SPLASH!® milk science update
October 2017 Issue

This month's issue features a report on the 14th annual IMGC Symposium, how cow's milk may improve cognition, the effects of Holder pasteurization on the milk digestion, and how an infant's diet influences the development of their immune system.

Milk Genomics and Human Health: A Report from the 14th Symposium

The 14th International Symposium on Milk Genomics and Human Health was held in the warm and welcoming surrounds of Quebec City, Canada. This was the first time the consortium has been held in Canada, and it was a great opportunity to experience the hospitality and learn from the wonderful dairy science culture of the Canadians and the international program that the committee had assembled.

The meeting opened with a retrospective on the 14 years of IMGC, with a personal perspective by Gerrit Hiddink. Gerrit was a founding scientist and illuminated the IMGC origins and the developments since its inception. The personal views from Gerrit reinforced what participants have come to expect and enjoy from the symposium, the multi-disciplinarity and tremendous breadth and depth of milk science and genomics. This expands the horizons of participants and challenges conventional views so everyone learns something new. This is at the core of the philosophy of the meeting and has become a sustaining driver of this symposium.

Danielle Lemay has been presenting updates on the metrics of the Symposium outcomes and participant outcomes for a number of years now. This year the presentation stepped up to another level with a deep analysis that demonstrated the astounding impact of the meeting on collaboration and quality research that is fostered by the symposium. Watch for the details of the analysis in future publications.

Danielle's talk was followed by the first of our invited speakers, Filippo Miglior from the Canadian Dairy Network. Filippo presented the state-of-the-art in genome prediction and phenotyping in the national Canadian dairy cattle improvement program. Genome prediction is now a major contributor to herd improvement and has contributed accelerated gain in recent years. He made the point that the dairy genome project had returned an estimated 250x return on investment since 2009. The power of this applied genomics methodology and a cost-effective access has fostered the drive for more valued phenotype analysis. Filippo described the Canadian program's efforts to capture in-depth analysis of milk composition from 750,000 samples. This tremendous resource will make milk a highly sophisticated natural product for decades into the future.

The next session covered aspects of lactation genomics. Monique Rijnkels has been leading a lactation annotation effort as part of the Functional Annotation of Animal Genomes global network. Monique and her colleagues have explored a detailed analysis of transcriptomes and genome modifications in an attempt to unravel the complexities of mammary gland function. This is a huge undertaking and continues to amaze the researchers in its complexity and technical challenges. The project will continue to develop the annotation to identify non-coding DNA in the bovine genome that contributes to the production of milk.

Nina Poulsen from the Danish-Swedish Dairy Genomics Initiative presented their latest analysis of milk oligosaccharides. They found a significant effect of parity and breed on the relative proportion of acidic, neutral and fucosylated oligosaccharides. Genome-wide association analysis identified a complex pattern of potential genomic regions driving this trait, and candidates are under further investigation. However, the heritability estimates do suggest that there is room to select for oligosaccharide composition.

Klaus Lehnert provided a genomics perspective on the small but emerging dairy goat industry in New Zealand. Klaus has genotyped representative animals from the primary industry-contributing farms in NZ and found an extremely structured goat population. A single chromosomal region characterized the selected animals, suggesting that farmers had established the industry on the basis of a very narrow genetic base. It will be interesting to see how the farmers engage with genetic selection strategies to develop the industry in coming years.

Rachel Gervais, an animal nutritionist from Univesité Laval, provided a detailed analysis of milk fatty acid profiles and the influence of feed composition and rumen microbiota. Lipid composition of milk has been re-evaluated in recent years, and there is a lot of interest in beneficial and bioactive properties of fatty acids. Rachel provided a detailed overview of the variation in milk lipid composition that is found in different production systems. The composition is not only relevant to consumers and processing but is an accurate biomarker of rumen function.

Darryl Hadsell continued on the theme of milk composition but in an entirely different context, consistent with the IMGC diverse

systems approach. He showed a detailed genetic analysis of milk composition in the mouse diversity panel, highlighting a range of genome regions associated with milk components, and specific regions of interest that were potential causal candidate genes. However, as he pointed out, there was an apparent gap in the linkage between peak signals and key candidates in two primary associated regions, suggesting an apparent but unannotated functional association between gene and intergenic sequence. Nevertheless, there was clear evidence of concordance between 8/19 associated regions between the mouse model and those described in dairy cattle.

Alexandra Carrier from Université Laval provided an interesting insight into efforts to conserve the Canadienne breed of dairy cattle. This resilient breed was brought to Canada in the 19th century, and by 1850 there were an estimated 300,000 individuals on-farm. The numbers dropped in the 20th century as other breeds replaced dairy herds so that only 10,000 were left by 1970 and were crossed with Swiss Brown. Alexandra estimated the effective population size at around 43 and described the strategy to save the breed and its characteristic traits by increasing diversity and improving udder conformation.

The milk metabolome is now within reach of detailed analytical profiling using NMR. Ulrik Kræmer Sundekilde described the variation in milk metabolites in human milk. The study was focused on the comparison of pre-term milk and full-term milk at various stages of lactation. There were notable differences in oligosaccharide and branched amino acid composition. After approximately 5 weeks, the pre-term samples resembled full-term milk.

Kasper Hettinga has been interested in milk proteomics throughout his career in The Netherlands. This year he explored the variation in the proteome of cow's milk at different stages of lactation and with varied diets. The most significant variation was between individual cows, with 50% of variation happening in proteins with immune or protease or protease inhibitor functions. There was a strong correlation between these groups of proteins.

André Marette from INAF at Univesité Laval described a series of studies on the effects of milk products, especially fermented products, on metabolic and cardiovascular markers. His group was particularly interested in examining whether the products would influence the mice via changes to gut microbiota. He showed data to demonstrate some effect of fermented milk-derived peptide supplements on improving insulin sensitivity in LDL-knockout mice and an effect on improvement in plasma triglycerides. However, triglycerides in liver were increased and there was an unresolved issue of whether there was a direct relationship between the two compartments. There were also some effects on inflammation as measured by circulating cytokines. When analyzing gut microbiota, his data indicated that dysbiosis was resistant to change, but there was an increase in gut microbes that have been positively associated with metabolic health.

Diana Taft from UC Davis continued on the theme of gut microbiota and described an analysis of samples from infants in a study in Bangladesh. The focus was on antibiotic-resistant strains, a concerning problem worldwide, especially in countries like Bangladesh where antibiotics are still cheap and available without a prescription. Using a sequencing approach, Diana examined seven samples with low numbers of bifidobacterial species and eleven with high numbers. She found a higher number of Enterobacteriaceae species, with up to nine antibiotic resistance genes in samples with lower numbers of bifidobacteria. There were a few bifidobacterial species with low antibiotic resistance.

Steve Frese received the most valued speaker award from the 2016 symposium. He presented an update on his work in developing a commercial product based on cultured *Bifidobacterium longum* infantis, and results of a trial of infant feeding as a supplement to breastfeeding. The goal was to positively influence gut colonization to avoid any dysbiosis that may affect health. The supplement resulted in a 10–100-fold increase in *Bifidobacteria longum*in the stool samples from the babies and a significant reduction in unfavorable bacterial species over the first year of life. There was a notable decrease in stool pH associated with metabolic changes related to gut microbiota composition. This was associated with anecdotal evidence of reduced defecation frequency, more firm stools, and mothers reported more "settled" babies.

Vanessa Dunne-Castagna studies infant gut microbiota and is interested in the protective effects of immunoglobulin A. The secreted form of IgA (sIgA) is found in breastmilk early in lactation, and Vanessa examined its role in gut colonization. She used a model of gut epithelial cell binding to test the effect of coating bacteria with sIgA. The coating did indeed increase binding of the bacteria to the intestinal cells and, which could provide a protective effect for colonizing bacteria.

David Dallas studied a series of samples from premature infant gastrointestinal tract. The preterm infant gut is immature and unable to produce an appropriate digestive microenvironment, so in this study, samples were analyzed for protein digestion and peptide composition. Peptides are first released within milk as a result of intrinsic proteases, but digestive enzymes are essential for nutrition and potentially for bioactivity directed towards infant development.

Søren Nielsen from Aarhus University, in collaboration with the Dallas lab, described the development of an online tool to search peptide sequences for known bioactivity. The tool draws on 994 database entries and 294 research articles with 606 known peptides. Many of these milk peptides (327) are inhibitors of angiotensin-converting enzyme. The tool provides an individual or file upload facility to search the database and return matches, either as exact or embedded sequences. The analysis is fast and can also provide additional data from public domain protein databases.

Vivi Gregersen, also from Aarhus University, has been quantifying specific milk proteins in dairy herds with an interest in heritability and the potential for selective breeding. There was reasonable heritability for osteopontin, but low values for beta-casein and lactalbumin. Expression analysis suggested a relationship between protein levels and osteopontin or a prolactin effect.

Peter Williamson, the author of this article, presented data from bioactive protein and peptide analysis of a processed whey stream. Following initial work to show that whey proteins have a marked effect on gut development in a mouse model, and a significant effect in gut repair following injury, his graduate student established a semi-automated assay for tissue repair and screened 25 whey proteins from a library of recombinants made from bovine mammary gland genes. Three of the proteins were identified in whey along with 57 free peptides. Thirteen of these peptides had predicted bioactivity when analyzed *in silico* with the milk bioactive peptide database.

Betty van Esch from Utrecht University gave an update on her work looking for mechanisms to explain observations in a childhood cohort that showed an association between cow's milk consumption (pasteurized vs unpasteurized) with atopic disease. Using a mouse model, she showed data that supported an immunomodulatory effect of milk consumption as measured by T-cell profiles, cytokine profiles, and mast cell activity. This effect was significant in mice fed untreated milk when compared with heat-treated milk. Disassociating bioactive properties of milk following heat treatment will hopefully provide clues to these effects in future studies.

Benoit Lamarche (INAF Univesité Laval) presented an analysis of evidence for the health impact of dairy with a focus on saturated fats. The premise that dietary guidelines assume independent actions of food components was challenged. Data from his own group showed no impact of dairy foods on risk factors for cardiovascular disease. The complexity of the food was shown to influence measurements of plasma biomarkers, so the matrix represented in cheese had a markedly different effect when compared with that of butter. He argued that simple measures of plasma cholesterol or lipid profiles are a poor measure of risk and that more relevant assays should be used, as provided by the example of macrophage cholesterol efflux. The data support an argument for evaluation of foods—not nutrients—in formulating dietary guidelines. The work of Benoit was reinforced by studies presented by Jean-Philippe Drouin-Chartier (INAF Univesité Laval) that focused on the release of nutrients from different cheeses. Harder cheeses significantly slowed the post-prandial levels of triglycerides and apoB-48.

Bruce German presented his latest discoveries and developments in milk lipids. He was driven by the analysis of Nurit Argov-Argaman of lipids in milk fat globules. A key factor in MFG size is phospholipid composition. In lactating women, the size of MFGs can affect mastitis. Bruce was particularly excited to present the discovery of nanostructures formed during the digestion of milk fats. Ben Boyd (Monash University Australia) found a remarkable nanostructure of milk-derived lipids that appears to facilitate both fat-soluble and water-soluble nutrient absorption through a unique three-dimensional arrangement that provides a dual purpose.

Deborah O'Connor (University of Toronto) presented a view from front lines of the neonatal intensive care unit. The extensive experience of herself and colleagues in nursing care for preterm babies provided a compelling argument for advancing studies of nutritional support for these babies. Data were presented to show a decrease in necrotizing enterocolitis when babies are fed breast milk, but effects on neurodevelopment were not measurable. Deborah argued that some very fundamental research would be enormously beneficial. Her most immediate concerns were with nutrient loss in protocols for handling breast milk of maternal or donor origin. She sees a marked loss in folate and vitamin C following freeze thaw and heat treatments, and a surprisingly high loss of lipid in feeding tubes.

Kevin Nicholas (Moansh Univ, Deakin Univ) and Christophe Lefevre (WEHI) gave a comprehensive summary of lactation biology and bioactivity in marsupial milk. Kevin's group has pioneered a comparative strategy to understand how the altricial young that grow in the pouch of Tammar wallabies utilize milk of different compositions to grow and develop. In the studies described, there was a focus on the development of a mature lung. Kevin and Christophe presented a wealth of data characterizing the factors involved in lung developmental stages based on studies using gene expression, an *in vitro* lung culture model and cross-fostering experiments. Milk from different stages of lactation assayed in a mouse model of lung development showed that bioactive factors from milk in the early stage of the lactation cycle were most potent. Interestingly, the extracellular matrix of the mammary gland was shown to control milk bioactive composition.

Paul McJarrow (Fonterra New Zealand) presented an analysis of breast milk oligosaccharides in two Asian ethnic groups of women. Overall there were no ethnic differences in breast milk oligosaccharides, with the possible exception of 3' fucosylated forms when compared with published studies on non-Asian populations, but there were isolated individual differences, particularly in 3' fucosylated content. This is consistent with a lack of a 1,3-transferase in some people. There were suggestions of differences in sialyllactose content, but it was difficult to distinguish in comparison with historical data.

Carrie-Ellen Briere has recently established a laboratory at the University of Massachusetts and launched a project to study breast milk-derived stem cells. She confirmed the presence of stem cells in her samples and is currently doing characterization studies using flow cytometry and gene expression analysis. She is particularly interested in translating the health-promoting properties of stem cells in applications related to health and well-being of pre-term babies.

As with previous symposia, selected students were strongly supported with generous travel awards to attend the meeting. This year we heard from Andrea Zukowski (University of Ottawa), Randall Robinson (UC Davis), Léa Guinot (Univesité Laval), Ryskaliyeva Alma (INRA, UMR GABI, AgroParis Tech and Univ Paris-Saclay), and Junai Gan (UC Davis). Andrea described her study on the milk of mastitic cows showing variation in exosome phenotype and size, with an update on analysis of a complex miRNA profile in progress. Alma Ryskaliyeva is a graduate student from Kazakhstan working with Patrice Martin in Paris. She introduced the meeting to the camel milk industry and described how the modern methods of genomics have allowed a very productive analysis of camel lactation. Randall Robinson (UC Davis) described the development of a multiplex assay for milk oligosaccharides. The mass spectrometry-

based method used accelerates the analysis of complex oligosaccharide mixtures many fold. He demonstrated its utility by analyzing samples from over 600 dairy cow milk samples. Léa Guinot (Univesité Laval) described a laboratory model for studying digestion and analyzed the release of nutrients from cheese matrices. Junai Gan (UC Davis) described her efforts to unite a team of young researchers in a project focused on milk bioactive peptide discovery. So far the work team has identified 85 peptides for further analysis.

Once again, it was an informative and diverse meeting. See you all next year!

Contributed by
Professor Peter Williamson
Associate Professor, Physiology and Genomics
University of Sydney, Australia

Cow's Milk May Improve Cognition in People with Higher Fasting Glucose

- **Foods such as cow's milk can influence cognitive skills over the long term, but their short-term effects on cognition are still unclear.**
- **A new study compared participants' ability to perform various cognitive tasks shortly after drinking either cow's milk, juice, or water.**
- **Participants who had higher fasting glucose performed better on certain cognitive tasks after drinking cow's milk compared with when they had juice, whereas the opposite was true for participants who had lower fasting glucose.**

What we eat is known to influence our cognitive skills, and consuming dairy, in particular, has been associated with improved cognition [1-4]. "There have been epidemiological studies or observational studies looking over the long term showing that people who ingest a higher amount of dairy products have better cognition in the long run," says Professor Mary Beth Spitznagel of Kent State University.

Rather than focusing only on long-term effects, Spitznagel became interested in studying the short-term effects of a meal on cognition. "We wanted to look at this very acutely, just to see if in the moment what we drink or what we eat is going to impact our thinking skills," she says. A couple of studies that previously looked at the acute cognitive effects of cow's milk had contradictory results, with one finding a beneficial effect on cognition and the other finding no effect [5,6].

In a new study, Spitznagel and her colleagues compared the effects of cow's milk, juice, and water on participants' ability to perform various cognitive tasks [7]. They found that participants who had higher fasting glucose performed better on certain cognitive tasks after having cow's milk compared with when they had juice, whereas the opposite was true for participants who had lower fasting glucose.

"There probably is an optimal glucose range for cognitive function," says Spitznagel. "So people who are starting out lower may need something to push them into that range, whereas the people who start out with that higher fasting glucose need something more balanced to prevent them from being pushed out of that optimal glucose range," she says. "If you're on the higher end for fasting blood glucose, milk might be better for your thinking skills, and if you're on the lower end, maybe juice would be better," says Spitznagel.

The study highlights the importance of knowing fasting glucose levels even in healthy people. "One of the things I like about this study is that we specifically brought in healthy young people with no problems with glucose tolerance, blood sugar, hypoglycemia, or hyperglycemia," says Spitznagel. "Potentially it would be important for people even if they consider themselves healthy to know what their typical fasting glucose range is and eat accordingly," she says. "If you are a student who is about to take a test, and you know that you need to really be focused for that brief period of time, then knowing what your fasting glucose is might actually help you choose what to consume," says Spitznagel.

Spitznagel became interested in how diet affects cognition a few years ago. "As a clinical neuropsychologist, I'm really interested in finding ways that people can try to improve or optimize their cognitive skills," she says. "In general I have an interest in non-pharmacological interventions, things like exercise and diet, the minor changes that we might be able to make in our lives that could potentially boost performance on a daily basis," says Spitznagel.

In 2016, Spitznagel and her colleagues conducted a literature review and found a lot of research on the effects of diet on cognition [8]. But the results of many of the studies were inconsistent. "We were interested in understanding what might be contributing to some of those inconsistencies," she says. "We noticed in the course of doing that review that the papers that were focused more

specifically on people who have impaired fasting glucose or impaired glucose tolerance or diabetes, in general their findings were very consistent," says Spitznagel. "Higher carbohydrate meals are not good for cognition if you have diabetes, which is not surprising," she says.

The review made Spitznagel consider the potential importance of glucose regulation and fasting glucose levels on post-meal cognition. Previous work had shown that baseline glucoregulation could affect cognition after a meal [9,10]. "There's a little bit of work showing that people with really excellent glucoregulation do benefit from something that's a slightly higher carbohydrate meal," says Spitznagel. "For those who maybe had slightly higher fasting glucose, the hypothesis was that they would need something that was a little bit more balanced," she says.

In the new study, Spitznagel and her colleagues gave healthy young adults 8 ounces of either 1% milk, apple juice, or water, and had them perform various cognitive tasks 30, 90 and 120 minutes after the meal. They also measured participants' fasting glucose. "We're using it as a proxy measure for glucoregulation," says Spitznagel.

Spitznagel chose to study the effects of milk, apple juice and water because these are common beverages. "I wanted this to be something that would actually apply in the real world," she says. "We're kind of looking at the apple juice condition as our very high carbohydrate condition, and milk in terms of its macronutrient profile is more evenly balanced," she says. "The water control condition was just to keep people hydrated, as there's been some literature that suggests that when someone's dehydrated their cognition is not optimized," says Spitznagel.

The researchers hypothesized that juice might improve cognition in those with lower fasting glucose, while milk might improve cognition in those with higher fasting glucose. "It did pan out the way we expected, so that was exciting," says Spitznagel. "People who had higher fasting glucose did perform better in terms of working memory or complex concentration tasks after milk compared to juice, and the opposite was the case for people who had lower fasting glucose," she says.

One of the innovations of the new study is that it considered glucoregulation as a continuous variable. "In the past when glucoregulation has been considered, it's most typically been in a way where the researcher is splitting people up into two groups, high fasting glucose and low fasting glucose, and sometimes dividing those groups at 100 mg/dL, and sometimes at 90 mg/dL," says Spitznagel. "Looking at it in a continuous way allows us to more specifically detect what's going on," she says.

"I was especially excited that we were able to look at and pinpoint a specific range," says Spitznagel. "When fasting glucose was above 106 mg/dL, those folks did better after milk in that working memory task, and if fasting glucose was below 77 mg/dL, they performed better with juice," she says. "What our work is showing is that we see these differences not necessarily at 100 mg/dL, not necessarily at 90 mg/dL, but we're seeing them at 77 mg/dL and 106 mg/dL," says Spitznagel.

Spitznagel wants to replicate the findings in other study populations, and is currently working on a follow-up study in children. "I think this is work that should also be done in older adults, as we know that some of the things we're looking at in terms of glucose tolerance and some of the hormones that are involved may differ across different ages," she says.

Spitznagel also plans to look at the effects of diet on a greater variety of cognitive tasks. "I think it's also going to be important for future work to look at other cognitive domains like learning and memory to identify the optimal glucose ranges for different beverages by cognitive domain," she says.

Future studies could also look at the effects of larger serving sizes. "We used a single serving size, 8 ounces, but especially in this country oftentimes we are not just consuming 8 ounces of beverage but instead consuming 24 or 32 ounces," says Spitznagel. "I think there's good potential that the effects could be larger with a larger amount of beverage than we're looking at, and that potentially might be more reflective of how people are consuming beverages these days," she says.

The fact that cow's milk may improve cognition in people with higher fasting glucose may become even more significant in the future, given the increasing prevalence of high fasting glucose levels. "I think increasingly our body mass indices are on the rise, and associated with that we're having increased incidence of diabetes or impaired fasting glucose, and so I think this is going to become increasingly important," says Spitznagel.

1. Nyaradi A., Li J., Hickling S., Whitehouse A.J., Foster J.K., Oddy W.H. Diet in the early years of life influences cognitive outcomes at 10 years: a prospective cohort study. Acta Paediatr. 2013 Dec;102(12):1165-73.
2. Rahmani K., Djazayery A., Habibi M.I., Heidari H., Dorosti-Motlagh A.R., Pourshahriari M., Azadbakht L. Effects of daily milk supplementation on improving the physical and mental function as well as school performance among children: results from a school feeding program. J Res Med Sci. 2011 Apr;16(4):469-76.
3. Crichton G.E., Murphy K.J., Howe P.R., Buckley J.D., Bryan J. Dairy consumption and working memory performance in overweight and obese adults. Appetite. 2012 Aug;59(1):34-40.
4. Crichton G.E., Murphy K.J., Bryan J. Dairy intake and cognitive health in middle-aged South Australians. Asia Pac J Clin Nutr. 2010;19(2):161-71.
5. Galioto R., Alosco M.L., Calvo D., Gunstad J., van Dulmen M., Spitznagel M.B. Neurocognitive response to dairy milk versus fruit juice in obese and lean individuals. Transl Issues Psychol Sci. 2015 Sep;1(3):250–61.
6. Brindal E., Baird D., Slater A., Danthiir V., Wilson C., Bowen J., Noakes M. The effect of beverages varying in glycaemic load on postprandial glucose responses, appetite and cognition in 10-12-year-old school children. Br J Nutr. 2013 Aug 28;110(3):529-37.
7. Anderson J.R., Hawkins M.A.W., Updegraff J., Gunstad J., Spitznagel M.B. Baseline glucoregulatory function moderates the effect of dairy milk and fruit juice on postprandial cognition in healthy young adults. Eur J Nutr. 2017 Jul 13.
8. Galioto R., Spitznagel M.B. The effects of breakfast and breakfast composition on cognition in adults. Adv Nutr. 2016 May 16;7(3):576S-89S.
9. Nabb S., Benton D. The influence on cognition of the interaction between the macro-nutrient content of breakfast and glucose tolerance. Physiol Behav. 2006 Jan 30;87(1):16-23.

10. Nabb S.L., Benton D. The effect of the interaction between glucose tolerance and breakfasts varying in carbohydrate and fibre on mood and cognition. Nutr Neurosci. 2006 Jun-Aug;9(3-4):161-8.

Contributed by
Dr. Sandeep Ravindran
Freelance Science Writer
Sandeepr.com

Holder Pasteurization May Alter the Digestion of Human Milk

- **One way to monitor how a type of food is digested is to mimic the physiological process in the lab.**
- **Researchers have monitored raw and Holder pasteurized human milk for when the digestion process imitates that of a term-born infant as well as a premature infant.**
- **Although Holder pasteurization alters milk's digestive progress in the lab, results form a small clinical trial suggest these differences are slight—if they exist at all—in hospitalized infants.**
- **More clinical research in this area is required before conclusions can be drawn.**

There is a laboratory in Rennes, the capital of Brittany, France that seeks to mimic the interior of the human gut. It has a machine with a compartment that pretends to be a stomach, full of acid and enzymes. Another compartment replicates the conditions of the small intestine. A computer modulates how food, in its various stages of digestion, flows through this system, by altering the activity of peristaltic pumps. In the past couple of years, scientists operating this system have put it to work digesting human milk. And because every aspect of digestion can be finely tuned, they can speed up gastric emptying, lower certain enzymatic activities, and raise gastric pH—as per a preterm (relative to a term-born) infant's system. The main question these scientists seek to answer is how pasteurizing milk by heating to 62.5 °C for 30 minutes alters how well it is digested.

Of course, the machine is not a perfect replica of nature—there's no absorption, nor hormonal feedbacks, for example—but because samples can be removed and analyzed at any point, it provides a microscope on the step-by-step molecular processes of digestion [1].

Why might Holder Pasteurization (HoP), which is the most common method of pasteurization for milk banks everywhere, change how the infant body processes human milk? Simply put, milk contains proteins that assist an infant's digestion. This is a useful property because newborn infants do not have fully developed guts—and those of infants born prematurely are even less developed. Heating complex, active proteins tends to mess with the proteins' three-dimensional structures, which is crucial to their ability to catalyze chemical reactions, such as those that occur during digestion. Therefore, pasteurize human milk using a lot of heat, and you might be taking out some of its digestive aids. Moreover, freezing and unfreezing milk can disrupt the structure of its fat globules, making them more prone to stick to the sides of feeding equipment. So what has to be digested, as well as the biochemical tools available to do the job, may change as a result of HoP.

There are a few clear candidates for the digestive aids that might be affected by heat treatment. An enzyme in human milk called bile-salt-stimulated lipase, for example, facilitates the breaking up of fats and the absorption of some vitamins. Lactoferrin plays various roles, including in digestion, and, as discussed in another *SPLASH!* article about HoP's impact on immunological proteins, is affected by the heating process. Then there are molecules like α1-antitrypsin and α1-antichymotrypsin; instead of speeding things up, these proteins slow down digestion by interacting with protease enzymes that are produced by an infant's body [2].

A couple of years ago, the team in Rennes—most of whom work for France's national agricultural research agency, INRA—set up their machine to imitate the conditions of a term-born infant's gut [3]. They fed it donor milk from the milk bank at the University Hospital Center in Rennes. This milk was pooled from five different women. They repeated the experiment several times, sometimes using milk that had not been pasteurized, and other times with HoP-treated milk, with every run of the experiment, they withdrew a sample from the "stomach" and another from the "small intestine" after 30, 60, 90, and 120 minutes.

Comparing the pasteurized and raw milk results, they found that fat digestion progressed more slowly whenever the machine had been given HoP-treated milk to digest, probably because of damage to bile-salt-stimulated lipase as well as the formation of clumps of protein around the milk-fat globule membranes in the pasteurized milk. The machine was also slower to break down lactoferrin in the pasteurized milk than it was in the raw milk, yet quicker to digest the protein beta-casein.

Having established what the differences were for term infant digestion, last year the group in Rennes reset their machine to imitate the digestive abilities of an infant who was born after 28 weeks' gestation, four weeks after he or she was born [3]. That meant the compositions of gastric and intestinal fluids were slightly different than in the term-infant experiment, and the machine's gastric emptying time was lowered from 47 to 36 minutes.

This time, the team again found that the fat in the pasteurized milk samples was harder for the machine to digest and that some proteins were broken down faster than they were in the raw milk samples, while others were broken down more slowly. The worry with the fat result is that pasteurization might be reducing the useable calories in milk, not only by causing fat to adhere to milk containers but also by reducing the activity of enzymes that can break it down. The impact of the results for proteins is less obvious.

What did concern the researchers, however, was the rate at which milk disintegrated in the machine. (Although milk might seem pretty disintegrated as a foodstuff, more complete mixing helps ensure that digestive enzymes can attack from every angle.) The pasteurized milk formed smaller aggregates in the machine's
"stomach" than the raw milk did, and then it formed larger ones in the "small intestine". The larger gastric aggregates of the raw milk samples tended to cream in the stomach compartment because they were less dense. Because this kind of thing is linked to more rapid gastric emptying in adults [4], the authors worried that slower-than-usual gastric emptying associated with digesting pasteurized milk could raise the risk of gastrointestinal infections.

Most recently, the team in Rennes has started to monitor premature infants in the local university hospital, and has conducted a randomized controlled clinical trial [5]. These infants acted as their own control group by consuming their own mothers' milk raw for a few days in a row, and also consuming it after it had undergone the HoP process for a few days in a row.

The study was small—involving only a dozen infants—and was also limited in that the researchers could not study the biochemistry of digestion beyond the first 35 minutes. But the results were nonetheless reassuring. Even though HoP did inactivate bile-salt-stimulated lipase in this trial, the infants were just as good at breaking down the fat in their mothers' HoP-treated milk as they were at breaking down the fat in raw milk. Moreover, HoP appeared to enhance lactoferrin digestion, rather than reduce it. And there was no difference in the gastric emptying time when the infants consumed raw versus pasteurized milk.

The researchers in Rennes warn that inactivated bile-salt-stimulated lipase could still have consequences for fat absorption in the intestine, which matters for premature infants in particular because they are unable to properly make pancreatic lipase. Partly because of this, they conclude that the clinical impact of human milk pasteurization using HoP remains uncertain. Given the ever-growing number of very young infants who consume Holder pasteurized milk from milk banks, surely further clinical studies of this nature—if possible, with more than a dozen infant participants—would be helpful for doctors and policymakers.

1. Ménard O., Picque D., and Dupont D. 2015. Chapter 8: The DIDGI System. In: 73
K. Verhoeckx et al. (eds.), The Impact of Food Bio-Actives on Gut Health. Chapter 8, 73–81. Doi: 10.1007/978-3-319-16104-4_8.
Available: https://link.springer.com/content/pdf/10.1007%2F978-3-319-16104-4_8.pdf
2. de Oliveira S. C., Bourlieu C., Ménard O., Bellanger A., Henry G., Rousseau F., Dirson E., Carrière F., Dupont D., and Deglaire A. 2016. Impact of pasteurization of human milk on preterm newborn in vitro digestion: Gastrointestinal disintegration, lipolysis and proteolysis. Food Chemistry 211, 171–179.
3. de Oliveira, S. C., Deglaire, A., Ménard, O., Bellanger, A., Rousseau, F., Henry, G., and Bourlieu, C. 2015. Holder pasteurization impacts the proteolysis, lipolysis and disintegration of human milk under in vitro dynamic term newborn digestion. Food Research International 31, 88:263–275.
http://dx.doi.org/10.1016/j.foodres.2015.11.022 (in press).
4. Berni Canani, R., and Terrin, G. 2010. Gastric acidity inhibitors and the risk of intestinal infections. Current Opinion in Gastroenterology. 26, 31–35.
5. de Oliveira, S. C., Bellanger A., Ménard O., Pladys P., Le Gouar Y., Dirson E., Kroell F., Dupont D., Deglaire A., and Bourlieu C. 2017. Impact of human milk pasteurization on gastric digestion in preterm infants: a randomized controlled trial. American Journal of Clinical Nutrition 105, 379–390. doi: 10.3945/ajcn.116.142539.

Contributed by
Anna Petherick
Professional science writer & editor
www.annapetherick.com

Infant Diet Influences Gut Microbiome and Immune System Development

- **Microbial colonization of the infant gut is required for normal immune development.**
- **Researchers used rhesus macaque monkeys as a model to determine the influence of formula and mother's milk on the development of the gut microbiome.**
- **Formula-fed macaques had distinct gut microbiomes and immune profiles from milk-fed macaques, and many of the differences persisted through the first year of life and into the juvenile period.**
- **Early life events, such as infant diet, have long-lasting effects on immune function in rhesus macaques and potentially other primates, including humans.**

The relationship between bacteria and the immune system is usually viewed as antagonistic, but bacteria and immune cells are not always on opposite teams. In fact, the bacteria that colonize the gastrointestinal tract during infancy actually shape the development of the immune system. Research in humans and nonhuman animals indicates that the types and quantities of immune cells that the infant produces are influenced by the strains of bacteria that take up residence in the infant's gut [1–4]. The infant's diet is an important source of gut bacteria—does this mean that differences in early diet (i.e., mother's milk vs. formula) could result in differences in immune function?

A team of researchers from UC Davis and UC San Francisco tested this intriguing hypothesis in a population of rhesus macaques

(*Macaca mulatta*) housed at the California National Primate Research Center (CNPRC) [1–3]. Rhesus macaque monkeys share many biological traits with humans, including similarities in immune function and milk microbial populations [1]. Moreover, animal models allowed the researchers to control for more confounding variables than would be feasible in a human study (e.g., all monkey mothers and weaned infants consumed the same diet, a commercially-produced high-protein chow, and all monkeys were housed indoors) [1–3].

A 2013 study on CNPRC rhesus macaques demonstrated that formula-fed infants developed gut microbiomes that were different from mother's milk-fed infants [1]. However, this study only followed the monkeys through 12 weeks of age.

"Previous work showed that the microbiota is powerfully shifted by early diet, but nobody knew how persistent those differences might be" explain study authors Dennis J. Hartigan-O'Connor, M.D. Ph.D., an Associate Professor of Medical Microbiology and Immunology at UC Davis, and Nicole R. Narayan, a graduate student in the same department. "We knew that the bacteria in the gut were important for immune function, and also that many characteristics of the immune system are stable throughout life. Putting these ideas together made us wonder if the microbes of infants might leave an indelible imprint on immunity."

Hartigan-O'Connor and colleagues looked for this indelible imprint from gut microbiomes on immune function by following infant macaques into the juvenile period. Newborn macaques were randomly assigned to be nursery-reared (and formula-fed) or dam-reared (and milk-fed), with six infants in each group. Stool samples collected at 5, 6, 9, and 12 months were analyzed for microbial profiles [2], and blood samples taken at 5, 6, 9, and 12 months, 3 years, and 5 years were analyzed for immune cell profiles [2, 3]. The immune cell of interest was TH17 (short for T Helper 17). They selected this cell because its development is induced by gut commensal bacteria [2, 3]. Additionally, newborn macaques have very few TH17 cells. Sometime between birth and 18 months, macaques develop a TH17 cell population, which then remains stable throughout adulthood. Importantly, how many TH17 cells an adult monkey has and how quickly those populations develop varies across the population; some monkeys get high numbers quickly (about 10% of all circulating cells), others reach similar numbers more slowly, and others still make relatively fewer cells (about 1% of all circulating cells) [2]. Taken together, these observations suggest a link between adult TH17 populations and early life events, making them a prime candidate for testing hypotheses regarding diet, gut bacteria, and immunity.

Corroborating results from the 2013 study, Hartigan-O'Connor and colleagues identified distinct microbial communities between the formula- and milk-fed macaques at 6 months of age [2]. Additionally, they demonstrated that these differences remained at 12 months, even though both groups were consuming identical diets at this time point [2]. Indeed, the differences between the two groups were greater and more statistically significant at 12 months compared with 6 months, highlighting the importance of the earliest microbial colonizers to subsequent gut microbiome composition [2].

"The early postnatal life is a window of opportunity," says Amir Ardeshir, D.V.M., M.P.V.M., Ph.D., one of the study's lead authors and an affiliate scientist at CNPRC. "It is the key time [for beneficial bacteria] to colonize the host and establish a robust and healthy immune system."

These findings are similar to those from a recently published study on breastfeeding and the development of the human gut microbiome [5]. Differences in gut microbiome strains between formula- and breast-fed human infants were identified before and after the introduction of solid foods, supporting the proposition that diet may make its largest impact on the gut microbiome during early infancy [5].

Immune profiles of rhesus monkeys followed the same trend as the gut microbiome, with differences between formula- and milk-fed monkeys detected at 9 months of age becoming more pronounced and statistically significant at 12 months [2].

"Milk-fed monkeys developed a healthy immune system. In particular, these infants developed robust populations of memory T cells, including memory TH17 cells, while formula-fed infants did not," explains Ardeshir.

Memory cells are what give the adaptive arm of the immune system its name and are the reason that vaccines are so effective in providing immunity. When the body is exposed to an antigen (e.g., bacteria, virus, fungus), it mounts an immune response. Part of the adaptive arm's response involves the production of memory cells that are specific to that antigen. If the body ever encounters that antigen again, it has an experienced team of memory cells at the ready. Because they've fought this antigen before, memory T cells mount a more efficient and faster immune response than do naïve T cells that would be starting from scratch.

Remarkably, many of the differences in immune cell types among infant macaques were maintained into the juvenile period [3]. Although not as dramatic as identified among infants, immune profiles generated at 3–5 years old were still distinct between the formula and milk groups. This finding suggests that infant diet modulates the strength of particular immune responses during infancy and the juvenile period, and perhaps even into adulthood [3].

Unfortunately, it is not possible to say with the data at hand that infant diet was solely responsible for the differences in gut microbiomes reported by Hartigan-O'Connor and colleagues [2, 3]. Mother's milk may be an important source of baby's first

microbes, but it is not the only source. In the previously mentioned study on the milk microbiome [5], researchers reported that 30% of the microbes in infant's guts matched those from milk. This means the majority of gut microbes come from sources other than the infant's diet, including the maternal skin, maternal fecal material, contact with other group members, and numerous other factors in the environment [1, 5]. Formula-fed and milk-fed infants had different diets and different exposures, as the latter were in constant contact with their mothers, whereas the former were separated at birth. It would be interesting to repeat this experiment with a third group of infants that are nursery-reared but bottle-fed with macaque milk instead of formula to try to quantify the effect of diet compared with environmental microbial exposure.

Even without a definitive source (or more likely, sources) for the infant gut microbiome, the results have important implications for understanding human immunity. Hartigan-O'Connor and Nayaran highlight the fact that their work "may help explain why genetic studies alone are insufficient to understand why certain diseases develop only in some people." And their co-author Ardeshir agrees that the rhesus model points to the strong influence of the microbiome on responses to infection. Indeed, Ardeshir believes that the influence of the microbiota on the immune system is so profound that you could actually use features of an individual's intestinal microbiome to predict how effective a vaccine will be. "This can be a game changer in the fight against infectious disease if we are able to improve vaccine efficacy by alteration of the microbiome with pre- or probiotics."

1. O'Sullivan A., He X., McNiven E.M., Haggarty N.W., Lönnerdal B., Slupsky C.M. 2013. Early diet impacts infant rhesus gut microbiome, immunity, and metabolism. J Proteome Res 12: 2833-2845.
2. Ardeshir A., Narayan N.R., Mendez-Lagares G., Lu D., Rauch M., Huang Y., Van Rompay K.K.A., Lynch S.V., Hartigan-O'Connor D.J. 2014. Breast-fed and bottle-fed infant rhesus macaques develop distinct gut microbiotas and immune systems. Sci Transl Med 6: 252ra120.doi:10.1126/scitranslmed.3008791. Available here: http://stm.sciencemag.org/content/6/252/252ra120
3. Narayan N., Mendez-Lagares G., Ardeshir A., Lu D., Van Rompay K.K.A., Hartigan-O'Connor D.J. 2015. Persistent effects of early infant diet and associated microbiota on the juvenile immune system. Gut Microbes 6: 284-289. Available here: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4615596/.
4. Hooper L.V., Littman D.R., Macpherson A.J. 2012. Interactions between the microbiota and the immune system. Science 336: 1268-1273.
5. Pannaraj P.S., Li F., Cerini C., Bender J.M., Yang S., Rollie A., Adisetiyo H., Zabih S., Lincez P.J., Bittinger K., Bailey A., Bushman F.D., Sleasman J.W., Aldrovandi G.M. 2017. Association between breast milk bacterial communities and establishment and development of the infant gut microbiome. JAMA Pediatr 171: 647-654.

Contributed by
Dr. Lauren Milligan Newmark
Research Associate
Smithsonian Institute

Funding provided by California Dairy Research Foundation and the International Milk Genomics Consortium