



SPLASH!® milk science update **March 2020 Issue**



This month's issue features PMS and dairy, milk sugars and bone stunting, climate-friendly herds, and human milk microbes.

PMS Symptoms Improve with Daily Recommended Dairy Intake

- **Pre-menstrual syndrome, described by depressive symptoms, muscle cramps, and headaches experienced by women in the 2 weeks preceding menstruation, may be caused by calcium deficiency due to changes in the concentration of estrogen.**
- **Calcium is required to synthesize serotonin, and calcium supplementation has been shown to improve symptoms related to pre-menstrual syndrome.**
- **A new study investigated whether meeting the recommended daily intake of dairy, which provides calcium and other important micronutrients, would improve symptoms of pre-menstrual syndrome.**
- **After two months of meeting their daily dairy intake, study participants reported an improvement in pre-menstrual syndrome symptoms and quality of life.**

Dairy foods are probably best known for their beneficial effects on bone health. But the same vitamins and minerals from dairy that help to build and maintain strong bones—calcium, phosphorus, magnesium, zinc, and riboflavin—may also have a positive influence on the symptoms of pre-menstrual syndrome (PMS). Expanding on previous research [1-4] that found an association between increased calcium intake and decreased risk of PMS symptoms, a new paper [5] from a team of Turkish researchers suggests the suite of micronutrients provided by dairy may be successful at alleviating both the emotional and physical symptoms of PMS.

Nutrients, Neurotransmitters, and Hormones (Oh My!)

Most people probably know that the nutrients from the foods they eat can influence their blood pressure and cholesterol. It might be less obvious, however, that there is a link between nutrition and emotional wellbeing. But nutrients, including vitamins and minerals, can influence (and are, in turn, influenced by) the action of hormones and neurotransmitters [5, 6].



Calcium plays a role in the synthesis of serotonin, a neurotransmitter that is involved in regulating mood [1-5]. Hormones, particularly estrogen, have an indirect link to serotonin production because estrogen regulates how calcium is metabolized and absorbed [1-5, 7]. Estrogen levels increase just before ovulation, which occurs approximately 14 days into the menstrual cycle. This dramatic increase in estrogen is associated with a decrease in calcium, which in turn leads to a drop in serotonin [7]. As a result, many women experience symptoms during the pre-menstrual part of their cycle (approximately days 14–28) similar to those of hypocalcemia, including depression and anxiety [8].

This link between estrogen, serotonin, and calcium prompted numerous studies that investigated the efficacy of calcium supplementation to treat PMS symptoms. Overall, calcium supplementation was associated with a decrease in PMS symptoms, particularly those related to emotional wellbeing [1-5, 7]. These results suggest that the cause of PMS may be calcium deficiency due to changes in ovarian hormones during the menstrual cycle [8].

Dairy and PMS Symptoms

But calcium is not the only micronutrient linked to depression; depressive symptoms have also been linked to insufficient intake of phosphorus, magnesium, and several B vitamins [5]. For example, riboflavin also influences serotonin production because it activates vitamin B6, which is required to convert the amino acid tryptophan to serotonin [8]. Because dairy foods provide many of these other potentially important nutrients, and also calcium, a new study [5] investigated how effective an adequate dairy intake was at alleviating symptoms of PMS.

The 31 study participants were all women between the ages of 20 and 28 that had doctor-diagnosed PMS and were consuming less than the recommended daily amount of calcium. Sixteen women were randomly placed into the intervention group, where the goal was to meet the recommended daily intake of calcium (1000 mg) for two months by consuming at least 80% of the calcium from dairy products. To meet this goal, the participants were instructed to consume at least 400 ml of cow milk (just over 1.5 cups) and 150 g of yogurt (a little over 0.5 cup each day, and were supplied daily with 50 g (approximately 2 ounces) of kasseri cheese (a Turkish cheese made from cow's milk). The control group (15 women) was instructed to maintain their usual diet.

PMS symptoms were assessed at both the beginning and end of the two-month intervention using a Premenstrual Syndrome Evaluation Scale and a Quality of Life Scale [5]. Between baseline and post-intervention, scores on the Premenstrual Syndrome Evaluation Scale significantly decreased, whereas those on the Quality of Life Scale significantly increased. In contrast, no significant difference in either scale was found for the control group [5].

Food as Medicine

Certainly, these differences may be the result of only the difference in calcium intake. Based on a three-day diet assessment at the end of the study, the average calcium intake for the intervention group was just over 1200 mg, whereas the control group was just under 500 mg [5]. But the authors believe that the potential influence of magnesium, phosphorus, zinc, iron, and B vitamins supplied by the various dairy products should not be overlooked. Magnesium is known to reduce pain from menstrual cramps and tension headaches by relaxing muscles and ensuring vasodilation, low zinc intake is believed to be a risk factor for PMS, and riboflavin and iron are required micronutrients in serotonin synthesis [5]. The daily intake of all of these micronutrients increased in the intervention group compared with the controls.

Unfortunately, this study did not include a calcium supplement group, making it difficult to determine if the improvement in PMS symptoms and overall quality of life achieved in the dairy group was similar to, less than, or greater than what has previously been observed for calcium supplementation. But for the 30% of reproductive-aged women that suffer from PMS (and the nearly 90% who report having occasional PMS symptoms) [5, 9] this study's positive findings suggest that lifestyle changes as simple as meeting recommended daily intakes of vitamins and minerals offer the potential to improve their emotional and physical wellbeing.

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How Human Milk Oligosaccharides Can Influence Bone Biology

- **Undernutrition can cause reduced bone growth and stunting in children, and efforts to reduce stunting through nutritional interventions have been largely ineffective.**
- **A new study finds that treating stunted children with therapeutic foods reduced bone degradation activity but did not affect bone production activity, and this effect was insufficient to improve their severe stunting.**
- **Mice raised in a sterile environment colonized with a stunted infant's gut microbiota and fed diets supplemented with human milk sugars containing sialic acid exhibited decreased bone degradation, unchanged bone production and increased weight and bone volume.**
- **The findings identify a mechanistic link between milk sugars and the regulation of bone degradation, and could eventually help find potential therapeutic targets to overcome stunting in undernourished children.**

Undernutrition is a pressing global health challenge and contributes to the deaths of more than three million children under the age of five every year (1). Children who are considerably shorter than the median for their age are defined as stunted, and so far, nutritional interventions have been mostly unsuccessful at reducing stunting (2).



That's particularly concerning due to the myriad long-term consequences of stunting, which include poor response to vaccines, reduced cognitive ability, and complications in pregnancy (3, 4). Many research efforts have thus been dedicated to understanding the underlying causes of stunting and why current treatments are unsuccessful, with the goal of finding more effective interventions.

"Undernutrition is a pressing, vexing, and in many respects tragic situation, and we want to be able to understand those factors that create risks for and that have large effects on the healthy growth of children," says Dr. Jeffrey Gordon of Washington University School of Medicine. "We are

interested in the community of tens of trillions of microbes that collectively are referred to as the gut microbiota, and have been pursuing a hypothesis that there is a definable normal program of assembly of this community in infants and children and that undernutrition, which is a leading cause of death of children under five worldwide, may be caused in part by defects in the development of this microbial organ," he says.

Gordon and his colleagues have followed cohorts of children in low-income countries from birth through the first several years of life and have sampled their microbial communities. "We have found that there's impaired development of the microbiota in children with undernutrition," says Gordon. "In preclinical animal studies, we introduced immature microbial communities into so-called gnotobiotic mice that have been raised under sterile conditions, and also took microbial communities from children with healthy growth and put their communities in, and found that you can transmit many features of undernutrition growth faltering with immature microbiota," he says.

In a new study, Gordon and a postdoctoral researcher in his lab, Dr. Carrie Cowardin, set out to investigate one particular aspect of the growth faltering seen in undernourished children—the reduced bone growth that underlies stunting (5).

Previous work by Gordon's group has shown that certain human milk sugars, known as human milk oligosaccharides (HMOs), may influence bone growth (6). "We are very fascinated by what components of

breast milk may impact the functioning of the microbial community in the gut, and if health of mothers, their nutritional status and the status of their breast milk all relate to the development of this microbial community and the pathogenesis mechanisms underlying the development of undernutrition," says Gordon.

"We reported in 2016 in a study of breastfeeding mothers living in Malawi that those who had infants who were stunted were being breastfed by mothers whose levels of carbohydrates in breast milk differed from mothers whose children were experiencing healthy growth," says Gordon ("Stunted infants were consuming breast milk that had lower levels of certain types of human milk oligosaccharides, particularly sialylated sugars," he says.

Gordon and his colleagues set out to purify substantial quantities of these sialic acid-containing milk oligosaccharides. "It's not possible to get enough human breast milk to purify large quantities, so we turned to the production of cheese and whey," says Gordon. "The whey waste stream from cows contains sialic acid-containing carbohydrate structures that look very much like those in human breast milk," he says.

When Gordon's group administered these purified bovine sialylated milk oligosaccharides (S-BMOs) to gnotobiotic mice colonized with bacteria from a stunted Malawian infant, they found that it could affect their bone growth (6). "That's the entree to this deeper dive into bone biology and the immune system, the gut microbiota and bone growth," he says.

In the new study, Cowardin and Gordon looked at bone biology in a cohort of stunted Bangladeshi children with severe acute malnutrition. "There's sort of a lack of understanding of whether bone resorption, which breaks down bone, or bone production are more important during undernutrition," says Cowardin. When the children were given nutritional rehabilitation with therapeutic foods, they experienced a significant reduction in levels of a biomarker of bone resorption by the end of treatment. "This marker of bone resorption was elevated prior to treatment and then decreased after nutritional therapy," says Cowardin. This reduction was not accompanied by changes in bone production, and was also not sufficient to improve the severe stunting of the children.

Cowardin then used a gnotobiotic mouse model of undernutrition to explore the cellular and molecular effects of sialylated milk oligosaccharides. "Based on our previous work, there was an interest in understanding how sialylated milk oligosaccharides impact bone biology," she says.

Gnotobiotic mice were colonized with bacterial strains cultured from a 6-month-old stunted Bangladeshi infant. "We used the defined gut communities from a child with severe acute malnutrition and who is also severely stunted," says Cowardin. "Then we fed these animals a diet that's representative of what the donor infant would have been consuming," she says. "When we supplemented one group with the sialylated oligosaccharides, we saw an increase in weight gain, as well as an increase in bone volume," says Cowardin.

The researchers then looked in more detail at how S-BMO intake affected bone biology. "What we saw was that rather than influencing bone building, we saw a decrease in bone resorption and a decrease in bone resorbing cells on the bone surface as well as their progenitors within the bone marrow," she says. "So, this component of healthy human breast milk, sialylated oligosaccharides, can improve weight gain and bone growth by decreasing bone degradation in a pre-clinical model," she says. "It's really interesting to us because it kind of mirrors what we saw in children undergoing treatment for severe acute malnutrition."

Interestingly, the effects of S-BMOs were structurally specific, and were not evident in colonized mice whose diets were supplemented with 2'-fucosyllactose (2'-FL), a different type of HMO present in human milk. "Perhaps the most prominent component of breast milk is a series of carbohydrates that have a molecule called fucose linked to it, and adding a fucose-containing carbohydrate structure did not re-enact this set of complex interactions," says Gordon.

Cowardin then investigated the mechanisms by which a human milk sugar might be interacting with bone-degrading cells, known as osteoclasts. "Osteoclasts are very interesting because they're known to be regulated by the immune system, so we wanted to understand how S-BMO supplementation was influencing host immunity both within the gut as well as within the bone marrow," says Cowardin. "What we found was that animals supplemented with S-BMO had an increase in gut eosinophils, which are a type of immune cell that is not directly known to regulate bone building but that can influence osteoclast development," she says. "We also saw an increase in levels of eosinophil recruiting and an immune signal called eotaxin," says Cowardin. "To sort of tie this together, we saw an increase in gene expression within the gut that was indicative of an increase in tuft cells, which are really interesting cells in the intestine that sense luminal content and can orchestrate a type of immune response associated with eosinophils," she says.

The findings provide a mechanistic link between sialylated milk oligosaccharides and the regulation of osteoclast formation and function. "These tuft cells are very adept sensors of their environment and can taste, sense, and communicate that to neighboring cells," says Gordon. "This cell population was markedly affected by the presence versus absence of S-BMO in the diet, so that's interesting," he says. "Maybe we have a connectivity between this milk component, this very well-equipped sensory type of cell and a connection to the immune system, and the immune system in turn could communicate with these bone marrow-derived cells that are involved in degrading bone," says Gordon.

Cowardin also looked for a compound called succinate that is produced by the microbiota and that can increase tuft cell numbers. "We looked for succinate in the gut of animals supplemented with S-BMO and saw an increase in succinate, so increased succinate levels due to S-BMO supplementation drives an increase in tuft cell numbers and those tuft cells then orchestrate the immune response, culminating in a decrease in osteoclast numbers and a decrease in bone," says Cowardin. "Overall we found that there's an interaction between the diet and the microbiota to alter the immune system and influence bone and muscle growth," she says.

The results underscore the need to better understand the interactions between diet and bone biology in stunted children. Future studies could further explore the types of HMOs in human breast milk and how they affect the development of the gut microbiota and the interactions between the gut microbiota and key aspects of growth, including bone growth. "That's a major question in understanding infant development that we can explore using these animal models first and then test in humans," says Gordon. "There also needs to be more work looking at the complex set of interactions between maternal health status, breast milk and infant development, as they're all interrelated," he says.

Understanding the effects of milk oligosaccharides on the interplay between gut microbiota and bone biology may eventually have therapeutic implications. Identifying human milk components that influence infant development through their effects on the gut microbiota and immune system could provide new treatments for childhood undernutrition and stunting.

"I believe that as we gain more knowledge of these components of breast milk and how they act, how they interact with the developing gut microbiota, and how they interact through the microbiota or more directly with the infant, we will gain greater capabilities of ensuring healthy development of infants," says Gordon.

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How to Breed Climate-Friendly Dairy Herds

- **Researchers working across four countries have analyzed the microbiomes of cow rumens.**
- **The findings reveal remarkable consistency in the structure of the microorganism populations that compose the cow rumen, across different herds and breeds.**
- **Based on this evidence, the researchers propose breeding programs to ensure that the microbiomes of dairy cattle have minimal climate impact, in terms of methane emissions.**

When methane emissions that contribute to global warming are blamed on cows, they should, more precisely, be blamed on the microorganisms that live inside them. It stands to reason, therefore, that in seeking ways to reduce methane emissions from the dairy and beef industries, researchers' primary target should be cows' microbiomes. In line with this perspective, a group of researchers with teams in four countries recently carried out a detailed analysis of the microorganisms living in the rumens of different herds and breeds of cattle [1]. These researchers have identified a population of bacteria, protozoa, anaerobic fungi and archaea that consistently form the core population of the rumen microbiome. By linking microbiome components to phenotypes such as methane emissions, they propose the establishment of microbiome-led breeding programs as a means to make livestock farming more climate-friendly.



Conducting such investigations required analyzing the rumen contents of more than one thousand cows. In each participating farm in the UK, Italy, Sweden and Finland, the teams made sure that the feeding and living conditions of the animals were very similar. All of the cows were healthy and lactating, having given birth between 10 and 40 weeks previously. They all consumed maize or grass silage or grass hay.

But not everything was similar across the herds: 816 of the dairy cows, from two UK and three Italian farms, were Holsteins, whereas the remaining 200 from the farms in Sweden and Finland were Nordic Red dairy cows. Holstein cattle (also known as Holstein Friesians) are the most productive dairy cattle in the world, by liters of milk produced. This was the most important breed to assess, therefore, in terms of gathering the evidence for policy advice that keeps production up and methane emissions down. A second, less common breed was included in the study so that the researchers could get a sense of the generalizability of cows' microbiome components across breeds.

The teams collected samples for analysis by sucking a little fluid from cows' rumens after each cow was gently encouraged to swallow a flexible pipe—as humans undergoing some kinds of medical investigations often do. Some of this fluid was then sent to a laboratory where the ribosomal RNA of all of the microorganisms in the fluid was amplified (in other words, copied many times), in order to ensure there were sufficient amounts of RNA to genetically identify the microorganism species, and to look for common features across species. The research teams also recorded how much milk the cows produced, and the amount of methane in their breath.

Prior to this study, the broad categories of microorganisms that tend to appear in cows' rumens was known but with limited granularity—and without much understanding of the degree of similarity in the population structure of the microbiome across different herds and breeds. The laboratory analyses for this project discovered startling consistency in this sense. They revealed a particular group of microorganisms that compose only a quarter of a percent of the overall species pool in the cow rumen microbiome but are so abundant relative to other microorganism species that they consistently make up between 30 and 60% of the total cow rumen microbiome by mass. This is true across the two breeds, the many herds and the four countries in the study. The core group contains 512 types of microorganisms (or, 512 “operational taxonomic units” (OTUs), as types of microorganisms are referred to by experts). The vast majority of these OTUs—454 of them—are prokaryotic bacteria, 12 are protozoa, and 46 are fungi. At first blush, the core group may seem to have many members, but in light of the quarter-million OTUs that are present in the cow rumen, this group is in fact a very selective club.

Some interesting details emerged when the genetics of the core group were compared. First, these OTUs appear to be, broadly speaking, fairly closely related to each other. Second, the study demonstrated that the core group tends to be heritable—many of its members are passed down from cow to calf. Heritability of the gut microbiome is nothing new as a concept—indeed, there is evidence of this to some extent in humans—but the researchers behind this study found nearly 10 times as many heritable OTUs in cow rumens as was reported in a comparable human study [2].

Many members of the core microbiome were associated with the presence of products of fermentation in cow breath, such as the concentration of propionate. Fewer members—though some—could be linked statistically to patterns in milk production and to methane emissions. However, the fact that some OTUs were linked in this way, and the point about heritability, together suggest ways of leveraging knowledge about the cow rumen microbiome to reduce dairy farming emissions.

The researchers suggest selective breeding programs in which core group microorganisms that lower methane emissions are encouraged to populate the cow rumen over those that appear to contribute to methane emissions. Alternatively, the team proposes the use of precise probiotics—the inoculation of young cows with key core species associated with low methane emissions. If herds of dairy cows with low-methane-generating microbiomes could be developed, then, in theory, these cows will pass on their microbiomes to their young, and few or no additional interventions would be needed—meanwhile, the dairy industry, all things being equal, would be contributing less to climate change.

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Feeding Method Affects Human Milk Microbes

- **There are microbes in human milk, but their source is not yet known.**
- **In nearly 400 milk samples, feeding mode (direct at the breast vs. pumped) was the factor most consistently associated with milk microbial composition.**
- **Milk from mothers who pumped had lower species diversity and a higher chance of providing potentially opportunistic pathogens than did milk from mothers that directly breastfed.**
- **Microbes from the infant's oral cavity may be critical in shaping in the milk microbiome.**

It seems counterintuitive that breastmilk would be anything but sterile—human infants have a naïve and immature immune system and their first food should be free of potential pathogenic organisms, right? But study after study demonstrates that milk indeed contains microbes [1]. Precisely where these microbes originate and how they make their way into human milk, however, is still being worked out. There are two, non-mutually exclusive hypotheses to explain their origins: one argues that milk microbes originate from the mother's gut and are passed to the mammary gland (entero-mammary translocation) and the other that bacteria from the infant's oral cavity move back into the mammary gland and influence the types and quantities of bacteria passed via milk (retrograde inoculation) [1, 2]. Because not all milk microbes are equally beneficial for the infant, finding support for one or both of these hypotheses offers the potential of modifying the milk microbiome in ways that could improve infant outcomes.



A new study [2] of 393 mother-infant dyads simultaneously investigated both hypotheses by considering maternal and infant factors believed to influence the milk microbiome, including mode of delivery, maternal BMI, maternal diet, presence of older siblings, and indirect breast milk feeding (i.e., pumping). Milk samples were collected from healthy Canadian mothers between three and four months of lactation and were analyzed for milk microbiota composition as well as other milk components, such as oligosaccharides and fatty acids, that could potentially influence milk microbes [2].

There was a great deal of diversity across mothers, but the team identified 12 core microbes, defined as those that were present in at least 95% of milk samples with a minimum of 1% abundance. They then applied a statistical method to identify patterns among the 12 core microbes and found four clusters, each differing in the types of bacteria that were most abundant. Cluster 1 (C1) was made up of 42 milk samples and was enriched in *Moraxellaceae*, *Enterobacteriaceae*, and *Pseudomonadaceae*; C2 included 98 milk samples that were most abundant in *Streptococcaceae*, *Staphylococcaceae*, and *Comamonadaceae*; C3 was the largest cluster with 161 milk samples, and was enriched in *Oxalobacteriaceae* and *Comamonadaceae*; and finally, C4, from 92 milk samples, was enriched in *Streptococcaceae* and *Comamonadaceae* [2].

Cluster membership was most significantly associated with feeding method. C1 had the highest proportion of mothers that fed their infants pumped milk (86%), followed by C2 (66%), whereas less than half of the mothers in C3 and C4 engaged in indirect breastfeeding. Breastfeeding mode was also identified as the only consistent factor directly associated with milk microbiota in a more complex statistical model that considered all milk microbes [2].

With two different statistical approaches highlighting feeding method as an important determinant of milk microbiota, the researchers took a closer look at how milk from mothers that only engaged in direct feeding at the breast differed from that of mothers that occasionally or exclusively provided pumped milk. *Enterobacteriaceae* and *Enterococcaceae* were more enriched in indirect breastfeeding, as were the potentially opportunistic pathogens *Stenotrophomonas* and *Pseudomonadaceae*. *Gemellaceae*, *Vogesella*, and *Veillonellaceae*, a member of the oral microbiome, were more enriched with direct breastfeeding. In addition, species richness (i.e., the number of different species present in the milk) was greater in milks from mothers that provided breast milk directly.

Taken together, these findings offer strong support for the retrograde inoculation hypothesis. Pumping introduces exogenous bacteria to milk but also excludes an opportunity for contact with the infant oral cavity. The reduced species richness in pumped milk suggests a potentially critical role for infant oral microbes in shaping the milk microbiome [2]. Moreover, these findings also highlight the potential for pumped milk to contain harmful microbes that could expose infants to health risks and a lack of species diversity that could alter the development of their gut microbiome. These findings could be used to identify key taxa that may be missing from pumped milk that could potentially be supplemented and to educate parents on the importance of cleaning and disinfecting breast pumps after each use; pumping is

simply too integral to women's lactation plans to advise against providing pumped milk.

Interestingly, the study may have found additional support for the retrograde inoculation hypothesis by identifying sex-specific differences in milk microbial composition and diversity. For example, maternal BMI was found to be a significant factor on milk microbes in mothers with female offspring, whereas breastfeeding mode had a stronger association with male offspring [2]. Could hormonal differences between males and females influence their oral microbiota, which in turn could influence milk microbes? It is certainly an intriguing possibility, and sex differences in gut microbiomes have been identified. But the study authors also acknowledge that sex-specific differences have been found in [other milk components](#) and plan to integrate sex-stratified statistical tests in future studies in order to tease out just how infant sex might be influencing milk microbial communities [2].

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