Center of Excellence

Immune Supporting Properties of Milk
Part 3: Selected members of the innate immune system in milk – Oligosaccharides, glycosaminoglycans, glycomacropeptide, lysozyme and alpha-lactalbumin

ADPI® Center of Excellence (COE) team member Dr. David Clark shares his insight and knowledge on the immune supporting properties of milk.

Introduction

Milk really is a veritable treasure trove of components that provide support to the immune system. An overview of some aspects of the immune modulating activity of milk constituents is provided in this series of articles by COE member Dr. David Clark. Attention is drawn to any recently published findings on the effectiveness of milk components against the SARS-Cov-2 virus, the cause of the COVID-19 pandemic. In this third article in the series, the focus turns to a selection of milk components that contribute to the innate immune system.

Dairy-derived components in the innate immune system arsenal

In the first article in this series, we referred to the immune components in milk as an arsenal of weapons. Extending this analogy further, the components that are members of the **adaptive immune system** can be considered 'smart weapons'. An example of the latter are the antibodies in milk, which 'paint' infected cells and intruding organisms to assist the 'cavalry' in the form of natural killer cells and other lymphocytes circulating in the vascular and lymphatic systems to seek and destroy them.

Development and production of 'smart weapons' takes time, so nature provides a solution in milk through inclusion of multiple elements of the **innate immune system**. While these components are less targeted than antibodies, they stall progress of the infection to buy time for the adaptive system to join the fight. In this article, 5 members milk's innate system are discussed. They deploy a range of mechanisms to slow down and confuse the invader. For convenience, we will refer to these 5 milk components as the 3-letter innate squad comprising BMO, GAG, GMP, LYS and A-LA (or in long hand – Bovine Milk Oligosaccharides, glycosaminoglycans, glycomacropeptide, lysozyme and α-lactalbumin). Note that this list includes only about half the components in milk's innate immune system!

Returning to the arsenal metaphor, BMO, GAG and GMP confuse the invader by acting as decoy receptors, tricking the infecting agent by presenting 'fake' binding sites that mimic those found on cell surfaces, essentially 'neutralizing' their capability to infect. Oligosaccharides fulfil a further defensive role by selectively feeding probiotic organisms. These friendly bacteria could be considered analogous to 'mercenaries', that multiply to prevent colonization of the gut by weight of numbers, crowding out pathogenic organisms. Then we have lysozyme (LYS) that sabotages the integrity of the 'armored' cell wall of gram-positive pathogenic bacteria by enzymatic action literally causing these 'enemy' bacterial cells to explode. Finally, the lethal and indiscriminate nature of some of the 'chemical weapons' that are deployed by other members of the immune system – specifically reactive oxygen species (ROS), means that 'damage control' is essential. A-LA fuels damage limitation by providing a source of cysteine needed to produce glutathione (GSH). GSH is a powerful anti-oxidant, which helps contain the 'fires' i.e. inflammation caused by ROS produced by other elements of the immune system to kill invading or infected cells. Having introduced them, let's take a closer look at the members of the 3-letter squad!

Oligosaccharides – the innate immune system's first line of defense?

In some species, elements of the innate system appear to be more important in the early stages of neonate development than the adaptive system. For example, marsupials give birth to a very under developed neonate, roughly equivalent to an 8 week old human fetus! Following birth, assisted by olfactory means, the marsupial neonate navigates a path to the mammary gland in the mother's pouch and fuses to a nipple. Examination of the composition of early marsupial milk provides some hints as to which ingredients are likely to be most critical to the survival of these most vulnerable neonates.

It turns out that early milk in many species of marsupials, in common with that of humans, is highly enriched in oligosaccharides (Messer & Green, 1979; Newburg et al., 1999). This is suggestive that in the most primitive of live birth neonates, oligosaccharides may constitute the 'first line of defense' against infection for quite an extended period of their development. In contrast, oligosaccharides in the milk provided to mammalian neonates, which generally have a more developed immune capability, is only transiently dominated by oligosaccharides. For example, while cow's colostrum is a rich source of oligosaccharides, the content rapidly declines in the transitional milk and is much lower in mature cow's milk, compared to that of humans.

There are a dizzying array of different oligosaccharides in human and bovine milk, many with complex branched structures as illustrated in Figure 1. The complex branched structures of BMO and HMOs (human milk oligosaccharides) contrast sharply with the simple linear structure of GOS (galacto-oligosaccharide). GOS is comprised of a terminal glucose unit coupled to a string of galactose units and can be synthesized from lactose on a commercial scale by enzyme treatment. It is worth pointing out that GOS more closely mimics HMOs and BMOs, as it is composed of sugar molecules that occur naturally in these milk oligosaccharides, which is not the case with vegetable derived oligosaccharides such a fructo-oligosaccharide (FOS) and inulin (Figure 1).

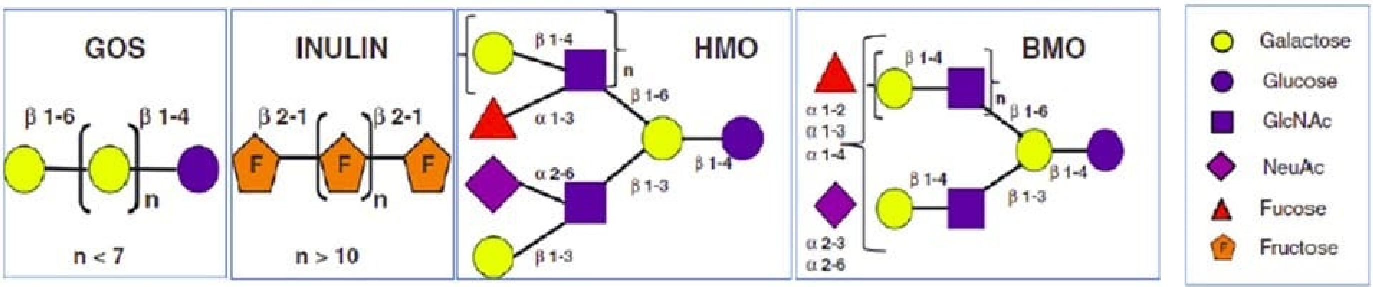

Figure 1: Schematic diagram comparing the linear structures of GOS and FOS with known HMO and BMO structural types. HMOs and BMOs comprise additional carbohydrate types and branched structures (Barile and Restall, 2013).

How do oligosaccharides support the immune system? Oligosaccharides are prebiotics and defend against infection by pathogens by at least two distinct mechanisms. One mechanism is called competitive exclusion. Oligosaccharides co-evolved with their hosts such that their structure can only be broken down and used as nutrients by limited classes of beneficial ‘probiotic’ bacteria – i.e. those that are equipped with the necessary enzymes. This explains the presence of the high degree of complexity found in BMOs and HMOs. It allows these prebiotics to behave rather like the selective culture media used in microbiology labs. Such selective media are chosen by microbiologists to stimulate growth of the target pathogen, e.g. Salmonella in a food sample to levels that practically guarantee detection should it be present. In case of milk oligosaccharides, individual variants have co-evolved with the host to selectively stimulate growth of beneficial probiotic bacteria to such an extent that pathogens are ‘crowded out’, so-called competitive exclusion.

The second mechanism of immune support displayed by oligosaccharides involves presenting a carbohydrate structure to pathogens that mimics that of the host’s – in this case human – mucosal cell (i.e. gut or respiratory tract) surface. Pathogens seek these surfaces as anchors from which to establish a colony or to penetrate and infect the cell. If the pathogen mistakenly binds to the oligosaccharide, it will fail to become anchored and will be flushed out through the gastrointestinal tract. In this mechanism, the oligosaccharide acts as a ‘decoy receptor’. In summary, oligosaccharide-based prebiotics indirectly protect the host by prevention of pathogen adhesion and stimulation of the growth of a beneficial microbiome.

The complexity and wide range of BMO structures and their low concentration in mature cow's milk makes them difficult to concentrate and isolate. To compensate for the low abundance compared to human breastmilk, the oligosaccharide content of cow's milk (BMOs) can be boosted by enzymatic treatment of lactose to produce galacto-oligosaccharide (GOS). This is of particular benefit in applications such as infant formula. In recent times, a few infant formula manufacturers have turned to supplementing their products with individual HMOs produced by genetically-modified organisms. However, this approach has limitations due to costs and to date, only a handful of the 200+ known HMOs are currently commercially available. As a result, supplementation with GOS is still widely utilized in infant formula and is starting to appear in other product segments.

While oligosaccharide is shown to be of benefit to a vulnerable neonate with an under-developed immune system, what can these prebiotics contribute to the immune system of an adult, especially with respect to COVID-19? There is strong evidence that GOS supports growth of probiotic bacteria, especially *Bifidobacteria* species (Chen and Vitetta, 2021) and several dietary supplement products containing GOS are available on the market (Figure 2). Establishing a cause and effect relationship between GOS-stimulated microbiome development and immune support has proven somewhat elusive in the small studies conducted so far (Wilms et al., 2021). However, there have been some encouraging observations with anti-viral vaccinations. For example, there are reports that supplementation with the combination of GOS and a live culture of Bifidobacteria significantly increased Bifido counts and sustained antibody titers for longer in subjects immunized with influenza A/H1N1 vaccine (Nagafuchi et al., 2015; Akatsu et al., 2016). These studies were included in the meta-analysis of Lei et al., (2017). They reported that probiotics and prebiotics, including GOS or FOS enhance the effectiveness of influenza vaccination in adults. Further evidence would be needed to determine whether there are similar beneficial effects of supplementation with GOS or milk-derived oligosaccharides on COVID-19 infections and vaccination effectiveness.

Figure 2: Examples of dietary supplements containing GOS. GoLive® which contains GOS in combination with a probiotic culture (Left); Klaire Labs® Galactomune prebiotic supplement based on GOS (Right). (Source: https://golivebewell.com/collections/ready-to-mix/products/berry-pomegranate; https://klaire.com/k-gal-galactomune-powders).

Glycomacropeptide – another dairy component with prebiotic activity

Glycomacropeptide (GMP) also exhibits prebiotic functionality manifested through stimulation of *Bifidobacteria spp* (Auestad & Layman, 2021; Córdova-Dávalos, 2019). GMP is the part of the κ-casein molecule released into the whey from the casein micelle during enzyme-induced curd formation in the cheese making process. In this case, the prebiotic functionality derives from N-acetylneuraminic acid (NANA), a member of the sialic acid group which is attached to specific amino acids in the GMP peptide chain. Influenza viruses are known to bind to NANA, which interferes with their ability to enter and infect cells. Recent publications show that NANA binds to the viral spike S protein of SARS-CoV-2. This first adhesion event facilitates later steps in virus multiplication and spreading of COVID-19. NANA has also shown potential as a SARS-CoV-2 virus sensor in a lateral flow test (Baker et al., 2020). Further research would be needed to establish whether NANA associated with GMP could support defense against COVID-19 infection.

Glycosaminoglycans – the least studied anti-microbial in milk

Glycosaminoglycans (GAG) are minor members of this group of immune-supporting components of milk and also one of the least studied. The majority (>90%) of GAG in cow's milk comprises chondroitin sulfate (CS), dermatan sulfate and fast-moving heparin (FM-Hep) (Coppa et al., 2011). CS and FM-Hep are dominant in human milk. The antiviral activity of these compounds was demonstrated in HIV research dating back to the mid 90s (Newburg et al.,1995), where it was found that multiple forms of GAGs inhibited the binding of HIV envelope glycoprotein to the host cell receptor, CD4, which a vital first step for HIV infectivity. It was further identified that this activity of GAG was unaffected by digestion with lytic enzymes, specifically for heparin, heparan sulphate and dermatan sulphate. The potential of cow's milk GAGs as an anti-viral agent for treatment of COVID-19 is unknown. However, high-sulfated GAGs, produced by chemical sulfation have been investigated as possible therapeutic drugs in in vitro cell studies. The authors claimed high potency against bovine CoV as well as alpha and beta variants of the SARS-CoV-2 virus, that is responsible for the current pandemic. The low concentrations of these components in human and cow milk means that ultimately, they would be unlikely sources of choice (Möller et al., 2022).

α-lactalbumin – the diminutive protein with the surprisingly strong nutrient punch

Before discussing how α-lactalbumin (A-LA) provides essential nutrients to the immune system, it is worth noting that A-LA also indirectly contributes prebiotic functionality. A-LA is synthesized in the epithelial cells of the mammary gland, where it is glycosylated (i.e. sugars are chemically attached). It appears that this glycosylation is a critical step in order to ensure the correct folding of the protein but also to allow A-LA to be transported across the cell membrane into the alveoli of the mammary gland Layman et al., 2018). During or immediately following this process, de-glycosylation occurs, releasing sialic acid into the milk, where it too can go on to act as a prebiotic and/or decoy receptor in the same manner as GMP.

Returning to nutrition, milk packs an enormous nutrient punch into a small volume and that is particularly the case with the whey protein, α-lactalbumin (A-LA). This protein contains much higher levels of critical amino acids, including tryptophan and sulfur-containing amino acids (SAA), than other dietary proteins such as found in beef, wheat gluten, soy and other sources (Layman et al, 2018).

SAAs are (conditionally) essential – methionine is an essential amino acid and cysteine can become essential under certain stressful situations, such as during illness. Cysteine is the limiting factor in glutathione (GSH) synthesis in the body. There have been several studies that show whey protein, which includes α-lactalbumin boosts the synthesis of GSH (Micke et al., 2001). GSH is the most abundant antioxidant present in the body and as it cannot be directly absorbed from the diet, must be synthesized. GSH plays a role in boosting levels of those 'behind the scenes' cells involved in the adaptive immune system, specifically T-cells and natural killer cells. GSH has also long been implicated in inhibiting the inflammatory response involving reactive oxygen species (ROS), including those generated to destroy infectious agents by the immune system. GSH was observed to inhibit viral replication and decrease levels of interleukin-6 (IL-6) in human immunodeficiency virus (HIV) infections. Based on this finding, ensuring maintenance of GSH levels could also be of benefit to COVID-19 patients (Guloyan et al., 2020). A surge in IL-6 levels (the 'cytokine storm) is a worrying symptom observed in serious COVID-19 cases. Frequently, it is associated with onset of acute respiratory distress syndrome (ARDS), the condition that frequently requires respiratory support using a ventilator (Notz et al., 2020). Thus, α-lactalbumin could be an attractive, safe source of SAAs which the body can use to synthesize GSH at times of acute need, as can be the case during COVID-19 infection.

Lysozyme – a ruthless ancestor of α-lactalbumin

Lysozyme (LYS) is another small, compact protein found in milk and many other biological fluids. LYS is closely related to A-LA. While these 2 proteins only show 40% homology in amino acid sequence, they are similarly sized, both bind calcium and they have very similar spatial structure. Indeed, it appears that A-LA evolved from a duplication of the type-C LYS gene some 300-400,000 years ago.

Despite these similarities, the proteins differ significantly in function and undertake rather distinct roles as enzymes. A-LA binds to galactosyl transferase forming the lactose synthase complex, responsible for the production of lactose in milk. In contrast, LYS is an enzyme in its own right, exhibiting bactericidal activity by hydrolyzing the cell wall of Gram-positive bacteria and can insert into and form pores in negatively charged bacterial membranes (Ragland & Criss, 2017). As a result, fluid levels and pressure increases inside the bacterial cell until is literally bursts. Thus, despite its comparatively low concentration level in milk, LYS is a potent contributor to the antibacterial defense system.

LYS also displays antiviral activity including inhibiting viral entry by binding to cell receptors or the virus itself. Here the cationic and hydrophobic nature of LYS is exploited rather than its enzymatic activity. The resulting inhibition of virus-induced cell fusion and separate effects on cell signaling reduce susceptibility to infection. It has been suggested that LYS could be a candidate for COVID-19 therapy but to date this is highly speculative (Mann & Ndung'u, 2020). There is a report from Eastern Europe of the successful use of LYS in a combination treatment for pediatric influenza infection and pneumonia (Luniakin & Bogomaz, 1977). However, in most studies, hen egg white is the preferred source of LYS due to its higher levels of concentration compared to milk.

Closing Remarks

Milk evolved not only to provide concentrated nutrition to neonates but to offer both specific and more general protection against infection. Ingredients that contribute to both the adaptive and innate immune systems are found in milk and are functional in both infant and adult subjects. Consumption of milk or products containing dairy ingredients flood the most vulnerable 'boundaries' between humans and the outside world – namely the gateway to the respiratory tract and the gut – with an array of immune modulating components. No other food that is available on the human menu evolved specifically to provide this protective shield! A fully functional and appropriately nourished immune system is not something that is only needed when we acquire an infection, it needs to perform 24/7 in the background to maintain health by reducing the risk of infection and fighting it once infection occurs!

References:

Akatsu H et al. Enhanced vaccination effect against influenza by prebiotics in elderly patients receiving enteral nutrition. Geriatrics and Gerontology, 2016, 16, 205-213. doi.org/10.1111/ggi.12454.

Auestad N & Layman DK. Dairy bioactive proteins and peptides: a narrative review. Nutrition Reviews VR 2021, 79(S2):36–47. https://doi.org/10.1093/nutrit/nuab097.

Baker AN et al. The SARS-COV-2 Spike Protein Binds Sialic Acids and Enables Rapid Detection in a Lateral Flow Point of Care Diagnostic Device. ACS Cent. Sci. 2020, 6, 11, 2046–2052. https://doi.org/10.1021/acscentsci.0c00855

Barile D and Restall RA. Human milk and related oligosaccharides as prebiotics. Curr. Opin. Biotechnol., 2013, 24, 214-219.

Coppa GV, Gabrielli O, Bertino E, Zampini L, Galeazzi T, Padella L, Santoro L, Marchesiello RL, Galeotti F, Maccari F and Volpi N. Human milk glycosaminoglycans: the state of the art and future perspectives. Italian Journal of Pediatrics 2013, 39:2. http://www.ijponline.net/content/39/1/2

Córdova-Dávalos LE, Jiménez M and Salinas E. Glycomacropeptide Bioactivity and Health: A Review Highlighting Action Mechanisms and Signaling Pathways. Nutrients 2019, 11, 598; doi:10.3390/nu11030598.

Chen J and Vitetta L. Modulation of Gut Microbiota for the Prevention and Treatment of COVID-19. J. Clin. Med. 2021, 10, doi.org/10.3390/jcm10132903.

Guloyan V, Oganesian B, Baghdasaryan N, Yeh C, Singh M, Guilford F, Ting Y-S and Venketaraman V. Glutathione Supplementation as an Adjunctive Therapy in COVID-19. Antioxidants, 2020, 9, 914; doi:10.3390/antiox9100914.

Layman DK, Lönnerdal B and Fernstrom JD. Applications for a-lactalbumin in human nutrition. Nutrition Reviews, 2018, 76, 444–460.

Lei W-T, Shih P-C, Liu S-J, Lin C-Y and Yeh T-L. Effect of Probiotics and Prebiotics on Immune Response to Influenza Vaccination in Adults: A Systematic Review and Meta-Analysis of Randomized Controlled Trials. Nutrients 2017, 9, 1175; doi:10.3390/nu9111175.

Luniakin AA and Bogomaz TA. Lysozyme in the overall treatment of children with an influenza infection and pneumonia. Pediatr. Akus. Ginekol. 1977, 1, 11–13.

Mann JK & Ndung'u T. (2020). The potential of lactoferrin, ovotransferrin and lysozyme as antiviral and immune-modulating agents in COVID-19. Future Virol. 2020, 15(9), 609–624.

Messer M., & Green B. 'Milk Carbohydrates of Marsupials. II. Quantitative and Qualitative Changes in Milk Carbohydrates during Lactation in the Tammar Wallaby *(Macropus eugenii).* Aust. J. Bioi. Sci., 1979, 32, 519 – 531.

Micke P., Beeh KM, Schlaak JF and Buhl R. Oral supplementation with whey proteins increases plasma glutathione levels of HIV-infected patients. Eur. J. Clin. Investig. 2001, 31, 171-178. doi.org/10.1046/j.1365-2362.2001.00781.x

Möller S, Theiß J, Deinert TIL, Golat K, Heinze J, Niemeyer D, Wyrwa R, Schnabelrauch M and Bogner E. High-Sulfated Glycosaminoglycans Prevent Coronavirus Replication. Viruses 2022, 14, 413. https://doi.org/10.3390/v14020413.

Nagafuchi et al., 2015. Effects of a Formula Containing Two Types of Prebiotics, Bifidogenic Growth Stimulator and Galacto-oligosaccharide, and Fermented Milk Products on Intestinal Microbiota and Antibody Response to Influenza Vaccine in Elderly Patients: A Randomized Controlled Trial. Pharmaceuticals 2015, 8, 351-365; doi:10.3390/ph8020351.

Newburg DS, Linhardt RJ, Ampofo SA, Yolken RH. Human milk glycosaminoglycans inhibit HIV glycoprotein gp120 binding to its host cell CD4 receptor. J. Nutr. 1995, 125, 419-424.

Newburg DS, Warren CD, Chaturvedi P, Newburg A, Oftedal OT, Ye S & Tilden CD, Milk oligosaccharides across species. Pediatr. Res. 1999, 45, 745.

Notz et al. Pro- and Anti-Inflammatory Responses in Severe COVID-19 – Induced Acute Respiratory Distress Syndrome—An Observational Pilot Study. Frontiers in Immunology, Front. Immunol. 11:581338. doi: 10.3389/fimmu.2020.581338.

Ragland SA, Criss AK. From bacterial killing to immune modulation: recent insights into the functions of lysozyme. PLoS Pathog. 13(9), e1006512 (2017).

Wilms E, An R, Smolinska A, Stevens Y, Weseler AR, Elizalde M, Drittij M-J, Ioannou A, van Schooten FJ, Smidt H, Masclee AAM, Zoetendal EG, Jonkers DMAE. Galacto-oligosaccharides supplementation in pre-frail older and healthy adults increased faecal bifidobacteria, but did not impact immune function and oxidative stress. Clin. Nutr. 40, 3019-3031.