



### SPLASH! milk science update

MAY 2013 issue

This month's newsletter tells the story of [protein nets found in cow's milk](#) that trap and kill bacteria, the immense task of [cataloging all bacterial species](#) found in milk, how [milk letdown evolved](#) and the hormones responsible for this critical physiological response, and a [review of Mary Roach's new book](#).

We hope you enjoy this action-packed issue of *SPLASH! milk science update*!

## Milk Cells Cast NETs to Snare Pathogens

- **Latest study identifies nearly 3000 unique proteins in cow's milk.**
- **Immune cells secrete a web-like network in the milk to thwart pathogens.**
- **These NETs (neutrophil extracellular traps) specifically bind and kill bacteria.**
- **NETs may have diagnostic potential for identifying subclinical mastitis.**

The list of proteins in cow's milk just keeps getting longer. Tim Reinhardt and colleagues have now discovered nearly 3000 unique proteins in cow's milk (1). While that would be a story by itself, they also reveal evidence for web-like nets that immune cells secrete into milk when pathogens are present. Yes, milk is cooler than fiction.

### Why does the list of milk proteins keep getting longer?

With each publication of the cow's milk proteome—a catalog of all of the proteins in cow's milk—the number of proteins in the catalog increases. Why? While mass spectrometry does improve, the biggest gains have occurred in fine-tuned separation of milk into fractions such that the highly abundant proteins in one fraction no longer eclipse the minorly abundant proteins in another. Reinhardt and colleagues have perfected this technique for cow's milk and published, for the first time, all of the proteins identified in all of the fractions (1). They also included milk from cows with a mammary gland infection (a.k.a. mastitis). With mastitis, an assortment of other immune-related proteins are secreted into milk, and these add to the long list of milk proteins.

### What do all these milk proteins do?

Dietary proteins provide a source of building blocks that can then be used by our bodies to build our own proteins. Unlike most plant proteins, milk proteins actually provide a well-balanced assortment of building blocks so that consumers can build the proteins they need without running out of a particular type of block. But milk proteins do even more than that. Some proteins form structures to escort calcium and fat, some are digestion-aiding enzymes, some are antimicrobial, and more. Perhaps the most fascinating milk protein is alpha-lactalbumin (LALBA), which transforms into HAMLET, a protein-fat complex that kills infected cells but spares normal ones (2).

### Bacteria-killing NETs found in milk

Undoubtedly, the most surprising finding in the latest paper from Reinhardt and colleagues, is the presence of neutrophil extracellular traps (NETs) in the fat fraction of milk. Neutrophils are a type of immune cell, and NETs are a web-like network of DNA and proteins that are secreted by these cells. NETs are able to trap bacteria and kill them (3). While a spider spins a web to catch a fly and then kills the fly with venom, NETs are like both the web and the venom together in one package.

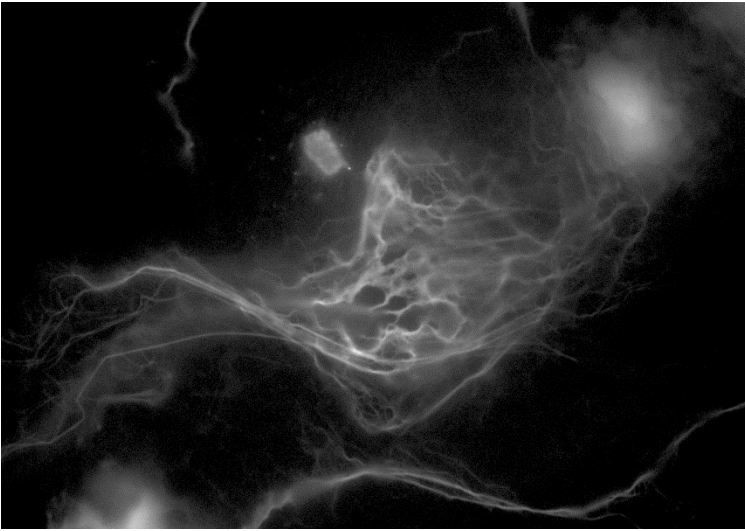


Image from Dr. Tim Reinhardt, Ruminant Diseases and Immunology Research Unit, Agricultural Research Service, USDA

In 2006, Lippolis, Reinhardt and others provided the first evidence of NET formation in milk (4). They incubated neutrophils in milk for up to six hours and then added the bacteria usually found in mammary gland infections. The neutrophils secreted NETs. In fact, neutrophils in milk were even more responsive to these bacteria than neutrophils in generic culture media.

After such compelling work, how is it that the next (and only other) paper on NETs in milk does not occur until 2013? In this next paper, the authors provide proteomic evidence consistent with NET presence and, more importantly, visual confirmation (1). The web-like structure of NETs is an image of beauty. Well, unless you're a bacterium.

### What is next for milk NETs?

The most obvious application for NETs in the dairy industry is in the area of diagnostics. Mastitis is currently tracked in dairy herds using the California Mastitis Test (CMT). The CMT is cheap and simply performed, but has imperfections as it is based on the number of immune cells present, which can change with age or noninfectious sources of inflammation (e.g., injury, irritation). Perhaps NETs, which are specifically secreted in the presence of mastitis-causing bacteria, could be used to design a new diagnostic to complement the CMT?

1. Reinhardt TA, Sacco RE, Nonnecke BJ, Lippolis JD. (2013) Bovine milk proteome: Quantitative changes in normal milk exosomes, milk fat globule membranes and whey proteomes resulting from *Staphylococcus aureus* mastitis. *J Proteomics* **82C**:141-154.
2. Svensson M, Düringer C, Hallgren O, Mossberg AK, Håkansson A, Linse S, Svanborg C. (2002) Hamlet--a complex from human milk that induces apoptosis in tumor cells but spares healthy cells. *Adv Exp Med Biol* **503**:125-132.
3. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, Weinrauch Y, Zychlinsky A. (2004) Neutrophil extracellular traps kill bacteria. *Science* **303**:1532-1535.
4. Lippolis JD, Reinhardt TA, Goff JP, Horst RL. (2006) Neutrophil extracellular trap formation by bovine neutrophils is not inhibited by milk. *Vet Immunol Immunopathol* **113**:248-255.

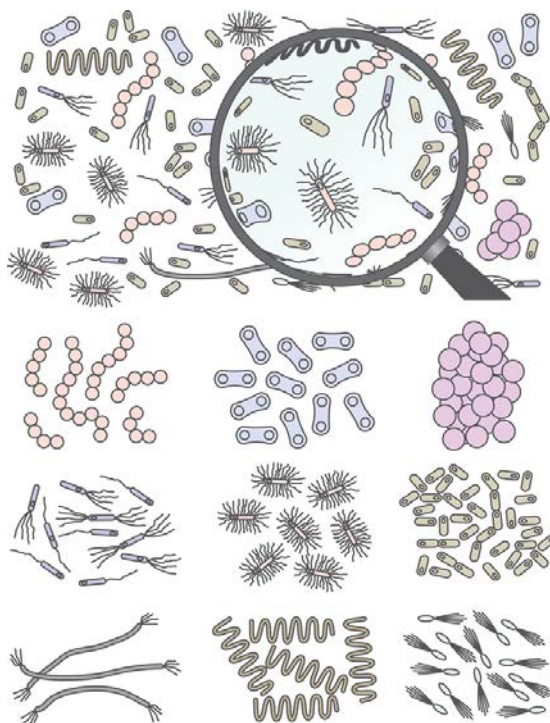
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## From Mother's Gut to Milk

- A new study scans breast milk for the different bacterial species found in it.
- Species that can grow whether or not there is air around them colonize a baby's intestine first, but are then overrun by other species that flourish in the absence of oxygen, like *Bifidobacterium infantis*.
- The class Clostridia can be found in breast milk, and probably travels there from mom's gut.

Milk enthusiasts probably all share a favorite bacterium: *Bifidobacterium infantis*, the species that coevolved with humans and promotes a healthy infant gut. Breast milk contains many other kinds of bacteria, but recording the full species register is a surprisingly tricky task. Recently, a team of Swiss researchers did the most complete job yet.



Ted Jost and others<sup>1</sup> in the Zurich-based group took milk samples from seven women at three intervals after giving birth, cultured the milk in various ways, and then sequenced the DNA in the milk using multiple techniques. That should cover all the bases. Their culture methods, numbering nine, catered for bacteria of every lifestyle choice. They laced agar jelly with all manner of nutrient mixtures and provided airy compartments for species that like to grow in oxygen. Meanwhile species that flourish despite an absence of oxygen (facultative anaerobes) or can't handle life in its presence (obligate anaerobes) were given the chance to grow in an anaerobic chamber.

Usually, the first kinds of bacteria to set up camp in a baby's intestine are facultative anaerobes like *Escherichia coli* species. As expected, *Streptococci*, another facultative anaerobe, was one of the most common categories identified in Jost et al.'s study. Then, after a few days, when the facultative anaerobes have used up the oxygen in a baby's gut lumen, populations of obligate anaerobes, like *Bifido* species, start to take off<sup>2</sup>. And *Bifidobacterium infantis*, by far the best at chomping through the oligosaccharides in breast milk, grows especially fast. Its growth aids the development of the baby's immune system and makes life difficult for any pathogen trying to find a spot. This sequence of events is fairly well understood.

Jost et al.'s results largely confirmed the above. But they are interesting for another reason. For the first time, several genera from the class of obligate anaerobes known as *Clostridia* were identified in breast milk. Some of these—*Faecalibacterium* and *Roseburia*—release relatively large amounts of a substance, butyrate, which keeps colons healthy. Butyrate is a short-chained fatty acid that reduces inflammation along the lining of the gut, possibly by inducing the death of immune cells called T cells. (*B. infantis* also has an anti-inflammatory influence. When fed the complex sugars found in breast milk, it binds especially well to intestinal epithelial cells and causes them to make more cytokines.)

The fact that hardcore obligate anaerobes like *Clostridia* and some species of *Bacteroidetes* popped up in their breast milk samples led Jost et al. to propose that these bugs originate in mom's gut. That is to say, they traveled from her intestines to her mammary glands in, probably, immune cells known as dendritic cells and macrophages. Once in her mammary gland, they found their way into breast milk.

This idea has been proposed before by others, but the evidence has always been hard to pin down. To really prove the point, researchers would ideally find a macrophage with a labeled gut bacteria tagging along, and then find the same labeled bacteria inside the host mom's breast milk. Jost et al. haven't managed that, but their paper certainly adds to indications that this route of bacterial transfer exists.

1. Jost T, Lacroix C, Braegger C, Chassard C. (2013) Assessment of bacterial diversity in breast milk using culture-dependent and culture-independent approaches. *Br J Nutr* **14**:1-10.

2. Garrido D, Dallas DC, Mills DA. (2013) Consumption of human milk glycoconjugates by infant-associated bifidobacteria: mechanisms and implications. *Microbiology* **159**:649-664.

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## The Evolutionary Origins of Milk Letdown

A hormone called oxytocin causes muscle cells in the mammary gland to contract and release milk (letdown).

- **Oxytocin (OXT)**—the milk letdown hormone—also influences social behavior.
- **Researchers identify vasotocin (VT), a water maintenance hormone--as OXT's ancestral origin in jawless fishes.**
- **Mesotocin (MT) evolved from VT, retaining VT's functionality with the addition of social behavioral influence.**
- **OXT evolved from MT, possibly three separate times, with the change of just one amino acid!**
- **One tiny change in the genetic code appears essential to the rise of placental mammals.**

The production of milk by the mammary gland is a key adaptation of mammals. But simply producing the milk is not enough; that milk needs to get from the mammary gland to the nipple to be ingested by the infant. But how is this movement of milk accomplished? In eutherian (or placental) mammals, a branch of mammals that includes primates, whales, rodents, and bats, infant suckling stimulates the pituitary gland to release a hormone called oxytocin into the maternal circulation. Once oxytocin reaches the mammary gland, it causes smooth muscle cells around the gland to contract and release milk in a process known as milk letdown. Oxytocin also causes contractions in the eutherian mammal uterus, which permits successful childbirth. Its essential role in both lactation and pregnancy suggests that the evolution of eutherian mammals was dependent on the presence of the oxytocin hormone. We know that mammals evolved from reptiles - but where did oxytocin come from?

### Embarking on a molecular excavation

When paleontologists want to reconstruct the evolutionary history of a species they turn to the fossil record. Fossilized remains of extinct species demonstrate how evolutionary processes modify existing anatomical features, resulting in new features with different functions. But whole organisms are not the only entities with evolutionary histories of interest. Molecular biologists investigating the origins of proteins, and the genes that code for them, follow a surprisingly similar process as paleontologists. To reconstruct the evolutionary origins of the oxytocin hormone, Yamashita and Kitano (2013) went digging into the genomes of species representing all of the major vertebrate classes (e.g., fish, birds, amphibians, mammals) in search of small chemical changes in the DNA code of the oxytocin gene that might represent an ancestor of this hormone. DNA consists of four letters (C, G, T, and A), each representing a nucleic acid (cytosine, guanine, thymine, and adenine). The genetic code is read in a series of three letter "words" (ACT or GGC, for example), each word coding for an amino acid, the building blocks of proteins. Just like small changes in the size and shape of bones influences the function of an appendage, changes in the order of amino acids for a particular protein, such as a hormone, can have major impacts on that protein's function. Yamashita and Kitano's molecular "excavations" identified numerous amino acid substitutions in the oxytocin genetic family tree, suggesting quite ancient roots for the hormone responsible for successful childbirth and lactation in humans and other eutherian mammals.

### Identifying the common ancestor

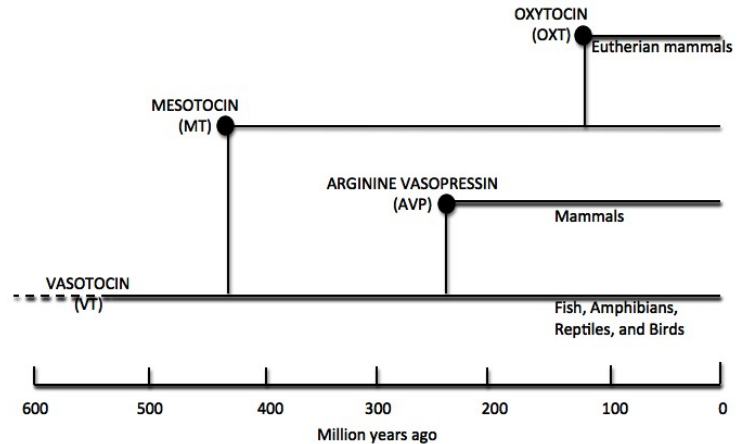
Yamashita and Kitano's evolutionary approach utilized genetic data from several eutherian mammal species (including humans, chimpanzees, macaques, mice, and cows), as well as a marsupial (an opossum, a mammal with a pouch), monotreme (a platypus, an egg-laying mammal), bird, amphibian, fish, and even a lamprey, a jawless fish believed to have shared a last common ancestor with humans approximately 550 million years ago (mya). Not all of the species they surveyed have the oxytocin gene (OXT), but all except the lamprey have at least one gene that is similar in amino acid sequence to OXT as well as another hormone called arginine vasopressin (AVP). AVP is responsible for maintaining water in the body, constricting blood vessels, and, like OXT, may influence social behavior (Heinrichs and Domes, 2008). In humans, these hormones are located on opposite ends of chromosome 20. Their similarity in function, structure (i.e., amino acid sequence), and location suggest they also have a similar evolutionary history.

Yamashita and Kitano followed this lead and looked for the origins of OXT in hormones similar to AVP. Here is where the lamprey becomes critical to the story; unlike all of the jawed vertebrates, lampreys only have vasotocin (VT), a hormone related to AVP with similar antidiuretic effects. The researchers concluded that VT must be the common ancestor of AVP and OXT.



## From sea to land

The next major branching event on the oxytocin family tree is estimated to have taken place approximately 400 mya with the appearance of mesotocin (MT). MT evolved from VT and is currently found in jawed fishes, amphibians, birds, reptiles, and most marsupials (Acher et al., 1995). Research in birds suggests MT may influence social behavior, including female flocking behavior (Goodson et al., 2009). In monotreme and marsupial mammals, MT plays a role in milk production. The sex-specific effects on social behavior and its role in lactation suggest that MT is closely related to OXT, and genetic data confirm that MT is structurally similar to OXT in noneutherian vertebrates.



## Oxytocin: Ancient ancestry or evolutionary convergence?

The evolutionary origins of OXT appear more complicated than MT. This hormone is present in all eutherian mammals and also in sharks and platypus. There are two possible scenarios to explain its distribution. The first is that it originated just after the appearance of MT, approximately 400 mya, and then subsequently disappeared in bony fish, amphibians, birds, and marsupials. The second scenario has oxytocin possibly evolving more than one time. Independent evolution of this hormone in three separate lineages (eutherian mammals, sharks, and platypus) is an example of convergent evolution. Genetic data support the second scenario. Although it appears to be evolutionarily ancient, oxytocin in sharks and platypus are unique to these evolutionary lineages, arising separately from MT by a mutation that changed only one amino acid. Amazingly, the same amino acid change in the MT gene produced OXT in all three separate lineage at different points in time. Yamashita and Kitano estimate that the mutation that produced the eutherian OXT from MT occurred approximately 24 million years before the appearance of the first eutherian mammals in the fossil record.

## Oxytocin: Where would eutherian mammals be without it?

The evolutionary history of oxytocin is an elegant example of how small changes in the DNA code over long periods of time can result in profound differences in the function of a protein. Yamashita and Kitano hypothesize that the ancestral hormone vasotocin was a neurotransmitter directed toward the central nervous system in jawless fishes. Oxytocin retains this ancient function, and numerous studies have demonstrated its influence on maternal (and paternal) behaviors and pair bonding in humans and other eutherian mammals. But small changes in amino acids in VT, and then MT, over nearly 400 million years of vertebrate evolution brought about new functional roles for oxytocin in milk letdown and uterine contractions. These functions of oxytocin are essential to the eutherian mammalian niche but are not physiological features that appear in the fossil record. Through molecular excavations, their origins can be traced to nearly 24 million years before the appearance of eutherian mammals, leading Yamashita and Kitano to suggest the evolution of oxytocin was essential to the evolution of eutherian mammals.

Acher R, Chauvet J, Chauvet MT. (1995) Man and the chimaera. Selective versus neutral oxytocin evolution. *Adv Exp Med Biol* **395**: 615-627.

Goodson JL, Schrock SE, Klatt JD, Kabelik D, Kingsbury MA. (2009) Mesotocin and nonapeptide receptors promote estrildid flocking behavior. *Science* **325**: 862-866.

Heinrichs M & Domes G. (2008) Neuropeptides and social behaviour: effects of oxytocin and vasopressin in humans. *Prof Brain Res* **170**: 337-350.

Yamashita K & Kitano T. (2013) Molecular evolution of the oxytocin-oxytocin receptor system in eutherians. *Mol Phylogenet Evol* **67**: 520-528.

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## A Delectable Gobble

The last time I read Mary Roach was on holiday, in the evenings that followed long hikes up, along, and down tabletop mountains in southern Venezuela. I read aloud from *Stiff: The Curious Lives of Human Cadavers* because my tent buddy was too careful a packer to have allowed himself the additional mass of a book. As he sensibly tended to sore blisters and swollen bites, I enriched the moment by, for example, loudly vocalizing Roach's prose about the precise details of how and when death occurs in people who are unlucky enough find themselves inside planes that explode in the sky.

This time I'm digesting *Gulp: Adventures on the Alimentary Canal* in the tearoom of the University Library in Cambridge, England. Although this is the sort of canteen that lumps all formulations of coffee that are not 'filter coffee' into the single category 'specialty coffee', the students here call the library 'Alcatraz' because of its décor, not the quality of the refreshments.

I find myself paying close attention to the mastications of those around me. The way you chew, according to Roach, is 'as unique and consistent as the way you walk or fold shirts'. Indeed, the best predictor of how long people spend chewing before they swallow is who's doing the chewing, not any attribute of the food that they're eating. Before today, I think I've only consciously considered how I chew during moments of inappropriate inelegance.

This is one of Roach's many gifts: she arouses readers' sense of intrigue in the mundane and then satisfies their curiosity using simultaneously crisp, graceful, and puckish turns of phrase. Chewing comes after her discussions of feeding preferences, part of a narrative arch that goes on to follow the entire structure of the alimentary canal. The book is replete with yuck-but-wow anecdotes that are tempting to inflict on those around you as you read. Yesterday, as I was having my hair cut, I informed the person doing the cutting that human hair is in fact kosher, should she ever get hungry at work. Pet food, I let the cat know this morning, has a higher nutritional value (for the humans who eat it – and a surprisingly large number apparently do) than sirloin steak, McDonald's burgers, bacon, fried chicken, ham, or shrimp. (I was not enthused enough to try it, though.)

Roach weaves such details, digressions, and sub-stories together deftly. And what seem like tangential or ridiculous questions – Can chewing lower the national debt? Can the eaten eat back? – are answered engagingly, and linked back to the book's fixed path with some carefully applied literary peristalsis.

She has dug up some real gems. The story behind that photo taken in the Florida everglades, of a dead alligator and the burst python that made the fateful decision to swallow it, probably involved the alligator kicking its way out, the experimental evidence suggests. A chapter titled 'I'm all stopped up' describes her interview with Elvis's longtime doctor, George Nichopoulos, and suggests that the singer died from straining so hard on the toilet that he threw off the rhythm in his heart. The autopsy of Elvis's body found no obvious cause of death, although it did reveal that his colon was two to three times normal size. Today, 'megacolon' is commonly associated with colon inertia, but back in the 1970s medical practitioners didn't realize that, in certain cases, constipation can kill.

Roach offers only a few brief notes about milk, where plenty more would have been apt for this book. She mentions that lactose intolerance can lead to flatulence and that baby saliva contains lots of lipase, which tapers off as infants are weaned. But those examples aside, her observations are sociological, not biological. Roach reports that the consumption of five glasses of a woman's breast milk is sufficient, according to one Islamic scholar, for a man to be the woman's 'breast milk son', and therefore left alone with her, even though she is not a member of his immediate family. Another scholar writes that the man must drink from her breast. 'Which is crazier,' asks Roach, 'that Saudi courts, in 2009, sentenced a woman to forty lashes and four months in prison for allowing a bread deliveryman inside her home, or the notion that she might have avoided punishment by letting him suckle on her breast?'

I finish my reviewing session fully satiated with weird and wonderful—and disgusting—facts, and head to the toilet where someone once scratched on the back of the door 'Graffiti, spontaneous and anonymous, is the purest form of art. Discuss.'



Sitting there, I flick back to Roach's chapter on Elvis and wonder whether to add to Alcatraz's decoration with a passage from the autopsy report: 'this [Elvis's] megacolon was jam-packed... the impaction had the consistency of clay and seemed to defy Florendo's efforts with the scissors to cut it out'. Or with Roach's introduction to a late chapter: 'Should circumstance prevent a man from carrying his cigarettes and mobile phone in his trousers pocket, the rectum provides a workable alternative. So workable that well over a thousand pounds (400kg) of tobacco and hundreds of mobile phones are rectally smuggled into California state prisons every year'.

I decide against it. If there is a take home message here, it's to relax as you go.

Roach, M. (2013) *Gulp: Adventures on the Alimentary Canal*. W. W. Norton & Company, Inc.

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