access to more commercially-available products.
Co-author: Jennifer T. Smilowitz, University of California Davis

Student Award Presentation: From Colostrum to Mature Milk: Key Changes in Bovine IgG N-Glycosylation
Inge Gazi, PhD Student, Utrecht University

The total protein content and the milk proteome dramatically change during lactation, particularly in the first few days after calving. Bovine milk colostrum is defined by an unusually high protein content, half of which consists of immunoglobulins of the IgG class. Changes in the N-glycosylation conserved on the second constant heavy chain domain (CH2) of all IgG subclasses are linked to differential biological functionality. We investigated longitudinal changes in milk IgG content and N-glycosylation microheterogeneity using emerging analytical technologies and specialised methods for glycoprotein analysis. Bovine milk samples were collected from individual dairy cows at 1-28 days in lactation. Proteolytic digests of whole bovine milk and of IgG captured therefrom were analysed by LC-MS/MS. N-glycopeptide analysis was performed with dedicated methods employing glycan-derived oxonium ion-triggered hybrid fragmentation. The data acquired was analysed with a search engine specialised in glycopeptide identification. The immunoglobulin content of milk decreased from nearly 50% in the colostrum, to less than 1% of total protein in the mature milk. The relative abundances of immunoglobulin isotypes IgG, IgM and IgA to each other remained similar throughout lactation, with IgG being the dominant isotype. The IgG CH2 N-glycosylation also showed longitudinal dynamics, with a more diverse repertoire in the colostrum than in mature milk. The N-glycan repertoire shifted from mainly sialylated glycans in the colostrum, to more neutral glycans in the mature milk. Sialylation with both N-acetylneuraminic (Neu5Ac) and N-glycolylneuraminic acids (Neu5Gc) was detected in colostrum, whereas the sialylation of mature milk IgG CH2 N-glycans was exclusively with Neu5Gc. The results of this work shed new light onto the dynamics of the bovine milk proteome and the complexity of the key component, i.e., IgG. These findings lay the ground for future studies into the relevance and importance of bovine milk IgG and its N-glycosylation.
Co-authors: Albert Heck1, André Groeneveld2, Jan Bastiaans2, Karli Reiding1, Thom Huppertz2,3
Affiliations: Utrecht University1, FrieslandCampina2, Wageningen University3

Knowledge to Practice: Lactation as the Guide for Biotechnology in the 21st Century
Bruce German, Distinguished Professor, University of California Davis

The century of Biotechnology is emerging and will change the world, reshaping everything from the impact of human activity on the environment to the impact of the environment on the people in it. The IMGC is providing important examples of how this revolution can be achieved safely and effectively. Lactation is the blueprint for biotechnology. Scientific research and its translation to commercial utility can use the principles learned under this relentless selective pressure of Evolution. Mammalian mothers are the penultimate bioreactor for nourishment, generation after generation, lactation traits selecting for maximum nourishment at minimal cost. Bringing these lessons to practice is becoming a reality with inspiring new initiatives in milk structures, functions and cellular production. IMGC is the convening platform for sharing discoveries of molecules with the technologies of isolation and production; sharing the mechanisms of action with the diagnostics of their intervention in infants and adults; sharing the genetic regulation of mammary tissue with the support and bioproduction of immobilized mammary cells in culture. Innovations in the new biotechnology era will require highly integrative scientific initiatives and clear demonstrations of intervention efficacy and safety. Lactation research is coming into focus as the inspiration and actualization of biotechnology's bold future.

Anti-SARS-CoV-2 Milk Antibodies Over the Course of Lactation, After Infection and Vaccination
Yarden Golan, Postdoctoral Scholar, University of California San Francisco

An important benefit of human milk is the presence of IgA and IgG antibodies that provide passive immunity to the infant. However, the function of these antibodies in protection of infants against COVID-19 is not fully understood. The COVID-19 Vaccine in Pregnancy and Lactation (COVIPAL) cohort study recruit participants from December 2020 to Match 2022. Eligible participants were actively lactating, planning to receive any COVID-19 vaccine, and willing to donate blood and/or milk samples. Anti- SARS-COV-2 S1 RBD antibodies were measured using ELISA or Pylon 3D automated

immunoassay system. High titers of anti-SARS-CoV-2 IgG were found in blood and milk after vaccination. In contrast, we found that IgA levels in milk were higher after SARS-CoV-2 infection (Omicron) compared to samples collected after vaccination (mRNA-based vaccines). In addition, we tested the present and of anti-SARS-CoV-2 in infant saliva after breastfeeding persistent (multiple time points until next feeding). We found that IgA was present in infant's saliva for longer time and in more infant compared to IgG. IgA have an important role in infant protection against invasion of pathogens through the mucosal surface. The antibodies type secreted to the milk after vaccination may have an important role in infant protection, and therefor further studies are needed to understand the mechanism of IgA secretion into human milk. In addition, mucosal immunity (in the mammary gland) and pathogen specific IgA secretion after vaccination should be considered as important factor to determine vaccination efficiency for the nursing infant.

Cellular and Transcriptional Diversity over the Course of Human Lactation
Brittany Goods, Assistant Professor, Dartmouth College

Human breast milk (hBM) is a dynamic fluid that contains millions of viable cells, but their identities and phenotypic properties are poorly understood, particularly over lactational time. In order to better understand cellular dynamics and longitudinal lactational heterogeneity, we sought to characterize the transcriptomics of hBM-derived cells using single-cell RNA-seq (scRNA-seq) on longitudinal samples. hBM was collected longitudinally from 15 human donors across various stages of lactation (3 to 632 days postpartum). For each sample, we collected a rich set of information about the mother-infant dyad, including vaccine history, illness, and daycare status. To our knowledge, we have generated the first single-cell analysis of hBM-resident cells over the course of lactation, with a dataset comprised of over 48,478 cells from 50 samples. We confirm that the majority of cells in human breast milk are lactocytes, a specialized epithelial subset, and that cell type frequencies shift over the full course of lactation yielding greater epithelial diversity at later points postpartum. Further analysis of lactocytes reveals a continuum of cell states characterized by transcriptional changes in hormone, growth factor, milk production, and tight junction related pathways. Generalized additive models suggest that one sub-cluster, luminal cluster 1 (LC1) epithelial cells, increase as a function of time postpartum, daycare attendance, and the use of hormonal birth control. We also identify several sub-clusters of macrophages in hBM that are enriched for tolerogenic functions, possibly playing a role in protecting the mammary gland during lactation. Our description of the cellular components of breast milk, their association with maternal-infant dyad metadata and quantification of alterations at the gene and pathways levels provides the first detailed longitudinal picture of hBM cells across lactational time. This work paves the way for future investigations of how a potential division of cellular labor and differential hormone regulation might be leveraged therapeutically to support healthy lactation and potentially aid in milk production. Future work should also better delineate how the hBM environment promotes tolerogenic functions of macrophages and in turn, how macrophages may specifically support healthy lactation.

Invited Presentation-- Feedomics: Omics-Based Nutrition to Improve Milk Production and Nutritional Composition
Leluo Guan, Professor, University of Alberta

Increasing the efficiency and sustainability of dairy animal production is an effective way to produce valuable proteins for a growing human population. The application of advanced omics approaches (genomics, epigenomics, transcriptomics, proteomics, metabolomics, metagenomics/metatranscriptomics) in livestock research has revealed regulatory mechanisms in milk production and nutrient quality. However, most studies to date have only focused on a single tissue or organ and/or one or two types of technologies, which cannot lead to a comprehensive understanding of the interactive and systematic mechanisms contributing to milk phenotypes. Recently, we have developed a research concept termed "omics-based nutrition" to translate multi-omics data to their applications for animal production and human health. Using a multi-omics assessment of different molecules (DNA, RNA, and metabolites) across the rumen, liver and mammary gland tissues, we identified the molecular regulatory mechanisms of milk production and quality

when cows were fed a diet consisting of low-quality forage. We identified omics-based individualised responses of cows and symbiotic microbial metabolism and beneficial bioactive components in milk in response to bovine diet composition. The findings from this study have led us to propose the concept of "feedomics", use of these powerful multi-omics technologies to identify and understand the mechanisms involved in multiple biological processes regulating the molecular, physiological, metabolic and animal level changes through the animal production system. In addition, the feedomics can help to elucidate the complex interactions among nutrition, environment, animal genetics, metabolism and physiology, and their symbiotic gut microbiome that determine animal productivity, product quality, health and welfare. Herein, we discuss the importance of feedomics in dairy research, with a view to ensure that dairy cattle exhibit the human desired production traits from system biology aspect.

Invited Presentation: B. Infantis EVC001 Colonization in Breastfed Infants Modulates Enteric Inflammation Leading to Improved Health Outcomes

Bethany Henrick, Vice President of Discovery Science, Infinant Health

The intestinal microbiome composition and specifically the functional genes that they carry are critical to acute and long-term immune modulation that impact the health of the newborn. *Bifidobacterium longum* subspecies *infantis* (*B. infantis*) carries the full repertoire of functional genes to utilize and convert all human milk oligosaccharides (HMOs) into useable fuel sources, as well as critically important immunoregulatory bacterial metabolites that are linked to improved health outcomes in term and preterm infants. Here we identify the enteric inflammatory profile as a root cause of many infant and childhood-related disease states that have acute and long-term health implications. Specifically, colonization of *B. infantis* EVC001 in infants led to significantly increased HMO utilization gene abundance, as well as improved HMO utilization in vivo, correlated with significantly altered metabolite profile, including higher production of key immunoregulatory bacterial metabolites, notably indole-3-lactic acid, and altered the functional capacity of allergic and autoimmune disease-related CD4 Th2 and Th17 cells, respectively, in vitro. Furthermore, altered microbiome composition by *B. infantis* EVC001 modulated enteric and systemic inflammatory profiles in preterm and term infants, which correlated to improved anthropometric scores in severely malnourished infants, and decreased necrotizing enterocolitis (NEC) in preterm infants. Taken together, these data show how a critical infant symbiont restores gut function leading to improved immune system function and ultimately health outcome in preterm and term infants.

Keynote Address: From Farm to Table – Gut Microbiome and Immune Development in Farming- Lifestyle Infants

Kirsi Jarvinen-Seppo, Associate Professor, Chief of Pediatric Allergy&Immunology, University of Rochester School of Medicine / Golisano Children's Hospital

Growing up on traditional, single-family farms is associated with protection against asthma in school-age, but the mechanisms against early manifestations of atopic disease are largely unknown. Our studies among the rural Old Order Mennonite (OOM) community of western New York have shown that also atopic dermatitis and food allergy are rare in this traditional farming population. Our studies have also shown that at a median age of 2 months, infant stool was enriched with *Bifidobacteria*, *Clostridiaceae and Aerococcaceae* in the OOM compared to Rochester infants from atopic families. *Bifidobacterium longum subspecies infantis (B. infantis)* was more abundant ($p<0.001$) and prevalent, detected in 70% of OOM compared to 21% of Rochester infants ($p<0.001$). Stool colonized with *B. infantis* had higher levels of lactate and several medium- to long/odd-chain fatty acids. In contrast, human milk was enriched with a distinct set of FAs including butyrate, IgA antibodies and a more diverse microbiome. In conclusion, a high rate of *B. infantis* colonization, similar to that seen in developing countries, is found in the OOM at low risk for atopic diseases. Our ongoing birth cohort is assessing the development of infant gut microbiome and metabolome as well as immune system development longitudinally within the first 2 years of life in these populations at different risk of atopic diseases. Understanding the effect of microbiome composition and function on immune development in populations at risk of and

in those protected against atopic diseases can inform us about future prevention strategies to address the allergy epidemic.

The Genetic Basis of Gene Expression in Human Milk and Effects on the Infant Gut Microbiome
Kelsey Johnson, Postdoctoral Scholar, University of Minnesota

Understanding the genetic and genomic basis of lactational variation is critical for identifying the mechanisms linking milk composition and production to infant and maternal health. Transcriptomics is a powerful tool to assess phenotypic variation across individuals at the molecular level. Identifying genetic variants associated with changes in gene expression (cis-eQTLs) can reveal the molecular mechanisms underlying genetic associations with traits like the infant gut microbiome. We performed RNA and whole-genome sequencing using one-month postpartum milk samples from 171 exclusively breastfeeding women, and used metagenomic sequencing to quantify the composition of their babies' gut microbiome. We tested for associations between maternal genotype and milk gene expression (eQTLs) using a linear mixed model. We compared milk eQTLs to those previously identified in 45 other human tissues using statistical colocalization, and tested for associations between maternal genotypes at milk eQTLs and infant gut microbial abundances using linear mixed models. We identified eQTLs for 2,686 genes, of which eQTLs at 668 genes were specific to milk (i.e. not observed in other tissues). Milk-specific eQTLs affected genes encoding milk-specific proteins (e.g. CSN3) but also ubiquitously expressed genes (e.g. circadian gene CLOCK). We observed an association (FDR<5%) between the lead SNP of the milk lactase eQTL and Collinsella, a beneficial microbe in the infant gut. This eQTL is linked to genetic variants that confer lactase persistence in adults. The lactase expression-increasing allele was correlated with decreased infant gut Collinsella. We report the first eQTL study of human milk, elucidating the genetic regulation of gene expression in the lactating mammary gland and placing it in context with other human tissues. We leverage our milk eQTLs to find evidence that changes in milk gene expression could potentially drive variation in the infant gut microbiome. Co-authors: Cheryl Gale1, Dan Knights1, David Fields2, Ellen Demerath1, Frank Albert1, Katherine Jacobs1, Michael Rudolph2, Ran Blekhman1, Timothy Heisel1. Affiliations: University of Minnesota1, University of Oklahoma Health Sciences Center2

Identification of Human Milk-Derived microRNAs Associated with Low Milk Supply, Reduced Infant Growth and Early Breastfeeding Cessation
Shannon Kelleher, Professor, UMass Lowell

To identify milk miRNAs associated with low milk supply in women and ascertain molecular pathways in the lactating mammary gland that are responsible. RNA isolated from foremilk collected from women with low (LMS, n=47) and adequate (AMS, n=123) milk supply was sequenced. Longitudinal changes in miRNA candidates were measured and relationships between milk miRNAs and milk production, breastfeeding outcomes, and infant weight gain, were assessed. Infants of mothers with LMS (20.6 ± 11.8 oz/d) had a lower mean weight-for-length z-score (0.05 ± 1.2) at four weeks compared to infants of mothers with AMS (28.1 ± 15.6 oz/d; p = 0.003) whose mean weight-for-length z-score was 0.50 ± 1.1 (p = 0.013). Mothers with LMS were also more likely to have ceased breast feeding at 24 weeks (38.2%), compared to mothers with AMS (17%; p = 0.0003). Five milk miRNAs were associated with suboptimal lactation; let-7a-5p, let-7g-5p and miR-22-3p levels were higher in milk of mothers with LMS compared with mothers with AMS one week after delivery, whereas miR-16-5p and miR-151a-3p levels were lower. Milk volume remained significantly associated with miR-16-5p (R = -0.14, p = 0.0088), miR-22-3p (R = 0.13, p = 0.011), and let-7g-5p (R = 0.12, p = 0.023) levels throughout 16 weeks. KEGG pathway analysis suggested cell cycle, fatty acid biosynthesis, adherens junctions, Hippo signaling and TGFb signaling were probable target pathways, indicated FUT9, ESR1, ELOVL6, SLC30A4, and IGFBP2 were downregulated, while PAPPA, COL25A1, FASN, and FGF2 were upregulated molecular targets in the mammary gland. Specific miRNAs are differentially expressed in milk from women with low milk supply and may identify molecular

pathways that impair milk production. We propose specific miRNAs may be used as non-invasive biomarkers for predicting risk for low milk supply and early cessation of breastfeeding.
Co-authors: Alexandra Confair1, Desirae Chandran1, Steven Hicks1
Affiliations: Penn State Hershey Medical Center1

Invited Presentation*: SPLASH!® Milk Science Update* - 10th anniversary: Highlights and Upcoming Hot Topics
Danielle G. Lemay, Research Molecular Biologist, USDA, ARS, WHNRC

In April 2012, the IMGC began publishing its translational publication, SPLASH!® Milk Science Update, which features four articles per issue on emerging topics in milk science. The IMGC conference in 2022 marks the publication's 10th anniversary for which 440 articles will have been published! This talk will reveal the editor's choice awards articles published in the previous 12 months. It will also include a behind-the-scenes tour of SPLASH!: who are the current writers and editors, who are our readers and how do they reach our website. The SPLASH! newsletter has helped to grow the IMGC with more than 200,000 annual visits to the website each year. The talk will also cover milk science topics expected to emerge in the coming year and beyond.

Determination of the Effect of High-Pressure Processing Conditions on Bile Salt-Stimulated Lipase Activity in Human Milk
Ningjian Liang, Postdoctoral Scholar, Oregon State University

Milk-derived BSSL ensures efficient milk lipid digestion and is important for the healthy growth and development of infants. Pasteurization of donor milk to ensure microbiological safety is a routine in human milk banks. A complication of this is the denaturation of heat sensitive bioactive proteins such as the BSSL. Alternative technology is needed to ensure the safety of donor milk while preserving BSSL activity. The aim of this study is to determine the impact of different high-pressure processing conditions on BSSL activity in pooled raw human donor milk. Pooled raw human donor milk was processed at 300, 350, 400, 450 and 500 MPa for 1, 3, 5, 7 and 9 min, respectively. Vat-pasteurization was at 62.5°C for 30 min. The BSSL activity was determined by measuring the capacity of the milk samples on converting p-nitrophenyl myristate to p-nitrophenol within the 15 min reaction period. Our results showed that the vat-pasteurization decreased more than 99.0% of the BSSL activity in the milk sample. 300 Mpa for 5 min treatment started to cause a significant decrease (16.19±7.88%) on the BSSL activity compared to the control. Extending the treatment time from 5 min to 7 or 9 min didn't cause further decrease in BSSL activity. 350 Mpa for 1 min caused 18.4±1.7% decrease in BSSL activity compared to the control. Extending the treatment time from 1 min to 9 min didn't cause further decrease in BSSL activity. 400 Mpa for 9 min caused 32.9±0.6% decrease in BSSL activity. No further decrease in BSSL activity was observed when increasing the pressure from 400 Mpa to 450, or 500 Mpa. In conclusion, HPP processing below 500 Mpa and 9 min is an effective technique to preserve BSSL activity in human milk.

Student Award Presentation: Microbiome Based Mastitis Therapeutics
Kevin Linehan, PhD Student, University College Cork

Bovine mastitis is a disease with a multi-etiological nature, defined as an infection and inflammation of the udder. The emergence of antibiotic-resistant, mastitis-causing pathogens has highlighted the need for alternative therapies to treat and prevent the disease. Here, we report findings on two potential alternative treatments, bacteriocins and bacteriophages (phages). Firstly, we discuss a recent a field trial utilising a live bio-therapeutic developed by our group, Lactococcus lactis DPC3147, producer of the bacteriocin lacticin 3147. Twenty eight cows with chronic mastitis were treated with emulsion-based formulations containing either viable L. lactis DPC3147 cells (15 cows) or heat-killed L. lactis DPC3147 cells (13 cows). The efficacies of the two formulations in stimulating a localized immune response (measuring interleukin-8 concentrations in milk) and cure rates (somatic cell counts reductions and pathogen absence) were

evaluated. We demonstrated that the presence of heat-inactivated bacteria (a postbiotic) was as effective as the live bio-therapeutic in eliciting a localized immune response in cows with chronic mastitis. Secondly, we discuss two novel Staphylococcus aureus phages isolated from bovine colostrum (42,222bp) and human breast milk (43,593bp), representing new species of genus Phietavirus and subfamily Azeredovirinae. Both phages are lytic against several human and bovine mastitis causing strains of Staphylococcus aureus (including MRSA). Furthermore, these phages display excellent characteristics for use in vivo experiments, including wide host range, stability to variations in pH (4 to 9), temperature resistant (up to 60 °C) and resistant to isopropanol and chloroform. In vitro characteristic tests, TEM micrographs and whole genome bioinformatics analysis are described. The two novel therapeutics described herein represent potential novel candidates for mastitis treatment in bovines and humans.

Outstanding Legacy Career Award & Keynote Address: Bioactive Milk Proteins and their Impact on Infant Health and Development

Bo Lonnerdal, Distinguished Professor Emeritus of Nutrition & Internal Medicine, University of California Davis

Breastfeeding confers many benefits to the newborn infant. Several proteins in breast milk have been shown to have bioactivities, such as lactoferrin, milk fat globule membrane (MFGM) proteins and osteopontin. Since clinical trials with human milk proteins are difficult due to a limited supply of these proteins, studies with bovine milk protein counterparts showing beneficial outcomes provide proof-of-concept that these proteins in human milk provide valuable bioactivities. *Lactoferrin* is a major protein in breast milk that binds to a specific lactoferrin receptor in the small intestine. In the gut, lactoferrin has bacteriostatic and bactericidal activities and the lactoferrin receptor will facilitate uptake of lactoferrin into the intestinal cell. Internalized lactoferrin binds to the nucleus and affects expression of genes involved in immune function. Clinical studies have shown that bovine lactoferrin can reduce respiratory disease in term infants and sepsis and NEC in preterm infants. *MFGM proteins.* These proteins surround the lipid droplets in human milk. Proteomics have shown many proteins having anti-infective properties. We conducted a clinical trial on infants fed regular formula or formula with bovine MFGM. Infants fed MFGM formula had better cognitive development at 12 months of age than those fed regular formula and there was no difference between them and breastfed infants. We also found that infections were lower in the MFGM group as compared to those fed regular formula and again not different from breastfed infants. *Osteopontin* is involved in immune function and brain development. Osteopontin can trigger signaling events via integrin, a protein in the intestinal mucosa, thereby affecting the immune system. Our clinical trial on bovine osteopontin added to infant formula resulted in an improved cytokine profile and immune parameters as well as less illness, making these infants different from infants fed regular formula and more similar to breastfed infants. In conclusion, human milk contains bioactive proteins that are likely to be involved in the better outcomes of breastfed infants as compared to those fed infant formula.

Bovine Milk-Derived Exosomes as a Novel Injury-Targeting Drug Delivery System

Spencer Marsh, Chief Scientific Officer, The Tiny Cargo Company

A novel protocol provides large amounts of highly purified small extracellular vesicles (also called exosomes) from bovine milk (Marsh et al, PMID: 34367882). We sought to examine targeting of these pure, highly concentrated bovine milk-derived extracellular vesicles (mEVs) to injured cells and tissues. Targeting of mEVs to injured cells and tissues was tested in vitro using a scratch assay on human dermal fibroblast (hDFs) and MDCK cell monolayers, and in vivo, using mouse models of skin wounding and cardiac injury - as we have reported in PMID:34246197; PMID:29351451. mEVs were isolated using our published approach, then fluorescently tagged with Cell Tracker Deep Red (CTDR). Labelled mEVs were then applied to cell cultures at 20 ug/mL for 15 minutes post wound; cells were then rinsed, fixed and stained for cell nuclei. Mice were provided 2 ug/kg loaded mEV's by oral gavage before skin surgery and induction of cardiac ischemic reperfusion injury. Mice were sacrificed 4 hours post-surgery, and fixed by perfusion with 4% paraformaldehyde and PBS rinsing, followed by cryosectioning, staining for nuclei and actin FITC-phalloidin. Imaging was

performed on a Leica SP8 laser scanning confocal microscope and quantification mEV uptake normalized to cell nuclei using ImageJ. mEV uptake was significantly increased in scratch wounded cultures of both Hdefs and MDCK cells ($p<0.001$), over uninjured control cells. Similarly, injured heart and skin tissues exhibited significantly increased exosomal uptake ($p<0.001$), relative to sham injury controls and skin tissues remote from the injury. Our experiments indicate that injured cells and tissues show increased uptake of milk derived exosomes, with in vitro data suggesting that this enhanced uptake occurs, at least in part, in a cell autonomous manner. The data also supports that our isolation protocol provides an mEV-based drug delivery system that may preferentially targets injured or diseased tissues.
Co-author: Robert Gourdie, Fralin Biomedical Research Institute, Virginia Tech

Student Award Presentation: Human Milk Oligosaccharide DSLNT and Gut Microbiome, but Not Breast Milk Microbiome, in Preterm Infants Predicts Necrotising Enterocolitis
Andrea Chiara Masi, PhD Student, Newcastle University

Necrotising enterocolitis (NEC) is a devastating intestinal disease primarily affecting preterm infants. The underlying mechanisms are poorly understood: mothers own breast milk (MOM) is protective, possibly relating to human milk oligosaccharides (HMOs), MOM microbiome, and infant gut microbiome interplay. In a cohort of 49 NEC and 62 control infants MOM microbiome was determined through 16S rRNA gene sequencing, of which HMO profiling of MOM was performed for 33 NEC and 37 matched controls. Longitudinal stool metagenomic sequencing was also performed in a subset of 48 infants (14 NEC; n=644). Finally, bacterial species isolated from preterm stool and probiotic bacteria were tested for their ability to grow on selected HMOs. Concentration of a single HMO, disialyllacto-N-tetraose (DSLNT), was significantly lower in MOM received by NEC infants compared to controls. On the contrary, no difference in MOM microbiome was observed. Metagenomic sequencing of infant stool before NEC onset showed significantly lower relative abundance of Bifidobacterium longum and higher relative abundance of Enterobacter cloacae in infants with NEC. Moreover, infants receiving low MOM DSLNT were associated with reduced transition into preterm gut community types dominated by Bifidobacterium spp. Numerous bifidobacteria isolated from preterm infants showed growth on selected HMOs. The B. infantis probiotic strain was confirmed to be the most efficient HMO utiliser among the bacteria tested, however, only a B. bifidum isolate could metabolise DSLNT. These results underscore the importance of HMOs and gut microbiome in preterm infant health and disease, and that DSLNT may improve outcomes by potentially microbiome-dependent mechanisms. The findings offer potential targets for biomarker development, disease risk stratification, and novel avenues for supplements that may prevent life-threatening disease.
Co-authors: Nicholas D Embleton1, Christopher A Lamb2, Gregory Young3, Claire L Granger1, Julia A Najera4, Lars Bode4, Janet E Berrington1, Christopher J Stewart5
Affiliations: Newcastle Hospitals NHS Trust1, Newcastle upon Tyne Hospitals NHS Foundation Trust2, Northumbria University3,University of California San Diego4, Newcastle University5

Keynote Address: Hot Topics in Human Milk Research – Milk as a Biological System and COVID-19 Update
Michelle (Shelley) McGuire, Professor, Idaho State University

Human milk is universally recognized as the preferred food for infants because it provides not only essential and conditionally essential nutrients in necessary amounts but also other biologically active components instrumental in protecting, communicating important information to support, and promoting optimal development and growth in the infant. Indeed, researcher have long known that milk is a complex fluid containing not only nutrients but also immune factors and cells, hormones, growth factors, and complex carbohydrates. More recent findings also confirm that milk represents a rich source of microbes, including bacteria, viruses, and fungi. Although human milk researchers often

study the presence and impacts of single milk constituents or groups, thereof, emerging evidence suggests that the holistic effects of human milk consumption during early life is not equivalent to simply the sum of its parts. Rather, it is likely that milk acts as a biological system functioning within the even more complex ecologies constituting the mother, infant, and the shared environment. In response, the Breastmilk Ecology: Genesis of Infant Nutrition (BEGIN) Project was launched in 2020 as an effort by federal and non-federal partners and extramural investigators and led by the National Institute of Child Health and Human Development. This project, which was completed in 2022 and will be reviewed and discussed during this presentation, was spearheaded by five working groups and culminated in the drafting of several reports which collectively summarized maternal, infant, and environmental factors impacting human milk composition; milk as a biological system; descriptions of "moonshot" studies that might be conducted to better understand milk's impacts using a systems biology approach; and translation and integration of human milk and lactation science to public health and recommendations. The current state-of-the-science related to human milk, breastfeeding, and COVID-19 – including compositional responses to vaccines – will also be summarized.

Influence of Human Milk Oligosaccharides on the Vaginal Microbiota and Colonization by Neonatal Pathogen Group B Streptococcus

Katy Patras, Assistant Professor, Baylor College of Medicine

Group B Streptococcus (GBS) colonizes the vaginal mucosa of a significant percentage of women and is a leading cause of neonatal bacterial infections. Currently, women are screened in the last month of pregnancy and GBS-positive women are given antibiotics during parturition to prevent bacterial transmission to the neonate. Recently, human milk oligosaccharides (HMOs) isolated from breastmilk were found to inhibit GBS growth and biofilm formation in vitro. Women that make certain HMOs are less likely to be vaginally colonized with GBS, but these associations have not been explored in an experimental model. Using in vitro human vaginal epithelial cells and a murine vaginal colonization model, we tested the impact of HMO treatment on GBS burdens and the composition of the endogenous microbiota by 16S rRNA amplicon sequencing. HMO treatment reduced GBS vaginal burdens in vivo with minimal alterations to the vaginal microbiota. HMOs displayed potent inhibitory activity against GBS in vitro, but HMO pretreatment did not alter adherence of GBS or the probiotic Lactobacillus rhamnosus to human vaginal epithelial cells. Additionally, disruption of a putative GBS glycosyltransferase (Δsan_0913) rendered the bacterium largely resistant to HMO inhibition in vitro and in vivo but did not compromise its adherence, colonization, or biofilm formation in the absence of HMOs. We conclude that HMOs are a promising therapeutic bioactive to limit GBS vaginal colonization with minimal impacts on the vaginal microenvironment. Ongoing work seeks to characterize the impact of HMOs on the human vaginal microbiota using in vitro cultivation of human vaginal communities and gnotobiotic mice colonized with human microbes. These findings will lay the framework to expand our knowledge of therapeutic applications of HMOs and support their continued development as a target for controlling vaginal pathogens such as GBS and promoting colonization by vaginal microbes associated with health.

Student Award Presentation: Simulated Preterm Infant Digestion of Human Milk Processed by High Pressure Processing and Holder Pasteurization Using the TIM-1 Dynamic Model

Michael Pitino, PhD Candidate, University of Toronto

High pressure processing (HPP) and Holder pasteurization (HoP) of donor human milk and subsequent in vitro preterm infant digestion was investigated. Proteins in HPP-treated milk were hypothesized to digest most comparably to raw milk, given HPP minimally impacts composition and bioactivity. Pools (N=3), each produced from 3 unique donors, underwent HPP (500MPa, 10min), HoP (62.5°C, 30min), or kept raw. Dynamic in vitro digestion simulated preterm infant gastrointestinal physiology and samples were collected at 15-, 30-, 45-, 60- and 180-min from various compartments. Semi-quantitative densitometry of native/reduced polyacrylamide gel electrophoresis was used to assess changes in the

protein profile and aggregation. Particle size distribution was determined by dynamic light scattering. Mixed-effects models with post hoc comparisons tested differences among treatments. Irrespective of treatment, gastric protein digestion was limited, except the progressive digestion of bioactive milk fat globule membrane (MFGM) whey proteins and caseins. In the duodenum, caseins were rapidly digested in all treatments and MFGM proteins that resisted digestion in raw and HPP, were absent in HoP milk. Relative lactoferrin in raw milk was higher than HoP ($P<0.03$) at all time points, but not HPP. Undigested lactoferrin and MFGM proteins persisted in the jejunum and ileum in HPP and raw milk. Treatment did not impact digestion of α-lactalbumin. Ileal particle size (D50, number distribution) was significantly larger in raw (80.7±45.9nm) and HPP (119.5±20.7nm) versus HoP (36.3±25.1nm). Ileal outflow particle size in raw (115.3±67.5nm) and HPP (80.3±27.2nm) was also significantly larger than HoP (39.4±11.7nm). While HPP-treated milk preserves undigested high molecular weight MFGM proteins and lactoferrin similar to raw milk, HoP increased proteolysis of these proteins, significantly reducing particle size in the ileum and outflow. HPP is a promising alternative to HoP as it better preserves bioactive components that resist digestion which may benefit recipient infants immunologically.

Co-authors: Sharon Unger1, Alain Doyen2, Yves Pouliot2, Akash Kothari3, Mélanie Sergius-Ronot2, Richard Bazinet3, Debbie Stone4, Deborah L. O'Connor3
Affiliations: Sinai Health Toronto1, Université Laval2, University of Toronto3, Rogers Hixon Ontario Human Milk Bank4

Comparative Profiles of SARS-CoV-2 Spike-Specific Human Milk Antibodies Elicited by mRNA- and Adenovirus-Based COVID-19 Vaccines

Rebecca Powell, Assistant Professor, Icahn School of Medicine at Mount Sinai

Numerous COVID-19 vaccines are authorized globally, employing various platforms. No evidence-based guidance has been provided to lactating individuals as to which vaccine provides a superior milk antibody response that may potentially protect human milk-fed infants from SARS-CoV-2. As such, the objective of this study was to compare Spike-reactive milk antibody profiles in milk obtained from those vaccinated by the mRNA-based Pfizer and Moderna vaccines and the adenovirus-based Johnson & Johnson (J&J) and AstraZeneca (AZ) vaccines. Fifty-four pairs of milk samples were obtained within 1 week before vaccination and 14-35 days after completion of the vaccine regimen, and stored at -80C. Samples were thawed, centrifuged to remove fat and cells, and tested in separate ELISA assays measuring IgA, IgG, and secretory antibody. 86% -100% of mRNA vaccine recipient milk exhibited Spike-specific IgG, which were 12 – 28-fold higher than those measured for adenovirus vaccine recipients. Adenovirus-based vaccines elicited specific milk IgG in only 33%-38% of recipients. Specific IgA was measured in 52%-71% of mRNA vaccine recipient milk and 17%-23% of adenovirus vaccine recipients. J&J recipient milk exhibited significantly lower IgA than Moderna recipients, and AZ recipients exhibited significantly lower IgA titers than Moderna and Pfizer. <50% of milk of any group exhibited specific secretory antibody, with Moderna IgA titers measuring significantly higher than AZ. Moderna appeared to most frequently elicit >2-fold increases in specific secretory antibody titers relative to pre-vaccine samples. These data indicate that mRNA and adenovirus-based COVID-19 vaccines elicit poor secretory antibody titers, which is notable due to the relative durability of this antibody class. Adenovirus-based vaccines weakly elicit antibody in milk compared to mRNA vaccines. mRNA vaccines are preferred for immunizing the lactating population. This study highlights the need to design vaccines better aimed at eliciting an optimal milk antibody response.

Student Award Presentation: Bovine Caseinomacropeptide: Mass Spectrometric Characterization, Digestive Survival and the In Vivo Bioactivity in Adults with IBS

Yunyao Qu, PhD Candidate, Oregon State University

Caseinomacropeptide (CMP) is released from bovine kappa-casein after rennet treatment and is one of the major peptides in whey protein isolate. Previous studies demonstrate that intact CMP has in vitro anti-inflammatory and

antibacterial activities. Yet, the extent to which CMP survived within adults and thus has potential to exert bioactivity remained mostly unknown. Our objective was to develop an analytical method to fully characterize CMP composition and structure, to determine the extent to which intact CMP is digested into peptide fragments within the jejunum of human adults after consumption and to examine the effect of daily CMP consumption on numerous indices of gut health in adults with irritable bowel syndrome(IBS), including the composition of the microbiome, the microbial metabolome, fecal and blood protein markers of inflammation and gut-related symptoms. Whey protein isolate, purified caseinomacropeptide and adult digestive samples were analyzed using mass spectrometry-based top-down glycopeptidomics. Blood and stool from adults with IBS fed CMP were analyzed using, multiplex ELISA and 16S rRNA sequencing microbiome analysis. The liquid chromatography-tandem mass spectrometry spectra of CMPs were annotated to confirm peptide sequence, glycan composition and other post-translational modifications using automatic data processing. Intact CMP was dominant in whey protein isolate and CMP feeding materials. Intact CMP does not survive to the intestine, but releases an array of fragments with potential bioactivity. Results from our study on CMP feeding in adults with IBS are forthcoming. We developed a novel analytical method to comprehensively characterize of the multiple glycosylated forms of CMP within dairy products. We demonstrated that CMP is mostly digested in the human jejunum and released bioactive peptides. Our study on adults with IBS is ongoing. This work will help determine the biological relevance of CMP in consumers and help develop novel products.
Co-author: Jeewon Koh, Oregon State University

2021 Most Valuable Presentation Award: Structural Insights on Oligosaccharides in Commercial Infant Formula Products with Ion Chromatography-Mass Spectrometry
Neil Rumachik, Staff Scientist, Thermo Fisher Scientific
Tian Tian, Staff Scientist, Thermo Fisher Scientific

Interest in functional oligosaccharides has grown substantially because of their profound impact on the gut microbiome as prebiotics. The health benefits of functional oligosaccharides often lead to their supplementation in infant formulas. Understanding the structural complexities in oligosaccharide profiles is crucial, as many structural features are deterministic of their biological function as prebiotics. This study presents a valuable tool combining ion chromatography and mass spectrometry to characterize functional oligosaccharides in infant formula products. Powdered formula products were dissolved in water as per manufacturer instructions on the product label. Samples were diluted with water and centrifuged to defat. The middle aqueous layer was collected. Proteins were precipitated with ethanol at -30°C overnight and separated by centrifugation. After purifying oligosaccharides with porous graphitized carbon, oligosaccharides were analyzed using a Thermo Scientific™ Dionex™ CarboPac™ PA300-4µm analytical column in a Dionex™ ICS-6000 HPIC™ system outfitted with an electrochemical detector operated in the pulsed amperometric detection mode and a Q Exactive™ HF-X hybrid quadrupole-Orbitrap™ mass spectrometer. Before MS injection, the column effluent was passed through a Dionex™ ERD 500 electrolytically regenerated desalter for salt removal. Data were processed with Xcalibur™ software. Ion chromatography-mass spectrometry (IC-MS) was utilized to analyze dairy-based and soy-based formulas fortified with functional oligosaccharides. This method takes advantage of the power of IC to revolve heterogeneous mixtures of oligosaccharides, especially isomeric structures. The high-resolution Orbitrap MS analysis facilitates the sequence and linkage characterization of oligosaccharides. We confidently identified a diverse mixture of oligosaccharides, including 2'-fucosyllactose, sialyllactose, galactooligosaccharides, fructooligosaccharides, and maltooligosaccharides comprising up to 17 monosaccharides units. Structural isomers were well resolved. The IC-MS workflow features easy sample preparation, premium isomer separations, and high-quality MS data for structural elucidation of oligosaccharides, thus, positioning it as a beneficial tool for the qualitative assessment of formula products.

Keynote Address: Colostrum as Nutrition and Therapy in Pediatrics
Per Sangild, Professor, University of Copenhagen

Bovine colostrum (BC) is increasingly used to improve gut function, body health and performance in humans. The high levels of whey and casein proteins, immunoglobulins and other milk bioactives in BC are adapted to meet the needs of newborn calves. However, BC may work across mammalian species, especially when immune and gut functions are immature or compromised in early life. In infants and children such conditions include immaturity or low weight at birth, and postnatal diarrhea, infections, slow growth, gut surgery and mucositis. Both human trials and animal studies (mostly in piglets) have been done to assess the safety and efficacy of BC as an antimicrobial and immunomodulatory nutritional supplement for some pediatric patient groups. The studies suggest that intact BC, or fractions thereof, are safe and effective when supplemented at an optimal age, time and level of intake. However, the specific conditions for inducing benefits, without risk or harm, are poorly defined for all pediatric patient groups. Especially for highly sensitive preterm infants, more research is needed to demonstrate consistent benefits when BC is supplemented to human milk or infant formula. Across patient groups, longer-term exclusive BC feeding, without nutritional adjustments, is not recommended because of the differences in nutrient composition between BC and human milk. On the other hand, adverse effects (e.g. maldigestion, allergies, intolerance, dysmetabolism) are unlikely as long as BC is supplemented within normal nutrition guidelines. Because immunoglobulins and other milk bioactive factors in BC work in synergy with nutrients to improve health and growth, it is critical to preserve bioactivity with gentle processing and pasteurization methods. We conclude that BC may become a safe and effective nutritional supplement for several pediatric subpopulations. However, more research is required to define the optimal feeding and product conditions for the subgroups of infants and children that benefit from BC supplementation.

Invited Presentation: Human Milk Non-Protein Nitrogen is Utilized by Commensal Bifidobacteria and is Interconnected with Carbohydrate Metabolism within the Infant Gut Microbiome
David Sela, Associate Professor, University of Massachusetts Amherst

Human milk provides the nursing infant with molecules critical to infant development, homeostasis, and health. A fraction of these molecules are partially or fully indigestible and thus delivered intact to the infant colon. This includes human milk oligosaccharides (HMOs) and nitrogenous molecules that potentially provide functions beyond their intrinsic nutritive value. These human milk molecules interact with gut microbes to modulate the emergent physiology of the gut microbiome. Human milk non-protein nitrogen, including urea, is relatively poorly characterized with regards to their interactions with commensal microbiota. The genomics and molecular microbiology underlying reciprocal interactions between beneficial bifidobacterial populations and human milk molecules will be presented with an emphasis on HMOs and non-protein nitrogen.

Invited Presentation: Understanding the Function of Milk Components on Infant Health
Carolyn Slupsky, Professor, University of California Davis

Human milk is complex. The period after birth is the time when long-term programming in the neurologic, immune, and metabolic regulatory systems occurs. We have been assessing the impact of different milk components on the metabolic development of infants. Our work is showing that milk components work in concert, and that no single component can rescue a breastfed metabolic phenotype in a formula-fed infant. For instance, we showed that addition of milk fat globule membrane (MFGM) to infant formula resulted in a metabolic shift of formula fed infants toward breastfed infants. Additionally, changing the concentration of protein in a predominantly whey-based formula (80:20 whey:casein) resulted in lowering of insulin, HOMA-IR, as well as serum branched chain amino acids and urea to levels closer to breastfed infants. Reducing protein intake also resulted in increased levels of fecal microbes known to utilize complex carbohydrates. However, despite these changes to these components, altering these components in infant formula still resulted in a distinct formula-fed metabolic phenotype. Our results suggest that more work needs to be done to fully understand how milk components by themselves and in concert with other components impact infant development.

Closing Remarks: The Future of IMGC
Jennifer T. Smilowitz, Faculty Affiliate, University of California Davis

Spend the last few minutes of the symposium with Dr. Smilowitz as she highlights the symposium's sessions and discusses upcoming events on lactation and milk science hosted by the IMGC.

Industrial Large-Scale Isolation and Characterization of Milk Extracellular Vesicles for Utilization in Infant Nutrition
Marie Stampe Ostenfeld, Senior R&D Manager, Nutrition & Health, Arla Foods Ingredients

Milk extracellular vesicles (MEV) are gaining increasing attention due to their cargo of bioactive components, which potentially prime infant health development through recipient cell uptake. The biogenesis of MEVs and milk fat globules (MFGs) follows distinct routes. Thus, profiling of their content and understanding potential differential roles is warranted. Commercial infant formulas are largely depleted for MEVs as compared to human milk, making it timely to explore outcomes of adding bovine MEVs to infant formulas. Bovine MEVs were isolated in a dairy pilot plant production setup using skim milk subjected to acidification for casein precipitation followed by sequential filtration steps. Sub-fractions relatively enriched in MFGs, MEVs, small MEVs/proteins were obtained and analyzed by transmission electron microscopy (TEM) and for whey protein, fat, and miRNAs content. The industrial whey protein concentrate (WPC)-A - MEV fraction was further analyzed for purity of MEV vs. MFGM content using a relative quantification-based mass spectrometry method of tetraspanins (CD9, CD63, and CD81), butyrophilin, lactadherin, and xanthine oxidase. The fractions were used as emulsifiers in an in vitro infant lipolysis model and piglet model to test lipid bioavailability and impact on brain development. A novel industrial acid WPC-based fraction of MEVs was obtained with high MEV:MFGM purity (based on TEM and CD9:BTN MS ratio). The WPC-A-MEV fraction differed in content (proteins, fat, miRNAs) from the other sub-fractions. Emulsification with WPC-A-MEV elicited a significant increased lipolysis rate in vitro, and increased triglyceride bioavailability and hippocampus maturation compared to soy lecithin in neonatal piglets. A MEV fraction was successfully produced from a novel industrial-scale process. This MEV fraction applied as an emulsifier in an infant formula diet led to improved lipid bioavailability and brain hippocampus maturation. The results indicate preservation of unique bioactive MEV components and a potential for milk EVs in next generation infant formulas.
Co-authors: Anette Mullertz1, Jan Trige Rasmussen2, Soren Roi Midtgaard3, Thomas Thymann1, Xiaolu Geng4
Affiliations: University of Copenhagen1, University of Aarhus2, Arla Foods Ingredients3, Copenhagen University4

FinnBrain Birth Cohort Study – Human Breast Milk Metabolites Over 24 Months Lactation Associate with Infant Socio-Emotional Development
Ulrik Kræmer Sundekilde, Assistant Professor, Aarhus University

The objective of the study is to elucidate the human breast milk metabolome and how it changes during lactation in the first two years. Furthermore, we hypothesize that milk metabolites associate with child socio-emotional development. The FinnBrain cohort study population include 3808 women, 2623 fathers and 3837 children. A subset of the population delivered milk; 2-months: 419, 6-months: 178, 14-months: 82, 18-months: 41, 24-months: 19. Milk metabolites are profiled using NMR and 57 metabolites were profiled. Child behavioral symptoms were registered using the parent-report questionnaire Brief Infant Toddler Social Emotional Assessment (BITSEA) used to screen for social-emotional and behavioral problems and developmental delay at infant 2 years. PCA and Wilcoxon rank sum test with FDR correction for multiple testing was used. Secretor and Lewis phenotypes were established based on occurrence of 2FL and LNDFHII. Se+Le+ accounted 75%, Se-Le+ 15%, Se+Le- 9%, and Se-Le- 1%. Secretor status had a significant effect on total HMO identified as lower abundance at 2 months ($p=0.036$), and interestingly in higher abundance at 18 months ($p=0.007$). Le--mothers had significantly decreased total HMOs across timepoints ($p<0.001$). Furthermore, significant differences in non-HMO metabolites were identified between secretor and non-secretors at 2 months. Acetate, aspartate, methionine,

glutamine, leucine, and isoleucine were in significant lower abundance in secretor milk ($p<0.001$), whereas threonine was found in significant higher levels ($p<0.001$). Abundance of DSLNT at two months associated to BITSEA problem score, and when using the BITSEA problem score as classifier the metabolite that changed in the top 25% was lactate. Human milk varies from early lactation to late lactation. For the first time milk metabolites have been associated with infant socio-emotional development. This finding needs validation in a clinical trial to fully understand the mechanism of both lactate and DSLNT in human milk and infant development.
Co-authors: Alex Dickens1, Anna Aatsinki1, Hasse Karlsson1, Henna-Maria Kailanto2, Linnea Karlsson1, Santosh Lamichhane1, Ulrik Kræmer Sundekilde3
Affiliations: University of Turku1, International Flavors & Fragrances2, Aarhus University3

Keynote Address: Developing a Metabolic Taxonomy of Pregnancy and the Newborn-Scientific Rationale and Use Case
Karl Sylvester, Professor, Stanford University

Currently, anthropometrics, morphological measures and calendar-based dating are methods used to monitor the health trajectory of a pregnancy, provide insights to fetal development and project newborn health. Gestational age and birthweight are the principal measures utilized to stratify premature newborns for short-term disease risk. These "blunt" measures as leading indicators of adverse perinatal outcomes provide little pathologic or therapeutic insights. Moreover, age and weight-based risk factors do not reflect a specific underlying biology against which potential mitigating interventions can be measured for efficacy. Accordingly, the Sylvester laboratory and the Stanford Metabolic Health Center has been developing the tools and techniques to comprehensively profile the maternal and newborn metabolome in order to create the basis of a molecular taxonomy of pregnancy and the newborn. Specific use cases to be discussed include the development of a metabolic clock to date pregnancies and to predict adverse events including preterm birth and preeclampsia. Newborn metabolic modeling can be used to estimate "molecular age" of the newborn and risk for acquired disease that primarily effect the premature. Finally, novel mechanistic insights derived from combining metagenomic profiling and targeted functional metabolomics of newborn stool provides insights to the pathophysiology of Necrotizing Enterocolitis and rationale for therapeutic approaches targeting the newborn microbiota.

Human Milk Feeding Reduces Infant Carriage of Antimicrobial Resistance Genes, Even in Antibiotic Exposed Infants
Diana Taft, Assistant Professor, University of Florida

Antimicrobial resistance is a major public health challenge, therefore, we sought to determine if infants exclusively fed human breastmilk carry fewer antimicrobial resistance genes (ARGs) in their microbiome than their formula fed and mixed fed peers, and if this effect is observed even when infants are exposed to prenatal or intrapartum antibiotics. NovaSeq metagenomic sequencing was completed on age 2 months fecal samples from infants in the PREVAIL cohort. ARGs were identified using the MegaRes database. Quasipoisson regression was used to determine if the total number of unique ARGs differed by infant feeding status while adjusting for infant antibiotic use history (including maternal prenatal and intrapartum antibiotics) and sequencing depth. Samples from 205 PREVAIL infants were included. At sample collection, 78 infants were exclusively breastfed (EBF), 83 were partially breastfed (PBF), and 44 were not breastfed (NBF). 137 infants were exposed to prenatal antibiotics, intrapartum antibiotics, or both and 2 infants also received postnatal antibiotics prior to sample collection. Antibiotic use history was not associated with the number of ARGs ($p=0.38$). Compared to EBF, PBF and NBF infants had more ARGs ($p=0.03$ and $p=0.002$, respectively), when adjusting for sequencing depth. EBF infants had a median of 42 ARGs (range 1 to 162 ARGs), PBF infants had a median of 74 ARGs (range 0 to 238 ARGs), and NBF infants had a median of 83.5 ARGs (range 8 to 144). The incident rate ratio of ARGs for PBF vs EBF infants is 1.28 (CI 1.04-1.57) and for NBF vs EBF is 1.42 (CI 1.13-1.79). Exclusively human milk fed

infants carried a reduced number of ARGs compared to formula fed and mixed fed infants, even after adjusting for infant exposure to antibiotics. This suggests that more research into exclusive breastfeeding as a mechanism to control ARG carriage is warranted.
Co-authors: Alex Thorman1, Julia M Quinones2, Shannon Conrey1, David Haslam3, Ardythe L Morrow1
Affiliations: University of Cincinnati1, University of Florida2, Cincinnati Children's Hospital and Medical Center3

Keynote Address: Emerging Prospects of Bovine Milk-Derived Extracellular Vesicles for Arthritis Therapy
Fons van de Loo, Associate Professor, Radboud Institute for Molecular Sciences

There is compelling evidence that bovine milk-derived extracellular vesicles (nanosized lipid bilayer particles) contain an immunoregulatory cargo. To evaluate their potential therapeutic properties for rheumatoid arthritis we tested the oral application of bovine milk-derived extracellular vesicles mEVs) in two arthritis models. In both models, mEVs delayed arthritis onset and reduced cartilage loss and bone marrow edema. Evidence is emerging that mEVs can pass the intestinal tract, reach the circulation, and by this route may directly target cartilage and bone. We recently showed that mEVs exert a protective effect on human osteoarthritic cartilage explants by reducing proteoglycan release and expression of MMP1 and ADAMTS5, two cartilage-destructive enzymes. On bone cell precursors, mEVS accelerated osteoblastogenesis towards osteocytes and mineralization. Additionally, milk EVs and steered the osteoclast differentiation towards the formation of small osteoclasts with less bone resorbing activity. Milk EV treatment indeed increased the number of osteoclasts and osteocytes but the overall effect was less bone loss in both obesity-or ovariectomy-induced osteoporosis in mice. It must be emphasized that the site of action remains to be determined in our in vivo studies. During rheumatoid arthritis the intestinal barrier function is compromised and this makes the intestinal epithelium a prime target for mEVs in arthritis. The most consistent finding on HT29 epithelial cell-line was the induction of interleukin-8 (IL-8) by mEVs. IL-8 is a first response cytokine to environmental changes and a chemoattractant for rapid recruitment of immunosuppressive immune cells such as myeloid derived suppressor cells, dendritic cells, and regulatory T cells (Tregs). Milk EV can induce immune-suppressive Tregs and thereby suppress the osteoclastogenic Th17 cells in arthritis. These observations show the broad therapeutic potential of mEVs to reduce arthritis pathology. To translate this into a success story in humans, a large scale isolation method to obtain pure active mEVs will be pivotal.

Effects of 12-Month Supplementation of Term Infants with Bovine Milk Fat Globule Membrane on Neurodevelopmental Outcome Measured at 12 Months of Age
Bing Wang, Professor of Physiology and Nutrition, Charles Sturt University

Human milk is naturally enriched with milk fat globule membrane (MFGM), which promotes infant cognition and immunity development. We evaluated the neurodevelopment and growth of healthy term infants fed MFGM-enriched formula (MF), over 12 months. A prospective, multi-Centre, double-blind, randomized trial was conducted in Fuzhou, China. Healthy term infants (n= 212), aged <14 days, were assigned randomly to be fed MF or a standard formula (SF) for 6 months and then switched to stage 2 MF and SF formula until 12 months. A reference group (n= 206) contained healthy breastfed infants (BFR). Neurodevelopment was assessed with Bayley-III Scales. At 12 months, the composite social-emotional (+3.5) and general adaptive behavior (+5.62) scores were significantly higher in MF than SF (95% CIs 0.03 to 6.79 and 1.78 to 9.38; P= 0.048 and 0.004, respectively). Mean cognitive (+2.85, 95% CIs -1.10 to 6.80, P= 0.08), language (+0.39, 95% CIs -2.53 to 3.30, P= 0.87) and motor (+0.90, 95% CIs -2.32 to 4.13, P= 0.49) scores tended to be higher in MF than SF. BFR scored higher on Bayley-III than either MF or SF at 6 and 12 months. Cognitive scores were significantly higher in BFR than SF (95% CI 0.05 to 7.20; P= 0.045), but not MF (P= 0.74) at 6 months. Short-term memory was significantly higher in MF than in SF at 12 months (95% CI 1.40 to 12.33; P= 0.008). At 4 months, serum gangliosides were significantly higher in MF and BFR than in SF (95% CI 0.64 to 13.02; P = 0.025). Milk intake, linear growth, body mass, and head circumference were no different between formula-fed groups. MFGM supplementation in early life

supports adequate growth and improves some measures of cognitive development compared to SF, but not BFR in Chinese infants at 12 months of age.

Genetically Altered Milk Exosomes Facilitate Nutrition Research and Drug Delivery
Janos Zempleni, Professor, University of Nebraska-Lincoln

The objective of this research was to develop genetically altered milk exosomes suitable to interrogate milk-dependent pathways (nutrition research) and optimize the delivery of therapeutic cargo encapsulated in milk exosomes (drug delivery). Nanoparticle size analysis, immunoblotting, transmission electron microscopy and near-infrared imagining analysis were used to demonstrate that bovine mammary alveolar MAC-T cells secrete milk exosomes and are amenable to genetic engineering. Lentiviral protocols were used to express fusion proteins in MAC-T cells that localize to exosomes. Proprietary exosome modifications were used to with the goal to alter exosome uptake by bone-marrow-derived macrophages (BMDMs), uptake by glioblastoma multiforme brain tumor cells [(R132H)GBM], and homing to brain tumors in a mouse model of human GBM. Both exosome modifications UNL1 and UNL2 decreased by 50% the uptake of exosomes in BMDM cultures compared to wild-type exosomes ($P<0.05$, $n=3$). Exosome modification UNL3 increased by 40% the uptake of exosomes in (R132H)GBM cell cultures compared to wild-type exosomes ($P<0.05$, $n=3$). Exosome modification UNL2 increased the homing of exosomes to glioma: exosomes accumulated in tumors but not in healthy brains in a mouse model of human GBM. This proof-of-concept study provides experimental evidence that the genetic engineering of MAC-T cells affords investigators a tool to interrogate milk exosome-dependent pathways in nutrition and identify candidate modifications for improving drug delivery by nanoparticles.

Poster Presentations

Metabolomics and Microbiomics Integration Reveal Resilient Milk Metabolome, Microbiota and Infant Metabolism by Association with Pre- Gestational BMI
Julie Astono, PhD Student, Aarhus University

The objective is to investigate the relation between mother's pre-gestational BMI and the human milk (HM) metabolome and microbiome in a multi-omics approach. We hypothesize that infant metabolism and gut microbiota are affected by mother's pre-gestational BMI. The MaInHealth cohort study population include 168 mother-infant dyads. A subset of 60 HM samples collected at 1-month post-partum divided into three pre-gestational BMI groups (n=20); normal weight (BMI 18.5-24.99), overweight (BMI 25-30), and obese (BMI >30) were analysed with the Biocrates MxP® Quant 500 kit using a Triple Quadrupole LC-MS system operated in LC-MS/MS and flow injection analysis mode. Infant urine metabolites were quantified using 1H-NMR spectroscopy. The urine and HM metabolomics dataset were analysed using principal component analysis and univariate statistical test with correction for multiple testing. The bacterial composition of HM and infant feces was determined by 16S gene amplicon sequencing (using an Oxford Nanopore Technologies GridION MK1). Principal coordinate analysis with univariate statistical test using TukeyHSD and distance-based redundancy analysis using Holm correction was used to analyse the microbiome dataset. 431 metabolites were detected across all HM samples including phosphoglycerides, triacylglycerols, and amino acids among others. The number of detected metabolites in each sample ranged from 370-324. 286 metabolites were detected in all samples. Data analysis showed that milk and urine metabolite profiles were independent of pre-gestational BMI. Furthermore, the microbial alpha-diversity in HM did not differ between BMI-groups. Interestingly, we identified that maternal pre-gestational BMI affected infant gut microbiota alpha-diversity. Based on 431 milk metabolites and additional studies performed on samples from the MaInHealth cohort it is suggested that mother's pre-pregnancy BMI does not influence HM metabolite or microbiota composition or infant metabolism. However, infant gut microbiome seems to be associated with mother's pre-pregnancy BMI. Further studies will investigate this association.

Co-authors: Ulrik Kræmer Sundekilde1, Dennis Sandris Nielsen2, Rasmus Riemer Jacobsen2. Affiliations: Aarhus University1, University of Copenhagen2

Compositional Differences Between Plant and Dairy-Based Yogurts

Carolina Battistini, Postdoctoral Scholar, University of California Davis

Dairy yogurt is an excellent source of high-quality protein and contributes to the daily intake of essential nutrients. Yogurt, a potential probiotic food, also has the greatest evidence for health benefits among fermented foods. Nonetheless, plant-based, yogurt-like (PBYL) foods' market-share has significantly increased in the past three years. It has been suggested that PBYL foods are healthier alternatives to dairy. However, comparative studies are needed to examine this possibility because the food matrix composition, structure, and buffering capacity are related to the gastric emptying time, which in turn may influence the viability of probiotics and metabolites produced during fermentation and digestion processes. This study aimed to screen the most popular PBYL foods available in the US and compare their fat and protein contents with different yogurt styles. The sales volume of PBYL foods in the US was obtained between November 2020 and October 2021. The composition of 60 PBYL foods (19 brands) and 79 yogurts (23 brands) from plain, vanilla, and strawberry flavors were collected from the label or online. The most popular PBYL foods in the US were coconut, almond, and soy-based. Overall, coconut-based PBYL foods had a higher fat (4.4g/100g) and lower protein (0.5g/100g) content compared to dairy yogurts. Almond-based PBYL foods contained an average 6.6g fat and 3g protein per 100g. This was closer to whole milk Greek-style yogurts (4.3g fat and 5.5g protein/100g). Soy PBYL foods had similar fat (2g/100g) and protein (4g/100g) levels as low-fat yogurt (1.4g fat and 5.4g protein/100g). PBYL foods are heterogeneous, but almond and soy-based foods share similarities to dairy yogurts. Future studies should examine the digestion and nutrient release profiles and bacterial viability and stability of plant- and dairy-based yogurts to better understand the extent to which these foods are comparable for benefiting human health.

Relationships Among Milk Bioactives, Milk Microbiota, and Mammary Inflammation in Lactating Cows

Laurynne Coates, Postdoctoral Microbiologist, USDA ARS WHNRC
Mary Kable, Research Scientist, USDA ARS WHNRC

We aimed to determine the relationships among milk components (lactose, oligosaccharides (MO) and fatty acids (MFA)), milk microbiota, and somatic cell counts (SCC), in lactating dairy cows. Raw milk samples were collected from dairy cows at three timepoints each, ranging from early to late lactation. Lactose was determined by Fourier transform infrared spectroscopy, MO by nano-liquid chromatography quadrupole time-of-flight tandem mass spectrometry, MFA by fatty acid methyl esters analysis by gas chromatography with flame ionization detection, microbiota by 16S rRNA amplicon sequencing, and SCC (as a measure of mammary inflammation) by flow cytometry. Linear mixed effects modeling, generalized linear mixed effects modeling and repeated measures correlation were employed to determine correlations among milk components, milk microbiota, and SCC. No significant relationships between milk microbial taxa, or microbial alpha-diversity, and SCC were identified. However, there was a negative relationship between SCC and lactose concentration and a positive relationship between SCC and 6'-sialyllactose abundance. Although the microbial diversity was not correlated with MO diversity or MFA diversity, numerous MO, MFA, and lactose were predictors of microbial genera. Some of the most pronounced relationships between MO and milk microbiota were positive relationships between neutral fucosylated MO and mastitis-associated genera, and negative relationships between MO and beneficial genera, with and without lactose abundance as a fixed effect. Conversely, lactose had a negative relationship with Pseudomonas and positive relationship with Lactobacillus. Relationships between SCC, lactose and 6'-sialyllactose suggest a potential role of mammary inflammation in milk composition. The abundance of certain neutral fucosylated MO and unsaturated MFA may affect the abundance of microbes associated with mastitis. The correlation of economically important milk traits with mammary health suggests that we should not optimize for a milk trait in isolation; holistic optimization is needed.

Co-authors: Danielle Lemay1, Kenneth Kalscheur2, Sierra Durham5, Daniela Barile3, David Storms1, John Finley1, Laurynne Coates1, Mary Kable 1, Matthew Picklo1, Naomi Fukagawa1, Peggy Tomasula1
Affiliations: USDA ARS1, U.S. Department of Agriculture2, University of California Davis3

Proteomics Study of Cultivated Bovine Mammary Epithelial Cells Secretome

Jing Che, Ph.D. candidate, Aarhus University
Søren Drud-Heydary Nielsen, Assistant Professor, Aarhus University

In the present study, secretomes from cultivated primary bovine mammary epithelial cells (pbMECs) were analyzed by proteomics to investigate the protein profile of the secreted constituents and assess the presence of milk proteins. PbMECs were collected from mammary glands of cows (one in early-lactation and one in mid-lactation) and cultivated on Matrigel®-coated inserts in a transwell system, where the cells were treated by proliferation media with prolactin or without (control) in the lower chamber, while milk components were secreted into the upper chamber and collected for further analysis. The total protein content of secretomes was determined by the Bradford assay. The proteins were identified and characterized by bottom-up proteomics through SDS-PAGE, in-gel digestion and MALDI-TOF MS, as well as through in-solution digestion and LC-timsTOF Pro MS/MS. Identified individual protein was semi-quantified using label-free quantification. The total protein contents of secretomes were not significantly different between the non-hormone-treated (control) and the prolactin-treated pbMECs. Through SDS-PAGE and MS analysis, clear protein bands were observed and further identified as BSA, alpha-2-HS-glycoprotein, thrombospondin-1, etc. Furthermore, the major proteins identified by MS were confirmed by MS/MS. A total number of 805 and 729 proteins were identified in the secretomes of prolactin-treated pbMECs isolated from early- and mid-lactation cows, respectively, with BSA having the highest proportion. Importantly, the prolactin-treated pbMECs, however, showed secretion of major and minor milk components, including caseins, whey proteins, and various enzymes, in addition to a large range of other proteins. These preliminary findings indicate that the total secretion of proteins is not primarily influenced by the presence of prolactin itself, but rather by inter-animal variations, the lactation stage of cows, or the culture system. The analysis of secretomes indicates that this cell model is useful in the study of milk protein secretion.
Co-authors: Lotte Bach Larsen, Nina Poulsen, Stig Purup, Yuan Ye, Zahra Sattari, Søren Drud-Heydary Nielsen
Affiliation: Aarhus University

Impact of Different Dietary Fiber and Starch Levels on Bovine Milk Oligosaccharide Profiles

Sierra Durham. Postdoctoral Scholar, UC Davis

Bovine milk oligosaccharides (BMOs) have several demonstrated and hypothesized benefits including roles in cognitive development as well as prebiotic and anti-pathogenic activities, making them promising ingredients for infant formulas and nutraceutical applications. Oligosaccharide extraction from bovine milk for nutritional applications is challenged by low BMO concentrations relative to simple sugars like lactose. BMO abundances are known to vary with a cow's lactation stage, breed, and parity, but these characteristics are difficult to modify in existing dairy herds. In contrast, diet modification is an accessible target, and is already known to influence milk lipid, protein, and monosaccharide content. The goal of this study was to determine the impact of modifying the ratio of dietary fiber and starch on milk oligosaccharide abundances. Milk samples were collected from 59 mid-lactation Holstein dairy cattle in a crossover study design that included sampling during a 4-week pre-experimental baseline period and two subsequent 70-day treatment periods in which cows were fed either a low-starch, high-fiber diet or a high-starch, low-fiber diet. Milk samples collected from 2 consecutive morning milkings in the latter half of each period were pooled, and oligosaccharides were extracted, isobarically labeled, and analyzed by nano-liquid chromatography quadrupole time-of-flight mass spectrometry. 19 BMOs were identified across the sample set, including four large fucosylated compounds that are of particular interest due to the greater demonstrated bioactivities of such structures. Higher overall production

of 7 BMOs was found to occur during feeding with the low-starch, high-fiber diet compared to the high-starch, low-fiber diet. Additionally, this study afforded the opportunity to investigate the impact of other factors potentially influencing BMO abundances. Understanding how BMO profiles are impacted by diet will aid in developing dietary formulation practices that will positively impact milk composition and improve their potential for use as functional ingredients.
Co-authors: Danielle Lemay1, Kenneth Kalscheur2, Zhe Wei3
Affiliations: USDA ARC1, U.S. Department of Agriculture2, University of California Davis3

Human Milk Growth Factors and Hippocampal Growth During Infancy
Nneoma Edokobi, Medical Student, Virginia Tech Carilion Fralin Biomedical Research Institute

Human milk contains familiar macronutrients such as fats and carbohydrates that meet the infant's nutritional needs. In addition to these components of human milk, there are also bioactive factors that may impact other aspects of infant development such as neurodevelopment in brain regions including the hippocampus. Bioactive factors in milk shown to impact the hippocampus in animal studies include the growth factors, Neuronal Growth Factor (NGF), Insulin like Growth Factor 1 (IGF-1), and Erythropoietin (EPO). Although these growth factors have been identified in milk, changes in concentration across lactation have not been extensively studied. The goal of this study was to characterize the concentrations of these growth factors in human milk over 24 months of lactation, and to determine whether these concentrations correlate with hippocampal growth during infancy. A total of 420 human milk samples were collected in 280 women across lactation (range: 2 weeks to 3 years postpartum, range of 1-4 samples per individual). Growth factor concentrations were determined using ELISAs. Hippocampal volumes were calculated from automatic atlas-based segmentation procedures at each time point using a multi-atlas approach as implemented in the Auto-Seg software. Linear mixed effects models (lme; R v4.1.0) estimated the relationships of growth factor concentrations across lactation and hippocampal volume longitudinally. Within-participants, concentrations of EPO and NGF increased an avg of 7.4 mIU/ml and 440pg/ml per month respectfully, while IGF-1 decreased avg of 210 pg/mL per month. Average hippocampal brain volume increased as a function of age ($p<.001$), however no statistical correlations between of GF concentrations and hippocampal volume were observed. We found that for this cohort, human milk NGF and EPO concentrations increase throughout lactation, while IGF levels decrease. This study also suggests that there is not a relationship between the levels of growth factors in human milk and infant hippocampus size.

Yogurt Shapes the Gut Microbiota and Benefits Bone Mineralization in Ovariectomized Rats
Weiwei He, PhD Student, Aarhus University
Henrik Jørgen Andersen, Professor, Arla Foods amba

Being rich in calcium, intake of dairy products is beneficial to prevent bone loss in postmenopausal women, but recent research supports that dietary fibers also exert this capability. Milk matrix (e.g. casein micelles) is likely to benefit the controlled release of calcium into the intestinal lumen, thereby increasing calcium fractional absorption. Both yogurt and dietary fibers may improve bone homeostasis in postmenopausal women by modulating gut microbiota. However, the mechanisms underpinning these effects are scarce. This study aimed to compare the effects of milk, yogurt, and yogurt-inulin combination on the gut-bone associations. A 6-week dietary intervention study was conducted in ovariectomized rats. Calcium content, inulin, and milk/yogurt addition were the main differences in composition among the diets. Milk supplementation did not influence bone mineral density and content (BMD and BMC), femur mechanical strength, or femoral microstructure as compared to positive control receiving similar calcium dose from a calcium carbonate source, whereas yogurt supplementation significantly increased spine BMD. The serum metabolome revealed that yogurt also modulated endogenous glycine-related pathways with reduced concentrations of serum glycine, serine and threonine. No additive effects of yogurt and inulin were observed on the investigated bone mineralization parameters. Correlation analysis showed that increased lactobacilli and reduced Clostridiaceae members in the gut of the yogurt-supplemented group was linked with an increased spine BMD, while increases in some bacteria

(Bifidobacterium pseudolongum, Turicibacter, Blautia, and Allobaculum) and gut short-chain fatty acids in the yogurt-inulin supplemented group were not reflected in measured bone parameters. The present study demonstrated that yogurt intake changed the gut microbiota composition, serum metabolites related to glycine-related pathways with a concomitant increased spine BMD, suggesting that yogurt as a vehicle is superior to milk in terms of securing optimal conditions for bioaccessibility of calcium to enhance bone mineralization.
Co-author: Hanne Christine Bertram, Aarhus University

Using Untargeted Metabolomics to Understand the Metabolic Fingerprint of Human Milk
Zack Holmes, Bioinformatics Scientist, BIOMILQ

Human breast milk is a nutritionally and functionally complex biological fluid that varies in composition based on both maternal and infant health status, genetics, and environment. Characterizing breast milk composition using a metabolomics approach allows for a comprehensive understanding of the human milk phenotype and can provide insight into mammary physiology and the metabolic drivers of milk biosynthesis. While several studies using both NMR and MS approaches have been published on the human milk metabolome, these previous studies have primarily had the express analytical goal of comparing differences between groups, often among otherwise homogenous populations. The goals of this work are to characterize the metabolic profile of human milk from a diverse group of participants and to establish a reference for human milk compositional variability. In this study, breast milk samples were collected from 35 participants from diverse racial and ethnic backgrounds who represent different stages of lactation and diverse diets and were analyzed using untargeted UPLC-MS/MS. We demonstrate computational approaches to conceptualize the magnitude and meaning of compositional variability in human milk and to agnostically explore associations between metabolites and maternal and infant health history, lifestyle, and environment. This work provides insight into the scale of compositional variability of human milk from diverse participants and provides insight into the drivers of the human milk phenotype.
Co-authors: Claire O'Brein, Katariina Koivusaari, Leila Strickland
Affiliations: BIOMILQ

Human Milk Oligosaccharide to De-Inflame the Gut in Patients with End Stage Renal Disease
Sourabh Kharait, Founder/CEO, IGH Naturals

Constipation is very prevalent in patients with chronic kidney disease (CKD) and likely contributes to significant gut dysbiosis leading further to inflammation and its untoward complications such as poor cardiovascular health. Therapies to target constipation in CKD have limited data to assess if these improve gut and other functional outcomes. Current therapies such as lactulose can alter the gut microbiome further and may not have significant long-term benefits in this patient group. We assessed if 2'-fucosyllactose or 2'-FL (Human milk oligosaccharide) can be used to reduce constipation in patients on hemodialysis and if this can lead to a reduction in serum inflammatory markers. Five patients with end stage renal disease on hemodialysis who had constipation were advised to consume 2'-FL at a dose of 2 grams/day as a commercially available supplement. GI tolerance and consistency of bowel movements were tracked. Serum inflammatory markers (ferritin, C reactive protein (CRP)), hemoglobin and albumin were tracked weekly for a total of 3 weeks during the treatment. All 5 patients tolerated 2'-FL without any untoward effects and all had relief in constipation (at least one BM a day). 2'-FL significantly reduced inflammatory markers, both CRP and serum ferritin from baseline by 58% and 32% respectively by the end of week 3. Serum hemoglobin and albumin (marker of nutrition status) remained stable throughout the course of therapy and showed a slightly improving trend. 2'-FL seems to be well tolerated in patients with CKD on dialysis and can improve constipation as well as systemic inflammation. This can have beneficial effects on cardiovascular health, anemia and protein energy malnutrition. This preliminary study serves as a rationale for

a randomized control trial designed to clearly delineate the benefits of HMOs on the gut health and microbiome of patients with CKD.

Effects of Antibiotic Treatment on Cow Teat Skin and Milk Microbiota at Dry- Off
Mateus Lemos, PhD Student, UC Davis

Intrammary antibiotic therapy at dry-off is a common practice in intensive dairy farming systems to treat and prevent mastitis. However, there is the need to reduce antibiotic use due to antibiotic-resistance spread and because antibiotics may result in lasting negative consequences to the cow microbiota and milk quality. Therefore, we aim to understand the microbial changes of both milk and teat microbiota caused by antibiotic use over time after dry-off. For three dairies in California, teat swabs and milk samples were collected from cows with low and high SCC and given either Cephapirin, Ceftiofur, or no antibiotics at dry-off. Samples were collected at dry-off, 7 days later, and 6 weeks after the start of the next lactation period. Swabs and milk were processed for DNA extraction, high-throughput 16S rRNA gene DNA sequencing, and microbial community analyses. Corynebacterium (96.8% of all samples), Acinetobacter (85.5%) and Streptococcus (82.7%) were commonly present on the teat skin. Antibiotics had a minor effect on the teat microbiota at all time points. Location and time of sampling had the highest impact on the bacterial diversity in the teat microbiota (PERMANOVA, R2=0.05 and 0.11, p=0.001 respectively). By comparison, bacterial alpha-diversity was reduced in milk samples from cows with high SCC. The data also indicate there was an increase in bacterial diversity in the milk at 6 weeks after the start of the next lactation period for the antibiotic treated cows. Antibiotic treatment changed the bacterial diversity in milk, even though it had only a limited effect on the teat microbiota. A better understanding of the impact of antibiotic treatment for cows at dry-off may lead to better management practices to sustain cow health and milk quality. Co-authors: Ashley Niesen, Heidi Rossow, Wannes Van Beeck. Affiliation: University of California Davis.

High-Pressure Processing Preserves Bioactive Proteins in Human Milk to a Higher Extent Compared with Holder Pasteurization
Ningjian Liang, Postdoctoral Scholar, Oregon State University

Human milk contains many bioactive proteins which have numerous functions. Preterm infants are often fed donor milk as mothers frequently cannot provide a sufficient volume of their own milk for the infant's nutritional needs. However, preterm infants fed donor milk often have poorer outcomes than those fed mother's milk. To ensure microbiological safety, donor milk is Holder pasteurized by donor milk banks, but pasteurization degrades and denatures many of the bioactive milk proteins. The objective of the current study is to examine the effects of HPP on preservation of the milk bioactive proteins' structure and function. conditions on the structure of bioactive proteins in donor milk. Pooled donor milk was treated at Pooled raw human donor milk was processed at different HPP conditions. Preservation of lactoferrin, immunoglobulins (sIgA/IgA, IgG, IgM), osteopontin, polymeric immunoglobulin receptor (PIGR), α-lactalbumin, lysosome, vascular endothelial growth factor (VEGF), elastase were examined. 350 Mpa for 9 min significantly ($p<0.05$) decreased 24.9%, 12.1% and 39.5% of IgA, IgM and IgG, respectively, in donor milk. 400 Mpa for 9 min decreased 41.1%, 17.4% and 39.9% of IgA, IgM and IgG, respectively. 500 Mpa for 9 min decreased 43.6%, 53.2% and 58.6% of IgA, IgM, and IgG, respectively. Vat-PT decreased 66.2%, 73.2%, and 68.8% of IgA, IgM, and IgG, respectively. 300, 350, 400, 450, 500 Mpa for 9 min significantly decreased 21.9%, 22.7%, 19.6%, 46.5%, and 48.7% of elastase, whereas, vat-PT decreased all the elastase. All the tested HPP conditions did not significantly ($p<0.05$) decrease PIGR or osteopontin in donor milk, whereas vat-PT decreased 39.2% of PIGR and 48.4% of osteopontin. All the tested HPP condition and vat-PT preserved all the lysozyme, VEGF, and α-lactalbumin. High-pressure processing preserves a larger array of bioactive proteins in human milk to a higher extent compared with vat-PT.
Co-authors: Hussein Mohamed, Joy Waite-Cusic
Affiliation: Oregon State University

Antibiotic Administration in the Context of Iron Deficiency is Associated with Substantial Changes in Metabolism
Shannon Mcclorry, Postdoctoral Scholar, University of California Davis

Iron deficiency (ID) is the most common micronutrient deficiency worldwide. During infancy, a high-risk period due to low iron concentrations in milk, ID has been shown to increase susceptibility to infection. Due to the high burden of infection in children, antibiotics are often prescribed with an estimated 1.3 prescriptions/year/child in the United States. Here, we utilized a piglet model of ID to assess the alterations in systemic metabolism associated with ID and antibiotic administration in the context of ID. Piglets were litter-, sex-, and weight-matched then randomized into either control (CON; n=20), ID (n=10), or ID plus antibiotics (ID+Abx; n=10), and in a separate experiment piglets were randomized into control (CON*; n=6) or antibiotics only (Abx; n = 6). Piglets were allowed to suckle from their sow until PD25, then weaned to their assigned diets (CON, [Fe]=100 mg/kg; ID and ID+Abx, [Fe]=10 mg/kg; CON* and Abx, [Fe]=143 mg/kg) until PD43. Both Abx and ID+Abx piglets received antibiotics on PD34-36. Body weight, hemoglobin, and hematocrit were assessed throughout. Blood metabolites at weaning and sacrifice were assessed using 1H-NMR metabolomics. ID and ID+Abx piglets exhibited growth faltering and had lower hemoglobin and hematocrit compared to CON throughout. Prior to antibiotic administration, the overall metabolome of ID and ID+Abx piglets was similar, exhibiting elevated markers of oxidative stress compared to CON. After antibiotic treatment, Abx and Con* piglets had a similar metabolome; however, ID+Abx piglets exhibited a stronger metabolic response to the ID, with further increases in oxidative stress compared to ID piglets. Administration of antibiotics in the context of iron deficiency has a profound impact on the metabolome that persists at least a week after the conclusion of the antibiotic treatment. These results highlight that iron status in infants may need to be assessed regularly.
Co-authors: Peng Ji1, Mariana Parenti1, Carolyn Slupsky1
Affiliations: Department of Nutrition1, Department of Food Science and Technology2, University of California Davis

Quantification and Pathway Interplays of Processing-Induced Protein Modifications in Milk Proteins
Søren Drud-Heydary Nielsen, Assistant Professor, Aarhus University

Understanding the interplay between proteins and the food matrix molecules on protein changes occurring during processing and storage of milk proteins with impact on nutritional value. A liquid chromatography-mass spectrometry method using multiple reaction monitoring (MRM) acquisition was developed for absolute quantification of Maillard reaction products furosine, (N-ε-(carboxyethyl)lysine (CEL) and N-ε-(carboxymethyl)lysine (CML)), dehydroalanine (DHA) mediated protein cross-links (lanthionine (LAN) and lysinoalanine (LAL)), and the total lysine content. The method was applied to study micellar casein (MCI) and whey protein isolate (WPI) heated in presence or absence of lactose. As well as a storage experiment of UHT milk based on raw milk with different micelle size in relation to formation of sedimentation. In the model study, the Maillard reaction markers furosine, CEL and CML increased as expected during heat treatment in the presence of lactose, whereas the LAL and LAN increased in in both MCI and WPI both in presence and absence of lactose, although at lower levels in presence of lactose. The level of LAL and LAN was reflected by the amino acid composition of MCI and WPI, where the DHA and cysteine derived LAN was more abundant in WPI compared to the cysteine-poor MCI. All processing-induced markers increased in UHT milk during the 6-month of storage. In the corresponding sediment samples, a higher proportion of late-stage Maillard reaction markers and LAL was measured and in general, the milk pool with larger casein micelles developed a higher level of processing-induced protein modifications. The results confirm an existing competing state of the two pathways measured as well as an impact of amino acids, the food matrix molecules, and the native characteristics of the casein micelles. These results are important to understand the extent and driving forces of lower protein quality and nutritional value of milk after processing.
Co-authors: Lotte Bach Larsen, Nina Poulsen, Lotte J. Knudsen, Miguel A. Toro
Affiliation: Aarhus University

Integrative Analysis of Mammary Gland and Human Milk Cell scRNA-Seq Data
Sarah Nyquist, Post Doctoral Fellow, Gladstone Institute

During the process of lactation, live human cells are exfoliated into human breast milk (hBM) and can be used as a proxy in the study of the composition of the human lactating breast. Recent work has used single cell RNA-Seq (scRNA-Seq) to study the transcriptional profile of cells in hBM, but without direct comparisons to tissue from a human lactating breast using these methods, the potential of these cells to represent the composition of the lactating breast remains uncertain. We aimed to mine existing scRNA-Seq datasets to describe the potential and limitations in using hBM cells to study the lactating breast on a cellular level. Unlike human breast, the tissue composition of mouse mammary glands have been assayed via scRNA-Seq at all stages of life including the course of lactation. Through computational integrative analysis of scRNA-Seq data from human milk, human non-lactating mammary gland, and mouse lactating mammary gland, we investigate the similarities and differences in the cells derived from these compartments. We list the cellular phenotypes shared between human non-lactating breast tissue, cells exfoliated into breast milk, and mouse lactating mammary gland. We also describe potential avenues for direct comparison of mammary gland cells from different species through direct data integration and gene expression comparisons of key genes. The use of cells from human milk as a proxy for the study of cellular activity during lactation could allow for extensive and minimally invasive study of the influence of a wide variety of individual factors on the activity of the cells responsible for lactation -- for example, gene expression signatures that vary with milk production capacity, risk of breast cancer, individuals taking hormonal birth control, and many others. These results point towards promising avenues and limitations for the uses of these cells in future studies.

Thermal Stability of Milk Glycosidases and their Activities in Industrial Whey Preparations
Nina Poulsen, Associate Professor, Department of Food Science, Aarhus University

The study aimed to investigate the thermal stability of milk glycosidases, as well as assess the remaining activities of natural milk glycosidases in five commercial whey based preparations. Nine glycosidases (α-mannosidase, α-glucosidase, β-glucosidase, β-glucuronidase, α-fucosidase, NAGase, α-neuraminidase, α-galactosidase and β-galactosidase) were included in this study. Glycosidase activities were measured by assays based on 4-methyllumbelliferone. Unprocessed bulk milk was transferred to polymerase chain reaction (PCR) tubes and heat-treated using a PCR system. Milk samples were exposed to temperatures in the range from 45 ^{o}C to 90 ^{o}C with a holding time of 5 s to 120 s as well as assessing remaining activities of natural milk glycosidases in five commercial whey based preparations. The study revealed α-mannosidase and β-glucuronidase to be the most heat resistant glycosidases, which were still active after 80^{o}C/120 s, or 75^{o}C/30 s, respectively, and with inactivation energies (Ea) of 644.3 kJ/mol and 405.9 kJ/mol, respectively. Particularly α-mannosidase and α-glucosidase were found to remain active in the investigated whey protein- and whey fat concentrates after exposure to heat and ultrafiltration. In contrast, almost no activities remained in whey protein isolate or enriched whey protein concentrates due to the high purity of these products. No α-neuraminidase activity was detected in the whey products, and only low activity of α-fucosidase, which indicate low degradation of bioactive sugars containing the functional residues α-fucose and sialic acid. Our study highlights the significance of glycosidases in milk and reveals certain glycosidases to be active in whey preparations, which have been exposed to ultrafiltration as well as heat treatment processing steps. The glycosidase with the highest thermostability in the present study was the α-mannosidase. In particular, both α-mannosidase and α-glucosidase were found to be active in the studied whey-based preparations.
Co-authors: Lotte Bach Larsen1, Anne Vuholm Sunds2, Ida Schwartz Roland1
Affiliations: Aarhus University1, Arla Foods Ingredients2

Towards Cellular Milk: In-Vitro Induction of Lactogenesis and Comparison of Lactogenic Agents: an Optimization Step
Nina Poulsen, Associate Professor, Department of Food Science, Aarhus University
Zahra Sattari, PhD Candidate, Aarhus University

Cellular agriculture utilizes biotechnology to produce an abundance of animal products to sustainably feed a growing population. This study uses bovine primary mammary epithelial cells (bpMEC) to optimize of an in-vitro model for

production of the milk components. To induce lactogenesis in cells, prolactin or Bovine Pituitary Extract (BPE) can be used. These agents were compared by examining the expression of genes related to milk protein content and synthesis. bpMECs were isolated from the mammary tissue of a healthy cow upon slaughter. The cells were proliferated to passage 5 and their homogeneity was tested using flow cytometry. They were cultured on coated inserts of a trans-well system and treated with growth media (GM) until confluent. The cells were then treated for 4 days with differentiation media (DM) added to the lower compartment to simulate the blood-milk barrier. The DM was replaced daily and had the same composition as the GM except for being devoid of FBS and containing either BPE or prolactin. Finally the cells were lysed and gene expression measured for the following genes, using RT-qPCR: keratin 8, ribosomal protein S6 kinase, β-casein, κ-casein, αS1-casein, α-lactalbumin and fatty acid synthase. Data was analyzed as a one-way anova using treatment as a fixed effect. More than 90% of the cells were positive for the epithelial marker cytokeratin 18. Treatment with BPE significantly increased the expression of κ-casein and αS1-casein ($p \leq 0.001$) compared to the control and prolactin treatment. BPE also increased the expression of β-casein by 1.4 times. The BPE contains a variety of growth factors and hormones. It increased the expression of three milk protein genes in bpMECs compared to prolactin. Therefore, we selected BPE as the lactogenic agent for the study on development of an in-vitro model for milk component synthesis.
Co-authors: Lotte Bach Larsen, Nina Poulsen, Stig Purup, Yuan Yue, Rikke B. Kjærup
Affiliation: Aarhus University

The Interaction Between Bacteria and Milk Lipids is Affected by the Size of the Milk Fat Globule
Chen Raz , PhD Student, The Hebrew University of Jerusalem
Nurit Argov-Argaman, Professor, Hebrew University of Jerusalem

Milk lipids are organized in milk fat globules (MFG), ranging in size from 0.2 to15µm. MFG size is closely associated with its composition of fatty acids, polar lipids, sphingolipids, cholesterol and glycoconjugates. Differences in composition, together with the greater surface area of small MFG, was hypothesized to affect MFG interaction with microbial cells. Hence, we aimed to elucidate the role played by size in modulating the interaction between the MFG and the probiotic bacteria, Bacillus subtillis. Small (2.3µm) and large (7.0µm) MFG fraction were separated from raw milk of dairy cows. After pasteurization, the fractions used as a substrate for B. subtilis for 24h at 23oC. Bacteria growth was determined at 0, 2,4,6,12 and 24 h after incubation.Colony type biofilm formation was determined after incubation of bacteria with small or large MFG at 37oC for 5h on agar plates. Bundle biofilm formation measured using B. subtilis strain which harbors gene coding to cyan fluorescent protein under control of tapA promotor. A pro-proliferative effect was detected for the small MFG, expressed in a log difference in the number of B.subtilis cells compared with large MFG, and compared with skim milk or ultra-heated-homogenized milk. Additionally, specific phospholipids were added to the large MFG treatment to match their concentration to that found in the small MFG treatment. We found that supplementation of phosphatidylethanolamine to the large MFG treatment enhance the proliferation of the bacteria to a comparable level to that of the small MFG treatment. Accordingly, phosphatidylethanolamine supplementation to the large MFG suppressed biofilm formation of the bacteria. We suggest that the presence of polar lipids, closely associated with MFG size, governs the ability of B subtilis to utilize nutrients from milk fat, and affects the decision-making process leading to either biofilm formation or extensive bacterial growth.

Impact of Mammalian Milk Oligosaccharides on SARS-CoV-2 Infection In Vitro
Ishita Shah, Assoc. Proj. Scientist, University of California Davis

The SARS-CoV-2 pandemic continues to challenge health care and economic systems across the globe. It is imperative to have strategies to prevent and treat COVID-19 and develop effective therapeutics and prophylactic solutions for future viral threats. Shaped by millions of years of evolution, mammalian milk as the complete food for the newborn is both nutritive and protective. In fact, milk serves as mother-derived molecular PPE (Personal Protective Equipment) for the newborn. The viral protection afforded to infants through human milk creates an excellent landscape to look for specific

biomolecules that enable prevention of disease from a number of viral challenges. One such group of molecules are the prebiotic complex human milk oligosaccharides (HMOs). HMOs act as host cell receptor decoys and deflect pathogens away from the host epithelium. While evaluating the impact of HMOs on gastrointestinal tract cell lines, we recently discovered that HMOs also directly augment epithelial barrier function to alter bacterial pathogenesis and inflammation in intestinal epithelial cells. In infants, some HMOs are absorbed into the peripheral circulation potentially reaching all organs, including the lungs. Additionally, during consumption of human milk, HMOs bathe the laryngopharyngeal region and therefore could have a role in respiratory epithelial support including but not limited to barrier function improvement. We hypothesize that via receptor-decoy and barrier function modulation, HMOs will prevent and/or reduce the severity of viral infections in the lungs. Here, as proof-of-concept, using alveolar and bronchial epithelial cells, we show that human coronavirus HCoV-229E infection is dramatically altered in the presence of HMOs, specifically 2'-fucosyllactose (2'-FL) and 3'-sialyllactose (3'-SL). These HMOs improve barrier integrity resulting in reduced virus-mediated cell death and alteration in virus-dependent interferon signaling. Importantly, we also show that select HMOs led to a reduction in SARS-CoV-2 infection in lung epithelial cells as determined by viral titers during an infection assay. We propose that current, commercially available, HMOs can be repurposed via a novel functional formulation for direct delivery to nasopharyngeal and intra-bronchial epithelial surfaces that may elicit a protective response to SARS-CoV-2 infection. A community-wide application inspired by mothers' milk that is safe and scalable may serve to reduce health issues from virus-mediated respiratory diseases, for prophylaxis and possibly as therapeutics in early disease. This novel approach could result in a paradigm-shift in protection from future viral outbreaks, including the yearly flu, seasonal allergies and asthmatic symptoms.
Co-authors: Ana Maria Stoian1, David Mills1, Maciej Chichlowski2, Ruth Simmons2, Stefan Rothenburg1
Affiliations: University of California1, Reckitt2

Bacterial Load Ingested from Mother's Milk Increases Over Time among Hospitalized Very Low Birth Weight Infants
Sara Shama, PhD Candidate, University of Toronto

Mother's milk contains microbes with the potential to colonize the very low birth weight (VLBW, <1500g) infant gut. However, to support in-hospital growth, mother's milk is frequently nutrient-enriched with human (HMBF) or bovine milk-based fortifiers (BMBF), which are known to differentially displace the volume of mother's milk fed to infants. Currently, the bacterial load from mother's milk delivered to VLBW infants is unknown, particularly in the context of fortification. To determine the bacterial load ingested from mother's milk for hospitalized VLBW infants over time and in the context of different fortifiers. Human milk-fed infants (n=97) randomized to either a HMBF or BMBF in the OptiMoM Fortifier Study (NCT02137473) were included. Weekly mother's milk samples (n=400) were prospectively collected from birth until 8 weeks. The normalized abundance of the bacteria in milk (Log10 16S rRNA gene copies/mL) was determined from V4-16S rRNA gene sequencing and qPCR. Bacterial load ingested for each infant was calculated using these normalized abundances and the daily volume of mother's milk fed. Infants received a mean total bacterial load of 7.5 (SD ± 0.7) log copies from mother's milk, which increased over time (PFDR<0.0001); the temporal increase in bacterial load was more rapid among BMBF- versus HMBF-fed infants (p=0.04). Bacterial load of predominant genera in mother's milk (Staphylococcus, Acinetobacter, Pseudomonas, Corynebacterium, Streptococcus) also increased over time (PFDR=0.0017-0.0001) and was generally higher among BMBF-fed infants (PFDR=0.002-0.0001). Bacterial load of common vertically-transferred genera identified from the literature (Lactobacillus, Bacteroides, Bifidobacterium, Rothia) did not change over time (PFDR>0.05) and showed varied relationships with fortifier type. Bacterial load ingested from mother's milk increases over time and changes in response to fortifier displacement. Future studies are needed to establish how bacteria ingested from mother's milk shapes the microbial colonization of VLBW infants.
Co-authors: Adrianna Greco1, Akash Kothari1, Alain Stintzi2, Alex Kiss3, Amel Taibi1, Christopher Tomlinson4, Deborah O'Connor1, Elena Comelli1, James Butcher2, Julia Copeland1, Michelle Asbury1, Nicole Bando1, Pauline Wang1, Phillip Sherman1, Sharon Unger1

Affiliations: University of Toronto1, University of Ottawa2, Sunnybrook Research Institute3, The Hospital for Sick Children4

The Immunomodulatory Effect of Mesenchymal Stem Cells on Mammary Epithelial Cells Does Not Require Physical Contact

Lior Sharabi, MSc Student, The Hebrew University of Jerusalem
Nurit Argov-Argaman, Professor, Hebrew University of Jerusalem

Mesenchymal stem cells (MSCs) were shown to exert an immunomodulatory effect on different organs (e.g., lungs, heart, kidneys, and liver). These effects appear to be achieved by paracrine signals, including extracellular vesicles and cytokines released by MSC. We sought to study if such an effect is exerted in the context of lactating mammary epithelial cells (MEC) and whether these properties can change the production traits of these cells. Condition Medium (CM) was prepared from bovine Umbilical Cord-MSCs or from Bovine Fetal Fibroblasts (BFF, control), and was used as a pre-treatment for MEC in a basal or stressed conditions (5h incubation with 0, 1, 10µg/mL LPS). After treatment, the expression of genes encoding inflammatory cytokines (e.g., TNFα, IL-6), phase II enzymes (e.g., NRF2), proliferation-associated enzymes (e.g., HIF1α), and synthesis enzymes of milk fat (e.g., DGAT-1, ACACA, FASN, DES9), milk proteins (e.g., β-Cas) and milk carbohydrates (e.g., αLac) were determined. Under non-stressed conditions, MSC-CM reduced the expression of inflammatory cytokines (TNFα and IL-6) by 2-fold and increased the expression of NRF2 and HIF1α by 1.6-fold compared with control. Under stress conditions, MSC-CM pre-treatment decreased gene expression of TNFα and IL-6 by 1.3 and 3.6-fold, respectively ($P<0.0001$). While BFF-CM pre-treatment increased the expression of NRF2 and HIF1α upon exposure to LPS, no changes were recorded following MSC-CM pre-treatment. Interestingly, independent of LPS, MSC-CM pre-treatment increased the expression of lipogenesis-associated genes such as DES9 and FASN by 2.7 and 2.5-fold, respectively, compared with control ($P<0.0001$). Our results suggest that MSC-CM suppressed the inflammatory response of MEC to LPS and enhanced the lipogenic capacity of these cells. In-depth research is required to shed light on the mechanism of action responsible for the immunomodulatory and pro-lipogenic effects of MSC, as well as to decipher the CM's composition and reveal its active components.

Co-author: Sharon Schlesinger, Hebrew University of Jerusalem

Relative Abundances of Proteases, Inhibitors, Glycosidases and Glycosyltransferases in Human Milk in Relation to Lactation and Secretor Status

Martin Thesbjerg, PhD Student, Aarhus University

The purpose of this study was to investigate enzyme systems of proteases/protease-inhibitors and glycosidase/glycosyltransferases in the human milk proteome during early lactation and between secretors and non-secretors. Milk from six donors at two time points (months 1 and 3) was used; three donors represented secretors and non-secretors each. Human milk proteins were precipitated by ice-cold acetonitrile, digested with LysC/Trypsin, desalted using SPE columns before nLC TimsTOF Pro MS/MS analysis. The spectral data were searched using FragPipe. Sub-lists of enzymes and inhibitors were identified by functional analysis of the identified proteins using Uniprot.org. Across all donors, 1211 proteins were identified with their relative abundances. Total number of identified proteins per donor milk sample ranged from 809 to 352. Milk from month 1 had more identified proteins (647.5 ± 113.1) than milk from month 3 (487.5 ± 112.8). Secretors had more identified proteins (645.6 ± 113.1) than non-secretors (489.3 ± 98.4). Principal coordinates analysis revealed a separation of lactation stage, secretor status overlapped at month 1 only. A total of 44 proteases and 25 protease inhibitors were identified, among these were cathepsins D, B and G, metalloproteinase inhibitor 1, α-1-antichymotrypsin, α-1-antitrypsin and cystatin-C. The total relative abundance of proteases was stable across the dataset, with a slight decrease in relative abundance of protease inhibitors from month 1 (3.50 ± 1.00%) to 3 (2.89 ± 0.70%). In addition, 10 glycosidases and 5 glycosyltransferases were identified, among these were Lysozyme C, Neutral α-glucosidase AB, β-1,4-galactosyltransferase and α-N-acetylgalactosaminidase α-2,6-sialyltransferase 1. The relative abundance of glycosidases was significantly higher in non-secretors (1.17 ± 0.42%) than in secretors (0.58 ±

0.22%). The proteomic approach revealed 44 proteases, 25 protease inhibitors, 10 glycosidases and 5 glycosyltransferases. This study demonstrates that human milk contains a variety of enzymes and their inhibitors, which may vary in relation to lactation and secretor status.
Co-authors: Lotte Bach Larsen, Nina Poulsen, Ulrik Kræmer Sundekilde
Affiliation: Aarhus University

Streptococcus and Lactococcus are Dominant Viable Contaminants in Whey Powder
Wannes Van Beeck, Postdoctoral Researcher, UC Davis

Whey protein is an important by-product of cheese making. The economic value of whey is highly dependent on its microbiological quality. Presently, the origin of the viable microbial contaminants in whey powder is not well understood. We aim to determine the primary sources of culturable bacteria in whey by examining the microbial composition in raw milk and throughout whey processing. Raw milk and whey before and after HTST pasteurization and spray-drying were collected in sequence for microbial analysis. Samples were taken on three dates during two seasons, summer (August, 2021) and winter (February, 2022). Viable and total (viable and dead) cells were enumerated with and without propidium monoazide, respectively. 16S rRNA gene (V4) DNA sequencing was performed for total and viable bacterial community analysis. qPCR was used to enumerate Lactococcus, Streptococcus, and total bacteria. Raw milk samples contained approximately 10^3 cells/g and a diverse, viable microbial community which included Acinetobacter, Bacillus, and Pseudomonas. This changed after cheese making and viable cell numbers reached ~10^5 cells/g in the whey samples taken directly after cheese making, with Streptococcus and Lactococcus accounting for up to 79% and 21% of bacteria, respectively. Subsequently, throughout the whey processing pipeline Streptococcus and Lactococcus remained the dominant genera in the viable microbial community. Although qPCR showed that Lactococcus was abundant in the final whey powder (~10^3 cells/g), most cells were dead. By comparison, Streptococcus constituted ~99% of the viable bacteria (~10^3 cells/g) in the final whey powder. These findings were confirmed by both DNA sequencing and qPCR. Conclusions: Whey samples were dominated by Streptococcus, a genus found in raw milk but also added as a starter culture in cheese manufacturing. Metagenomic analyses and strain level characterization will be used to determine the origin of Streptococcus contaminants in the whey powders.
Co-authors: Iswandi Jarto1, Jessie Heidenreich1, Lei Wei2
Affiliations: Hilmar Cheese Company1, University of California Davis2

Transfer of Maternal Immunity Through Milk, Cord Blood and Amniotic Fluid, a Matter of Antibody Repertoire?
Arthur Van Stigt, MD, UMC Utrecht

Maternal vaccines are well-established vaccination strategy for infectious diseases such as influenza and pertussis. Antibody transfer through the placenta is assumed to be the most important route of maternal immunity transfer. Also human milk and amniotic fluid contain antibodies. Specific antibodies in breastfeeding are associated with protection from infections in neonates. Amniotic fluid also contains antibodies that neutralize RSV in vitro and protect mice pups. Little is known about the difference in antibody composition between these three media and their contribution in protection from infections. Insight into these three pathways of maternal immunity transfer is vital for improving maternal vaccines. The PRIMA human milk cohort is designed to study the role of antibodies in breastfeeding on infections during first year of life. We set up a nested subcohort to compare antibody repertoires between cord blood, breastfeeding and amniotic fluid. Amniotic fluid and cord blood are collected at birth, human milk and maternal blood are sampled in the first week postpartum. We anayzed the concentration of all human antibody isotypes. Total antibody levels are lower in cord blood, human milk and amniotic fluid compared to maternal serum. Human milk is enriched for IgA1 and IgA2 (77.06% and 15.60% respectively in human milk, 19.16% and 3.78% in maternal serum), cord blood and amniotic fluid are enriched for IgG1 and IgG2 (25.97% and 67.94% in cord blood, 27.63% and 54.94% in amniotic fluid, 11.90% and 46.70% in maternal serum). Amniotic fluid is also enriched for IgE and IgG4 (0.13% and 1.21% in amniotic fluid, 0.02% and 0.58% in maternal serum). Maternal antibodies in milk, cord blood and amniotic fluid display a

differential serotype repertoire, indicating selectively transport to these different locations. It is not yet know how these differences are related to protection of the neonates against distinct pathogens.