SPLASH!® milk science update
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INTERNATIONAL MILK GENOMICS CONSORTIUM

This month's issue features antioxidants in milk, non-food uses for milk casein, milk microRNAs, and glycogen in human milk.

The Ultimate Superfood? Milk Offers Up a Glass Full of Antioxidants

- **Antioxidants are molecules that neutralize free radicals and help prevent damage from oxidative stress that is associated with neurodegenerative diseases, diabetes, and cancer.**
- **Milk and other dairy foods provide fat-soluble antioxidants that are lacking in plant foods and are essential for combating oxidative stress in the brain, heart, nervous tissue, and lungs.**
- **Milk's fat- and water-soluble antioxidants work together synergistically to protect against oxidation damage throughout the body.**

Superman earned his "super" for his ability to outrun a speeding bullet and leap tall buildings in a single bound. Superfoods like açaí berries, kale, and tomatoes earned their "super" for their high concentration of nutrients, particularly those that act as antioxidants. The superlative name is appropriate. Antioxidants possess superhero-like powers that prevent damage to cells and DNA from oxidative stress, thereby reducing the risk of developing certain cancers, type 2 diabetes, heart disease, and neurodegenerative diseases.

Milk and other dairy foods are densely packed with antioxidants; nearly every milk ingredient displays antioxidant properties. But because many of milk's antioxidants are delivered in milk's unique fat and protein packages, they have a set of superpowers that other superfoods cannot offer [1-3]. Milk may not be the most exotic superfood, but it might be the most super of them all.

Can you spare an electron?

It is hard to think of oxygen as anything but helpful. It is in the air we breathe and the water we drink. But it is also highly reactive and will steal electrons when it reacts with certain molecules. The result of this electron theft is a free radical—an atom (or molecule) with unpaired electrons. Electrons do not like odd numbers; once free radicals form, they immediately start looking to steal electrons from their closest neighbor. When they do, it starts a chain reaction of electron stealing and free radical production.

If left unchecked, the free-radical chain reaction can cause damage (known as oxidative stress) to cell membranes, cell organelles, proteins, and even DNA. Oxygen may be necessary for life, but oxidation can be detrimental. Oxidative stress is associated with the development of several chronic diseases, including type 2 diabetes, neurodegenerative diseases, atherosclerosis, and cancer [1].

Free radicals are produced by normal physiological processes, including metabolism, cellular respiration, and immune function. Additionally, cigarette smoke, air pollution, ozone, and radiation (the usual suspects) can also result in the production of free radicals. [3,4].

The best defense is a good offense, and what better offense to offer up than antioxidants. Antioxidants are molecules that are stable enough to donate an electron to a free radical (or take one from it) without becoming a free radical themselves [4]. Produced by cells or provided in the diet, antioxidants give the free radicals what they are looking for (an even number of electrons), and in doing so put an immediate end to the electron-stealing chain reaction. It's a molecular win-win.

These foods are super

It is impossible to prevent production of free radicals (cellular respiration is necessary for life), but oxidative stress can be limited if a balance is maintained between pro-oxidants and antioxidants. Several lines of evidence suggest that the growing incidence in diseases like cancer and diabetes can be directly linked to diets low in antioxidants [1,2]. These diets tip the balance favoring the pro-oxidant side, leading to permanent changes in cells and cellular structures, including cell membranes, proteins, and DNA.

If too few antioxidants in the diet can lead to disease, can eating foods rich in antioxidants help prevent it? The negative correlation

between diets high in antioxidants and the incidence of diet-dependent diseases supports this hypothesis, as does in vitro and in vivo research demonstrating the mechanisms by which dietary-derived antioxidants neutralize free radicals and protect cellular components.

As a result of these findings, antioxidant-rich foods were given a promotion to "superfood." Little known fruits like açaí and goji berries became overnight celebrities, and kale salad was added to the menu of all the trendy health food restaurants and smoothie shops. These fruits and vegetables (along with many others) offer up a wide array of antioxidants, including some familiar names (vitamin C, vitamin E, and beta-carotene) and lesser-known plant-based compounds (ellagic acid, phytic acid, and lycopene).

Not all antioxidants neutralize free radicals in precisely the same way, nor are all equally effective in different types of cellular environments. For example, vitamin C (ascorbic acid) is water-soluble whereas vitamin E (tocopherol) is fat-soluble. As a result, vitamin C has antioxidant effects in water-based environments of cells and vitamin E is active in lipid layers.

Because oxidative stress occurs in both water and lipid environments of cells, it is essential to eat foods that supply both types of antioxidants. Moreover, the parts of the body most vulnerable to oxidative stress (cell membranes, the brain, nervous tissues, cardiovascular tissues, and respiratory tissues) are composed primarily of lipids whose oxidation can only be neutralized by lipid-loving antioxidants [1]. With a few exceptions (e.g., vitamin E, vitamin A), the antioxidants supplied from fruits, vegetables, and grains are primarily water-soluble. Where are the fat-soluble antioxidants hiding?

It's a collaborative effort

Milk rarely makes the list of top ten superfoods, and yet milk fat provides one of the most potent fat-soluble antioxidants, conjugated linoleic acid (CLA). CLA is a naturally occurring *trans* fatty acid found only in the meat and milk of ruminants (bacteria in the gut of ruminants convert polyunsaturated fatty acids from green forage into CLA by adding hydrogen atoms). Although industrially produced *trans* fats are associated with poor health outcomes (e.g., increased cholesterol and heart disease), natural *trans* fat like CLA have several demonstrated health benefits, including those from their antioxidant activity. For example, both in vitro and in vivo animal model studies demonstrate a direct effect of CLA on rates of lipid peroxidation and free radical scavenging in cell membranes [1].

The anti-carcinogenic and anti-inflammatory effects associated with CLA are given a boost from other antioxidants in the milk fat globule, including vitamin A, vitamin E (as the compound α-tocopherol), and coenzyme Q_{10} [1]. Vitamin A protects against DNA oxidation, which is implicated in the development and progression of cancer [1]. Vitamin E levels in milk fat are low, but α-tocopherol is the most active antioxidant form of this vitamin. In milk, α-tocopherol is believed to limit oxidation in cell membranes through free radical scavenging [1]. Coenzyme Q_{10} is also protective of cell membranes, as well as the membranes of the cell's mitochondria (which are directly affected by cellular respiration). In addition, coenzyme Q_{10}enhances the mechanisms by which vitamin E limits lipid peroxidation in cell membranes [1].

Milk may not be the best source for vitamins A and E, or coenzyme Q_{10}, but the delivery of these nutrients alongside CLA allows for synergistic mechanisms that are unique to milk fat. And the synergy does not stop there; fat-soluble antioxidants extend their collaborative behavior to milk's water-soluble antioxidants—what Grażyna et al. refer to as an antioxidant network [1]. In this sense, milk's antioxidants are more effective than the sum of their parts.

Encrypted codes

Even without the lipid-bound antioxidants and their synergistic activities, milk could still earn superfood status from its unique proteins. Both whey and casein milk proteins contain fractions—either individual amino acids or short chains of them called peptides—with antioxidant behavior [2,3]. The bioactive peptides that are contained in milk proteins are known to be highly active free radical scavengers, but to unlock these properties, digestive enzymes in the gastrointestinal (GI) tract must break down the proteins [2]. Researchers refer to these hidden antioxidants as "encrypted" within the protein structure, which gives them more cachet, don't you think?

In their in vitro simulation of milk protein digestion, Tagliazucchi et al. [2] found that many of milk's bioactive peptides demonstrated protective effects specifically toward tissues along the GI tract. They describe milk proteins as "carriers" for these gut-specific antioxidant compounds, and propose that they may be critical in reducing oxidative damage to the intestines [2]. This activity has potential applicability to the onset of inflammatory diseases of the GI tract and GI cancers that may result from free radicals supplied from the diet (e.g., fried foods and alcohol) and from metabolic activities of the intestines.

Tagliazucchi et al. [2] repeated their digestion simulation with full-fat, low-fat, and nonfat milks, identifying different antioxidant properties with each. This finding indirectly supports Grażyna et al.'s [1] suggestion that milk fat- and water-soluble antioxidants have synergistic relationships. However, it also suggests that all types of dairy products enjoyed by consumers can confer protective health effects.

What's better than super?

With such a long list of ingredients with antioxidant properties, many of which are unique to milk, the oft-used term superfood seems to come up short for milk. It may not be as trendy as açaí berries or kale, but milk is certainly a candidate for the ultimate superfood.

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Contributed by
Dr. Lauren Milligan Newmark
Research Associate
Smithsonian Institute

Dairy Paper, Plastic or Milk?: Non-Food Uses for Milk Casein

- **In 2016 USDA researchers developed an edible, food packaging bio-polymer from milk casein that may keep food fresher than traditional plastics.**
- **Casein bio-polymers can minimize waste and add nutritional value to foods.**
- **Casein has been historically used to make glue, paint, and textiles.**

"Paper or plastic?" This simple question, asked daily at grocery stores and markets around the world, has become increasingly complex over the past couple of decades. The choice between all-natural, biodegradable fibers and synthetic, single-use films has political and environmental consequences ranging from deforestation to endangered sea turtles. But a recent innovation in the development of food packaging may signal a new option altogether. Instead of recycling or wasting your bag, what if you could eat it?

To be clear, edible shopping bags are not what we're talking about, but at last August's meeting of the American Chemical Society in Philadelphia, Peggy Tomasula, Laetitia Bonnaillie, and their USDA research team presented their current progress in developing milk protein-based food packaging. Because of their molecular flexibility and emulsifying and stabilizing properties—as they are mostly random coil polypeptides—caseins possess good film-forming and coating abilities [1]. Thus there have been other attempts to produce a casein-based food film; yet earlier versions of these were not effective barriers to moisture, and therefore were not developed for widespread use. This latest iteration, however, highlighted in a video [2] featuring Tomasula and her team, combines milk casein with glycerol and citrus pectin to form a soft but structurally sound bio-polymer that protects food from light, oxygen, and some humidity.

Two of the most likely applications of these bio-polymers, according to Bonnaillie [2], are as single-serving wrappers and as dissolvable packets. String cheese and some refrigerated snack foods are often packaged in individual plastic wrappers, ideal for packed lunches. But the amount of waste for these items is disproportionate to their full-sized counterparts and is a concern. Likewise, dehydrated soup packets could be waste-free with casein film pouches replacing traditional paper or plastic packaging. Packets can be dropped into warm water and the film dissolves within seconds. In both of these instances, edible casein bio-polymers would minimize waste, and could also add nutritional value as vitamins and supplements can be added to the films during manufacturing. Many hope that this most recent advancement in developing casein-based bio-polymers will be a turning point for such edible packaging and that we soon may see them used commercially.

The same chemical properties that make casein effective in bio-polymers also enable its use in other ways. Dating as far back as the Middle Ages, records indicate that wood glues and cement-like materials were derived of casein [3], and many manufacturers from the nineteenth century through the present have relied on glues made of casein, sodium hydroxide, and calcium hydroxide. Though synthetic alternatives are available, certain niche industries and applications still prefer casein-based glues, including the labeling of bottles.

Another historical use for casein was as an additive to paint. Specifically because of its water solubility and ability to bind to pigments, casein-based tempera paint dates back to ancient Egypt and is still available today. Many artists have been attracted to its fast-drying properties, including Andy Warhol, who painted "Popeye" and "Dick Tracy" with it in 1960. Most contemporary artists prefer acrylic paint, which was developed in the late 1960s, though casein paint is still available and used by some.

Perhaps somewhat surprisingly, casein-based fibers and textiles were developed in the early 20th century, and their use peaked around World War 2 when they were combined with wool, cotton, and rayon. Aralac, the U.S. trade name for one such fiber, was not particularly strong, and so was often woven into fabric blends with pure wool, which was scarce during wartime, for added durability. It was also often a rather bland looking material, as it did not hold dye well. Though most have abandoned the idea of casein fibers, a German company is currently producing a milk-based fabric that has a silk-like texture [4].

A common feature of these casein-based non-food products—glue, paint, and fiber—is that each was superseded with a more durable, cost-effective, and/or marketable synthetic replacement. Yet with Tomasula and her team's recent production of an improved, edible casein-based film, perhaps this trend is shifting. As we look for safer and sustainable options for food packaging, it seems logical to turn to the staples of our diet, and their component parts, for inspiration and innovation. Hopefully, in the near future, we will be able to opt for not only paper or plastic but for milk.

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Contributed by
Dr. Katie Rodger
Managing Editor
SPLASH® milk science update

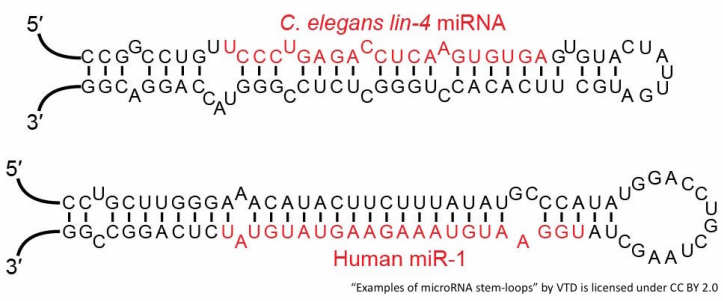
Milk microRNAs—Lots of Potential Functions but Conflicting Evidence

- **Milk contains microRNAs that are protected from degradation by a lipid coat.**
- **Milk microRNAs are altered in quantity and type by milk processing.**
- **There is conflicting evidence whether milk microRNAs can change offspring tissue functions.**

Controversy in science is good. It invites additional investigation generating new data, and ultimately there is either adequate proof or a lack of substantiation of an idea. The scientific idea then either swims into the future or sinks into oblivion. It is a proven but brutal, survival- of-the-fittest evolutionary process. This is how science works. In the intriguing case of the functions of milk microRNAs, this laborious scientific process is still underway.

What are microRNAs and what do they do?

A genome is the complete DNA code of an organism containing the instructions for the program of life and enabling the exquisitely efficient and accurate transfer of this coded information from one generation to the next. Most of the scientific focus on genomes over the last few decades relates to the identification of genes that encode for proteins within each species. Only about 1.5% of the genome codes for proteins. The neglected remainder of the genome is now revealing its many secrets.



"Examples of microRNA stem-loops" by VTD is licensed under CC BY 2.0

The production of a protein from a gene is complex and involves an intermediary called messenger RNA, the first cousin of DNA. Proteins form components of large cellular structures and make myriad biochemicals that are essential for everyday life. Changing the quantity of a protein often alters cellular and tissue functions. Only recently have scientists discovered that the genome also makes a huge variety of very small and very large RNAs that do not encode proteins. The functions of these RNAs are only partially known.

One group of very small RNAs is called microRNAs. Each microRNA functions by putting a brake on the amount of a specific protein being made within a cell, thus microRNAs are ultimately regulators of the quantities of target proteins being synthesized, and through that mechanism, microRNAs affect cell function. MicroRNAs have big reputations as they regulate many cellular processes, particularly the development of functionally specialized cells like muscle, nerve, and immune cells.

Dietary microRNAs

MicroRNAs are present in many foods [1, 2]. In particular, strong evidence from multiple sources shows that microRNAs are enriched compared to mammary cells in milk from cattle, humans, pigs, and rodents [1, 3–10]. This conclusion is not in dispute as it has jumped the rigorous hurdle of multiple detailed scientific investigations. It was initially thought that microRNAs should be vulnerable to destruction outside of cells, especially in milk; however, they are remarkably stable in body fluids [11]. This stability is due to a sphere of protective fatty acids surrounding multiple microRNAs [1, 7]. Perhaps microRNA stability is only a relative term as several investigations indicate that milk processing radically changes the quantities and types of microRNAs within milk [3, 7–9].

The biologically intriguing question is whether ingested milk microRNAs are stable in the gut and then regulate tissue development and function in the offspring. This is where the controversy begins.

Are dietary microRNAs present in offspring tissues?

The journey of ingested microRNAs to their target cells in the offspring is wrought with extreme difficulties. They not only have to survive the digestive activities of the gut, but microRNAs must pass through the gut cell barrier, enter into the blood circulation and then be absorbed by specific cells in a tissue. Here, sufficient quantities of the absorbed microRNAs must be present to regulate the quantity of the target protein within a cell and therefore its functions. This may be a tall order!

Several scientific investigations concluded that ingested microRNAs are stable and function in offspring. One study directly investigated the stability of two microRNAs under simulated gastrointestional conditions, and the investigators concluded that they can survive the perils of the gastrointestinal tract [12]. In addition, it is well established that the digestive functions of newborns are immature, which would also assist the survival of microRNAs in the gut. Other investigators demonstrated that bovine milk microRNAs are taken into human kidney, intestinal and immune cells grown in laboratory culture [10, 13, 14]. The cellular specificity and functional implications of this action are unclear. There is also scattered evidence that ingested milk microRNAs are taken up by offspring tissues where they alter tissue function, particularly immune system function [1, 2, 4, 10, 14–16].

The opposing conclusion that dietary microRNAs ingested by another species do not have biological effects after ingestion has been reached by a raft of recent scientific publications (summarized in [17–20]). Moreover, specific studies of humans ingesting bovine milk could not detect the transfer of bovine microRNAs into the blood circulation [20].

The contradictory information in these collective publications, however, cannot be easily reconciled. At this time, only more research using smart experimental designs can settle this scientific controversy. The urgency of this task is emphasized by preliminary reports that milk microRNAs may help some medical conditions, such as arthritis [14].

Conclusions

MicroRNAs are present in milk and have potential to promote the development of tissues in newborn offspring—a maternal inheritance offering a helping hand. However, the scientific jury has not yet received sufficient evidence to enable a decision regarding the effects of ingested microRNAs on an individual.

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Contributed by
Dr. Ross Tellam (AM)
Research Scientist
Brisbane, Australia

Human Milk Contains Glycogen

- **Researchers in Japan have demonstrated for the first time that human milk contains glycogen.**
- **The amount of glycogen reported is low, however it is higher in milk expressed within two months after birth, and among women with infected breast tissue.**
- **The source of glycogen in human milk is currently unconfirmed and its function unknown.**

Even though human milk is one of the most important substances to the healthy development of people everywhere, its basic composition is still the subject of entirely new discoveries. In a paper published in February this year, researchers from two institutions in Japan—the Institute of Health Sciences at Ezaki Glico Company and the University of Shiga Prefecture—report the presence of glycogen in human milk [1]. Over the years, there have been circumstantial suggestions that human milk may contain this carbohydrate. But this new finding is the first example of strong, confirmatory evidence of glycogen's presence.

Glycogen is best known as an energy store in the muscles and liver where it can be broken down into glucose. In muscle, the glucose that is released powers muscle contraction. That released in the liver circulates in the blood from which is it is delivered to all kinds of tissues in need of basic fuel for respiration.

Hiroko Matsui-Yatsuhashi, the lead author on the recent study, and her colleagues, first identified glycogen using several kinds of complex chromatography techniques—essentially tools to compare the relative sizes of molecules. These analyses showed that the substance they had extracted from human milk was indeed structurally very similar to glycogen found in cow's livers and in mussels (two dietary sources of glycogen), and gave the researchers an idea of the number of glucose monomers that were linked together in human-milk glycogen polymers.

Matsui-Yatsuhashi and her team then took a hint from nature: they used enzymes that only work on glycogen to hydrolyze it into glucose. This enabled them to measure the amount of glycogen in human milk that was donated by breastfeeding women who attended Nagao Clinic in Kansai region, Japan.

The results were not dramatic in their amounts—the concentration of glycogen in human milk tends to be low, and much lower than in cow's milk—but that does not preclude human-milk glycogen from having an important biological role. Over the course of lactation, the average concentration of glycogen in normal human milk was found to be 3.21 µg/mL, and the concentration was somewhat higher during the first two months of lactation than it was thereafter. The strongest indication of the glycogen's source came from measuring its concentration in the milk of women suffering from mastitis—inflammation of the breast. Milk from these women contained substantially higher levels of glycogen. Indeed, when the researchers analyzed milk from each breast of women with only one inflamed breast, they found that the milk from the inflamed breast had more than a dozen times the concentration of glycogen than the milk from the normal breast.

The source of glycogen in human milk is unknown, but the mastitis result offers a hint. Aside from muscle and liver cells, leukocytes (white blood cells) are known to contain glycogen—and leukocytes accumulate in the mammary gland during mastitis. Much higher quantities of leukocytes are present in the milk of mothers with mastitis compared to the milk of healthy mothers during peak

lactation. The source of glycogen in human milk might, therefore, be milk leukocytes.

While little is certain about the source of human milk glycogen, even less is known about its function. Given the high molecular weight of the glycogen that Matsui-Yatsuhashi and her team found, they suggest that some of it likely passes through the infant stomach unscathed and makes it all the way to the intestines. This is a prerequisite of a functional role, but unproven at the moment. How differing levels of glycogen might be affecting infant gut immunity in the first two months of life compared to later on—and in infants whose mothers suffer from mastitis—are surely questions worth answering.

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

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