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Milk-derived anti-infectives and their potential to combat bacterial and viral infection

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A B S T R A C T

Breastfeeding positively influences infant growth while providing protection against many diseases. Breast milk provides the ideal balance of nutrients for the infant and contains countless bioactive ingredients such as immunoglobulins (antibodies), fatty acids, oligosaccharides and others which function to protect against infection. Many of the anti-infective properties ascribed to breast-milk are not yet available to formula-fed infants. Infant milk formulas are predominantly based on bovine milk, which in some cases contain much lower concentrations of bioactives. However, bovine milk does contain a number of components which share homology with human milk bioactives which could imply common functionalities. Therefore, value may lie in extracting and concentrating select bovine milk components with a view to supplementing infant formula. This review will discuss the mechanisms of action of anti-infective milk components and their ability to decrease the risk of infection through their interactions with both bacteria and viruses.

1. Introduction

Human milk provides the first line of defence in the neonate and has a major impact on intestinal homeostasis. A wide range of immunological components are present in breast milk which offer a wealth of biological functions including defence against both bacterial and viral infections (Walker & Iyengar, 2015). One of the first lines of defence are antibodies, namely the immunoglobulins (Igs), transferred from the mother across the placental barrier to the neonate. Maternal antibodies do however, decline in the first 6–12 months after birth. Additional antibodies are supplied through breast milk with large quantities of secretory Ig A (sIgA) being provided to the breast-fed infant. sIgA antibodies are generated based on the mother's past exposure to infectious pathogens and may prevent similar infectious agents colonising the infant (Fouda, Martinez, Swamy, & Permar, 2018; Hanson et al., 2003). In addition to immunologic antibodies, breast milk provides an array of nonspecific components which have broad antimicrobial effects including oligosaccharides, nucleotides and proteins such as lactoferrin and α-lactalbumin, among others. Breast-fed infants are known to have a lower incidence of infection including gastrointestinal infections associated with diarrhoea, respiratory tract infection, otitis media and other extraintestinal infections compared to their formula-fed peers (Cushing et al., 1998; Duffy, Faden, Wasielewski, Wolf, & Krystofik, 1997; Duijts, Jaddoe, Hofman, & Moll, 2010; Oddy et al., 2003; Rebhan et al., 2009).

In an attempt to narrow the gap between the health outcomes of breast-fed and formula-fed infants, infant formula (IF) has advanced greatly due a plethora of scientific studies (Hernell, 2011). Examples of how IF has been modified include addition of key ingredients such as oligosaccharides (both fructo-oligosaccharides/galacto-oligosaccharides and human milk oligosaccharides such as 2′-fucosyllactose (2′-FL) and lacto-*N*-neotetraose (LNnT)), fatty acids (FAs) namely docosahexaenoic acid (DHA) and arachidonic acid (AA), nucleotides, lutein, natural vitamin E, taurine and an alteration to the whey:casein ratio. Although these changes to IF have resulted in very significant beneficial outcomes, differences still remain between breast- and formula-fed infants in response to bacterial and viral infections (Victora et al., 2016). For this reason, further optimisation of infant formula composition is warranted with the addition or enrichment of a range of milk bioactives aimed at preventing various infections.

Several constituents of human milk have displayed both antimicrobial and antiviral activity *in vitro*, however their incorporation into infant formula is limited due to the lack of clinical trials completed to date (Wada & Lonnerdal, 2020). In combination with this, the lack of large-scale commercial availability of many of these ingredients currently inhibits their use. Indeed, the study of certain bioactives found in breast milk (lactoferrin, osteopontin, immunoglobulins, glycosaminoglycans etc.) has largely been done through the use of their bovine milk counterparts which in many cases can be obtained in larger quantities.

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Although not identical, proteins derived from bovine milk share a high degree of homology with human milk proteins and may mimic the bioactivities observed with human milk proteins (Magnuson, Henry, Yip, & Hutchens, 1990; Su et al., 2017). Likewise, certain oligosaccharides are similar between both milks (Barile et al., 2009; Mariño et al., 2011; Silanikove, Leitner, Merin, & Prosser, 2010). In addition, the structure and concentration of milk fat globule membrane (MFGM) polar lipids is comparable between human milk fat and bovine milk fat (Fontecha et al., 2020).

Much remains to be understood regarding the antibacterial and antiviral activity of milk constituents. Specifically, the mode of action of many of these constituents requires further investigation in order to determine how they exert their anti-infective activities. Knowledge on the exact mechanism underpinning the observed bioactivity of milk ingredients is particularly important for their development and use in both IF and as potential antimicrobial agents against antibiotic-resistant microorganisms (Haney, Straus, & Hancock, 2019). In this review, we examine the *in vitro* and *in vivo* evidence associated with several components found in both human and bovine milk which are known to possess antimicrobial and/or antiviral activity. Their direct and/or indirect effects against pathogens and their mechanism of action are also discussed. The milk components discussed were chosen on the basis that they can be isolated in significant amounts with which to perform functionality studies and the majority of components described have been well characterised.

2. Mode of action

Anti-infective agents in milk exert their activities through several direct and indirect mechanisms, resulting in the blocking of pathogen adhesion and internalisation (as described in Fig. 1 and summarised in Tables 1–5). In order to colonise and multiply, pathogens must first adhere to epithelial cell surfaces often targeting specific receptors and cell infrastructure (Morrow, Ruiz-Palacios, Jiang, & Newburg, 2005). Many milk components share structural features identical to those found on epithelial surfaces as they are produced through similar enzymatic pathways (Smilowitz, Lebrilla, Mills, German, & Freeman, 2014). Consequently, these milk analogues directly inhibit pathogen adhesion (Table 1), through what is known commonly as the decoy receptor effect, whereby the milk factor binds to the pathogen and prevents its attachment to targeted receptors on the epithelial cell surface (Yu et al., 2014). This interaction may lead to the pathogen essentially being 'flushed out of the system' (Coppa et al., 2006; Feeney, Ryan, Kilcoyne, Joshi, & Hickey, 2017) or may lead to the breakdown of the pathogen either through opsonisation or through mediated perturbation of the pathogen's cell membrane (Kaufmann et al., 2005). Milk components also modulate the physicochemical properties of pathogens. Altered gene expression leading to reduced virulence and motility constrain specific interactions of pathogens with epithelial cells (Lawhon, Maurer, Suyemoto, & Altier, 2002). Another direct method of inhibition is the competitive binding of certain milk components to host cell surface

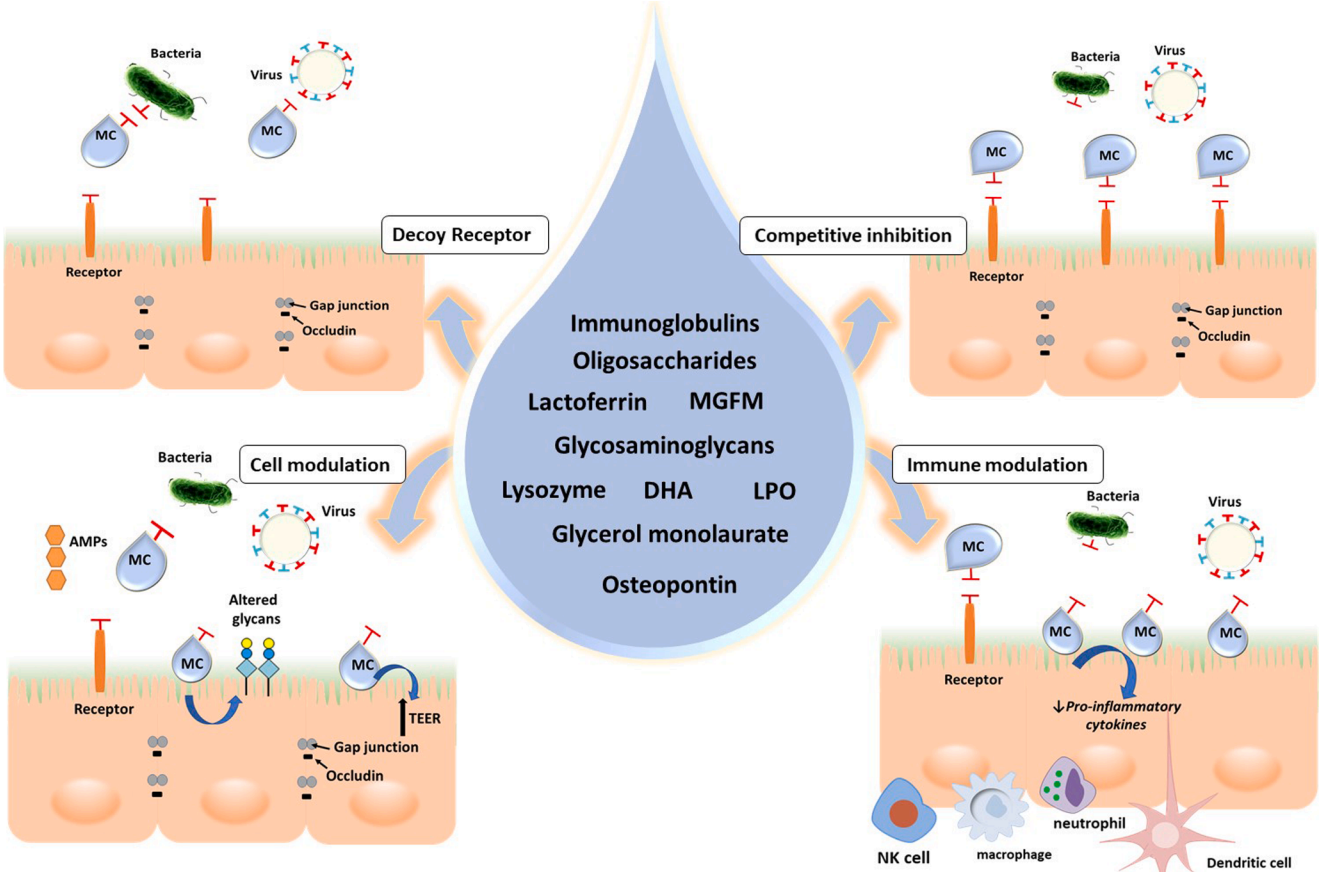

Fig. 1. Schematic overview describing the different mechanisms of actions which milk components (MC) possess to overcome bacterial and viral infection. Decoy receptor: Milk components have shown antimicrobial and antiviral effects via binding of viruses and bacteria. The milk component resembles structures which specific viruses and bacteria target and mistakenly bind the MC, never reaching the mucosal surfaces. Competitive inhibition: Milk components directly bind to cell surface receptors and subsequently block the attachment of pathogens, preventing the initial step required for subsequent colonisation. Intestinal modulation: Milk components modulate the epithelial cell surface leading to the secretion of antimicrobial peptides, changes in terminal carbohydrate moieties on membrane proteins and receptors, upregulation of tight junction proteins such as occludin and enhancing tight junction development, overall strengthening intestinal barrier function. Immunomodulation: Milk components modulate immune responses such as attenuating uncontrolled pro-inflammatory reactions due to infection preventing epithelial cell damage. Milk components may also induce phagocytosis of bacterial and viral particles by binding and activating phagocytic cells.

Table 1
Milk components demonstrating anti-infective activity against several bacterial pathogens via direct interaction.

Direct bacterial interaction			
Bacteria	Milk component	Activity	References
C. jejuni	α1,2-fucosylated HMO from human milk	Reduced bacterial adherence and binding to H-2 antigen on human intestinal mucosa	(Ruiz-Palacios, Cervantes, Ramos, Chavez-Munguia, & Newburg, 2003)
C. jejuni	2′-FL HMO (bio fermentation)	Reduced bacterial invasion of Hep-2 and HT-29 cell line	(Yu et al., 2016)
EPEC *E. coli*	Pooled HMO from human milk	Reduced bacterial adherence to HeLa and T84 cell line	(Manthey et al., 2014)
V. cholerae and *E. coli* O119	Pooled HMO from human milk	Reduced bacterial adherence to Caco-2 cell line	(Coppa et al., 2006)
H. pylori	Bovine 3′-SL, 6′-SL	Reduced bacterial adherence to HuTu-80 cell line	(Simon, Goode, Mobasseri, & Zopf, 1997)
Group B Streptococcus (GBS)	Pooled HMO LNT HMO (biofermentation)	Antimicrobial and antibiofilm activity	(Ackerman et al., 2018; Lin et al., 2017)
C. jejuni, EPEC *E. coli*, *S. fyris*, and *P. aeruginosa*	2′-FL, 3-FL HMO (biofermentation)	Inhibition of bacterial adherence to Caco-2 cell line	(Weichert et al., 2013)
E. coli, *C. sakazakii* and *S. Typhimurium*	bovine colostrum oligosaccharides	Reduced bacterial adhesion to HEp-2 cell line	(Maldonado-Gomez et al., 2015)
S. enterica	neutral and acidic bovine oligosaccharides	Inhibition of bacterial adherence to Caco-2 cell line	(Urakami et al., 2018)
N. meningitidis	human milk neutral or bovine milk acidic oligosaccharides.	Inhibition of binding of type IV pili to bovine thyroglobulin	(Hakkarainen et al., 2005)
ETEC *Escherichia coli*	Hyperimmune Bovine Colostrum (HBC)	Reduced bacterial motility and promotion of complement-mediated lysis	(Sears et al., 2017)
L. monocytogenes, *S. bovis*, *S. agalactiae*, *S. pyogenes* and *S. aureus*	Commercial HP	Reduced internalisation on HT-29 cell line	(Henry-Stanley et al., 2003; Henry-Stanley et al., 2005)
S. aureus, *S. epidermidis* and *S. saprophyticus*	Soluble HP, CS-B, CS-A and CS-C	Reduced bacterial adherence to Vero and Hep-2 cell line	(Hafez et al., 2008)
S. fyris and *Escherichia coli* O119	Purified GAG complex from human milk	Reduced bacterial adherence to Caco-2 and Int-407 cell line	(Coppa et al., 2016)
EPEC *Escherichia coli*, *P. multocida* and *S. aureus*	Porcine intestinal mucosa HP	Inhibited adherence to HT-29 cells	(Chen, Ling, Duan, & Zhang, 2012)
Staphylococcus spp. and *Streptococcus* spp.	Commercial HS & HS/CSA–CSC	Reduced adhesion to A549 and MRC5 cell line	(Rajas et al., 2017)
Cholera toxin	Bovine GMP	Prevented binding to CHO-K1 cell line	(Kawasaki et al., 1992)
E. coli 0157	Bovine GMP	Reduced adhesion to Caco-2 cell line	(Nakajima et al., 2005)
H. pylori	*N*-acetylneuraminic acid extracted from GMP	Reduced growth	(Kim et al., 2016)
EHEC *E. coli* (*E. coli* 12,900 O157:H7) and EPEC (*E. coli* O125:H32 and O111:H2)	Commercial GMP	Reduced adhesion to HT-29 and Caco-2 cell line	(Feeney et al., 2017)
VTEC and EPEC *E. coli*	Bovine GMP	Reduced adhesion to HT-29 cell line	(Rhoades et al., 2005)
S. flexneri, *S. typhimurium* and EPEC *E. coli*	Digested bovine GMP	Reduced association and internalization to Caco-2 cell line	(Brück et al., 2006)
ETEC *E. coli*	Commercial GMP	Prevent attachment to ileal mucosa tissue	(Gustavo Hermes et al., 2013)
E. coli, *K. pneumoniae*, *S. aureus*, *S. epidermis*, streptococci, and *B. subtilis*	Proteolytic digests of bovine α-La	Growth inhibition	(Pellegrini et al., 1999; Pihlanto-Leppälä et al., 1999)
H. influenzae, EPEC *E. coli* and *S. typhimurium* and streptococci	HAMLET α-La	Bactericidal and bacteriostatic activity	(Alamiri et al., 2019; Clementi et al., 2012; Håkansson et al., 2000; Marks et al., 2013; Meikle et al., 2019; Peso Echarri et al., 2012)
EPEC *E. coli*	Commercial bovine LF, OPN, LF-OPN complex	Reduced growth and adhesion to Caco-2 cell line	(Liu et al., 2019)
E. coli spp.	Commercial bovine LPO	Bactericidal effect	(Marshall & Reiter, 1980; Shin et al., 2001; Thomas & Aune, 1978)
S. lactis	Commercial LPO	Bacteriostatic effect	(Marshall & Reiter, 1980)
Salmonella spp.	Bovine LPO	Bactericidal/bacteriostatic effect	(Purdy et al., 1983)
B. cereus	Bovine LPO	Growth inhibition	(Tenovuo et al., 1985)
H. pylori	Commercial DHA	Growth inhibition	(Correia et al., 2012)
A. baumannii	Commercial DHA	Growth inhibition	(Jiang et al., 2019)
P. aeruginosa	Egg white Lysozyme and commercial DHA	Inhibit metabolic activity	(Martinez et al., 2009)
B. cenocepacia K56-2	Commercial DHA	Bactericidal/bacteriostatic effect	(Mil-Homens et al., 2012)
S. mutans, *C. albicans*, *A. actinomycetemcomitans*, *F. nucleatum* and *P. gingivalis*	Commercial DHA	Reduced growth	(Huang & Ebersole, 2010)
B. subtilis, *L. monocytogenes*, *S. aureus*, *E. aerogenes*, *E. coli* O157:H7, *P. aeruginosa*, *S. enteritidis* and *S. typhimurium*	Bioconverted commercial DHA	Reduced growth	(Shin et al., 2007)
E. coli, *S. aureus*, *C. perfringens* and *B. subtilis*	Synthetic GML and human, bovine milk GML	Growth inhibition	(Projan et al., 1994; Ruzin & Novick, 2000; Schlievert et al., 1992, 2019; Zhang et al., 2009)
L. monocytogenes and *H. pylori*	Commercial GML	Bactericidal effect	(Sun, O'Connor, & Roberton, 2003)
S. aureus and *E. faecalis*	Commercial GML	Prevent biofilm formation	(Hess et al., 2014)
E. faecalis	Commercial GML	Suppressed vancomycin resistance	(Ruzin & Novick, 1998)

Table 2
Milk components demonstrating anti-infectivity against pathogens through modulation of the intestinal surface.

Modulation of intestinal surface			
Bacteria	Milk component	Activity	References
EPEC E. coli	3′-SL HMO (biofermentation)	Pre-treatment led to reduced α-2,3- and α-2,6-linked sialic acid on Caco-2 cell surface. Reduced bacterial adhesion thereafter.	(Angeloni et al., 2005)
L. monocytogenes	HMO pooled from human milk	Reduced bacterial association to Caco-2 line due to differential gene expression of pre-treatment to cell line	(Chen et al., 2017)
S. aureus, *S. epidermidis* and *S. saprophyticus*	Commercial Heparinase, Chondroitinase AC and Chondroitinase B	Reduced adherence to Vero and Hep-2 cell line	(Hafez et al., 2008)
S. typhimurium	Human milk HA	Reduced adherence to HT-29 cell line	(Hill et al., 2013)
EPEC *E. coli*	Commercial CS sodium salt	Reduced adherence to T84 cell line	(Burge et al., 2019)
E. faecalis	Porcine HP &HS	Inhibition of adherence to Caco-2 cell line	(Sava et al., 2009)
EPEC *E. coli*	Commercial GMP	Delayed translocation	(Feeney et al., 2017)

Table 3
Milk components demonstrating anti-infectivity against pathogens through modulation of the immune response.

Immunomodulation			
Bacteria	Milk component	Activity	References
C. jejuni	2′-FL HMO (biofermentation)	Reduced expression of proinflammatory cytokines IL-8 and IL-1β and the neutrophil chemoattractant MIP-2 in Hep-2 and HT-29 cell line	(Yu et al., 2016)
NEC model	Commercial HA (Sodium Hyaluronate)	Reduction in pro-inflammatory cytokines TNF-α, GRO-α, IL-12p70, and IL-6	(Gunasekaran et al., 2020)
NEC model	2′-FL, 6′-SL HMO (biofermentation)	Reduction in pro-inflammatory gene expression: IL-1b, IL-6, iNOS, TLR-4; binding to TLR-4	(Good et al., 2016; Sodhi et al., 2021; Werts et al., 2020)
LPS	Commercial CS	Reduction in pro-inflammatory cytokines and attenuated NF-κB activity in THP-1 macrophages	(Stabler et al., 2017)
K. pneumoniae	Tripeptide GLF derived from residues of both human and bovine α-La	Enhanced phagocytosis	(Migliore-Samour et al., 1992)
A. baumannii	Camel milk LPO/Lf	Significantly higher levels of IL-4 and IL-10	(Mahdi et al., 2018)

receptors which also results in the blocking of the pathogen (Sava et al., 2009).

Indirect mechanisms of pathogen inhibition arise from the exposure of epithelial cell surfaces to milk components. Several milk components exert a protective effect on surface cells by preventing invasion, internalization and induced cytotoxicity by pathogens rather than by interfering with their initial attachment (Table 2) (Lin, Autran, Espanola, Bode, & Nizet, 2014). Epithelial barrier function is crucial to overall health for effective resilience against bacterial and viral challenge. Numerous studies have demonstrated that certain milk factors can enhance the barrier integrity of intracellular tight junctions that control permeability of the epithelial cells before and during pathogenic encounters (Anderson, MacGibbon, Haggarty, Armstrong, & Roy, 2018; Liu, Jiang, Liu, & Lonnerdal, 2020). Increased intestinal permeability plays a role in pathogenic intestinal diseases with prevention reducing the severity of infection and/or disease. Milk factors also exert immunomodulatory functions, attenuating pathogen-mediated inflammation and thus preventing the severity of epithelial cell injury and cell death (Table 3) (Schlievert, Kilgore, Seo, & Leung, 2019). Host cells treated with milk constituents may also directly modulate host epithelial cell responses inducing differential gene expression leading to a differential production of specific glycans and/or proteins on the host cell surface and indirectly affecting the availability of ligands for the invading pathogens to attach to (Table 2) (Angeloni et al., 2005; Hill et al., 2013). In summary, milk bioactives can exert their effects at several levels and this section will discuss the mechanistic evidence available to date on individual milk components and their role in defending against bacterial (Table 1–4) and viral infection (Table 5).

3. Carbohydrates

3.1. Oligosaccharides

One of the most remarkable features of breast milk is the diversity and abundance of complex carbohydrates known as human milk oligosaccharides (HMOs), which are indigestible to the infant and for this reason reach the colon intact (Kunz, Rudloff, Baier, Klein, & Strobel, 2000). Major innovations in the field of glycomics have resulted in the identification of over 200 HMO structures (Ninonuevo et al., 2006; Wu, Tao, German, Grimm, & Lebrilla, 2010). Concentrations of HMO are highest in colostrum (20 g/L) and decrease across lactation, with 16 g/L (5–15 g/L) (Bode, 2012) detected in 30 day mature milk (Coppa et al., 1999). HMOs vary in size, structure, and complexity and are composed of 5 monosaccharide building blocks, namely glucose (Glc), galactose (Gal), *N*-acetylglucosamine (GlcNAc), fucose (Fuc) and *N*-acetylneuraminic acid (Neu5Ac). With very few exceptions (Kunz et al., 2000), all HMOs are formed by a reducing lactose core that can be extended enzymatically by lacto-*N*-biose (Galβ-1,3-GlcNAc, type 1 LacNAc) or *N*-acetyllactosamine (Gal-β1,4-GlcNAc, type 2 LacNAc) motifs. These structures can be further elongated by the addition of Fuc residues in α1,2-, α1,3-, and α1,4-linkages and/or Neu5Ac residues in α2,3- and α2,6-linkages (German, Freeman, Lebrilla, & Mills, 2008). Inter-individual variation of HMO concentrations is pronounced, and is strongly dependent on maternal genetics and allelic variation in the Secretor (Se) and Lewis (Le) genes (Thurl et al., 2010). Although studies are limited, it is thought that maternal factors such as parity and body mass index (BMI) (Azad et al., 2018; Jantscher-Krenn et al., 2019), as well as environmental factors such as geographical location, also influence the composition of HMOs (Erney et al., 2000; van Leeuwen et al., 2018). Data from *ex vivo* and *in vivo* studies, including recent intervention studies in humans, highlight the importance of HMOs in the infant gastrointestinal tract (reviewed by Walsh, Lane, van Sinderen, & Hickey, 2020). HMOs serve as prebiotic components supplying metabolic

Table 4
In vivo studies demonstrating the anti-infective properties of milk components against pathogenic bacterial infection.

In vivo studies			
Bacteria	Milk component	Activity	References
C. jejuni	Neutral, fucosylated HMO from human milk	Significantly reduced campylobacter colonization in murine subjects orally challenged	(Ruiz-Palacios et al., 2003)
C. jejuni	2′-FL, total α1,2-fucosylated HMOs from human milk	Higher concentrations of 2′-FL, and total α1,2-fucosylated HMO in breast milk samples were associated with reduced susceptibility of *C. jejuni* associated diarrhea	(Morrow et al., 2005)
EPEC *E. coli*	Pooled HMO from human milk	Reduced EPEC colonisation observed in suckling murine subjects	(Manthey et al., 2014)
S. pneumoniae	LNnT HMO (biofermentation)	Reduced number of cells in rabbit model	(Idänpään-Heikkilä et al., 1997)
C. difficile	HBC	Reduced incidence of diarrhoea and colitis in piglet subjects	(Sponseller et al., 2015)
H. pylori	bovine antibody-based oral immunotherapy	Increased gastric bacterial clearance in human subjects	(Hu et al., 2015)
S. mutans and *S. sobrinus*	HBC	Reduced bacterial growth	(Ramezanalizadeh et al., 2017)
C. rodentium	Commercial HA	Protect murine subjects from infection. Higher expression of the antimicrobial peptide HβD2	(Kim et al., 2017)
NEC model	Commercial HA (Sodium Hyaluronate)	Increased expression of tight junction proteins. Reduction in pro-inflammatory cytokines. Reduced intestinal permeability and severity of intestinal injury.	(Gunasekaran et al., 2020)
Cholera toxin and enterotoxins LT1 and LT2 of *E. coli*	Bovine GMP	Reduced diarrhea in murine subjects	(Isoda et al., 1999)
ETEC *E. coli*	Commercial GMP	No increase in enterococcus counts. Reduced intestinal injury and permeability	(Rong et al., 2015)
ETEC *E. coli*	Commercial GMP	Prevention of colonisation of the ileum	(Gustavo Hermes et al., 2013)
No bacterial challenge	Bovine α-La	Enhanced the mucin layer of rat subjects	(Ushida et al., 2003)
EPEC *E. coli*	Bovine α-La	Enhanced ability to counteract with reduced diarrhea observed	(Brück et al., 2003)
EPEC *E. coli* and *S. Typhimurium*	Commercial α-La	Reduced levels of inoculated pathogens in infant faecal fermentation cultures	(Brück et al., 2003)
S. aureus and *S. agalactiae*	Bovine OPN	Induced phagocytosis	(Schack, Lange et al., 2009, Schack, Stapulionis et al., 2009)
No bacterial challenge	Bovine OPN	Differential gene expression including cell survival, proliferation, movement and communication	(Donovan et al., 2014)
NEC model	OPN	Reduced expression of the phosphorylated form of occludin in the colonic mucosa in murine subjects	(Woo et al., 2019)
LPS of *E. coli* O111:B4	OPN	Higher levels of IFN-γ in WT vs OPN-null mice	(Jiang & Lönnerdal, 2020)
A. baumannii	Camel milk LPO/Lf	Significant bacterial clearance in lung and blood cultures of *A. baumannii* pneumonia mouse model	(Mahdi et al., 2018)
LPS of *E. coli*	Bovine OPN supplemented (30 g/L) formula	Reduced incidence of diarrhoea in piglets	(Ren et al., 2019)
H. pylori	Commercial DHA	Reduced colonisation in infected murine subjects	(Correia et al., 2012)
B. cenocepacia	Commercial DHA	Reduced colonisation in infected larvae model	(Mil-Homens et al., 2012)

substrates necessary for beneficial bacteria to thrive (Bode, 2009; Gibson et al., 2017). These oligosaccharides have also been shown to improve gut barrier function (Chichlowski, German, Lebrilla, & Mills, 2011), promote immune development and tolerance (Donovan & Comstock, 2016), reduce respiratory viral inflammation (Duska-McEwen, Senft, Ruetschilling, Barrett, & Buck, 2014), modulate intestinal cell responses (Kong et al., 2019), reduce the severity of necrotizing enterocolitis (Sodhi et al., 2021), regulate gut contractions (Bienenstock et al., 2013) and may provide the infant with a source of sialic acid, an essential nutrient in brain development and cognition (Jacobi et al., 2016; Tarr et al., 2015) while fucosylated HMOs, such as 2′-FL, may improve cognition by the gut-brain axis (Oliveros et al., 2016; Vazquez et al., 2016).

One of the most studied attributes associated with HMOs is their ability to act as soluble decoy receptors (Laucirica, Triantis, Schoemaker, Estes, & Ramani, 2017). We refer the reader to the expansive literature that exists describing the action of HMOs against a variety of bacterial and viral pathogens as it is beyond the scope of this review to cover all anti-infective studies associated with HMOs (Hickey, 2012; Laucirica et al., 2017; Li et al., 2014; Manthey, Autran, Eckmann, & Bode, 2014; Morozov, Hansman, Hanisch, Schroten, & Kunz, 2018). Below and in Table 1 and Table 2, the most recent studies on the anti-bacterial and anti-viral properties of HMOs are summarised. Regarding bacteria, anti-*Campylobacter* effects have been observed in murine models, with the predominant human milk oligosaccharide, 2′-FL reducing invasion and intestinal inflammation associated with *Campylobacter* infection (Z. T. Yu, Nanthakumar, & Newburg, 2016). HMOs also function to protect against *E. coli* pathogenesis. Pre-incubation of enteropathogenic *E. coli* (EPEC) with pooled HMO fractions significantly reduced pathogenic colonization of cultured epithelial cells (Manthey et al., 2014), while HMO-treated mice demonstrated significantly less EPEC infection when compared to control pups (Manthey et al., 2014). In addition to reducing adhesion of whole pathogens, the HMOs, 2′-FL and lacto-*N*-fucopentaose-I (LNFP-I) may also reduce infectivity by binding to heat-labile enterotoxin type 1 (El-Hawiet, Kitova, & Klassen, 2015). Fucosylated HMOs are also efficient in reducing adhesion and infectivity of *Pseudomonas aeruginosa* (Weichert et al., 2013) while pre-incubation of Caco-2 cells with HMO alters gene expression of intestinal cells, resulting in a significant reduction in *Listeria monocytogenes* (Chen, Reiter, Huang, Kong, & Weimer, 2017) and *E. coli* adhesion (Angeloni et al., 2005). HMOs also have activity against group B streptococci (GBS) and may act as a substrate to modify growth of these bacteria (Lin et al., 2017). In another study, growth and biofilm assays showed that HMOs possessed antimicrobial and antibiofilm activity against three strains of GBS, antibiofilm activity against a methicillin-resistant strain of *Staphylococus aureus*, and antimicrobial activity against a *Actinobacter baumannii* strain (Ackerman et al., 2018). Moreover, HMOs have even recently been shown to act as adjuvants to enhance the function of antibiotics (Chambers et al., 2020; Craft, Gaddy, & Townsend, 2018).

HMO-mediated protection has also been described for viruses such as rotaviruses and noroviruses. Many studies have identified histo-blood group antigens (HBGA) as key binding sites for norovirus adhesion (Schroten, Hanisch, & Hansman, 2016). Some HMOs demonstrate

Table 5
Milk components demonstrating anti-infective bioactivity with several viral pathogens.

Direct viral interaction			
Virus	Milk component	Activity	References
Norovirus	2′-FL HMO (biofermentation)	Prevented viral binding to histo-blood group antigens (HBGAs)	(Koromyslova, Tripathi, Morozov, Schroten, & Hansman, 2017)
RV	2′-FL, 3′-SL, 6′-SL HMO (biofermentation), synthetic GOS	Reduced viral infectivity in African green monkey kidney epithelial cells (MA104 cells)	(Laucirica et al., 2017)
RV	3′ -SL, 6′-SL HMO (biofermentation) Neutral and Acidic HMO (pooled human milk)	Decreased viral replication in piglet subjects	(Hester et al., 2013)
RSV Influenza and *Haemophilus influenzae* type b (respiratory bacteria	Bovine IgG	Inhibited viral infection of HEp2 cell line	(den Hartog et al., 2014)
RSV	2′-FL, 3′-SL, 6′-SL, LNnT HMO (biofermentation)	Reduced viral infection and inflammation in airway epithelia	(Duska-McEwen et al., 2014)
RV	HBC	Reduction in duration of diarrhea, stool frequency, rotavirus excretion and need for oral rehydration solution	(Hilpert et al., 1987; Mitra et al., 1995; Sarker et al., 1998)
HIV-1	Commercial HP	Reduced viral adsorption to MT-4 cell line and viral destruction of cells prevented	(Baba et al., 1988)
HSV	Commercial HP	Inhibited binding of viral glycoproteins B and C to Vero cell line	(Laquerre et al., 1998)
SARS-CoV	Commercial HP	Infection inhibited by 50% on Vero cell line	(Vicenzi et al., 2004)
ZIKV	Commercial HP	Prevented virus-induced necrotic death of human neural progenitor (hNPC) and Vero cell line	(Ghezzi et al., 2017)
DENV and HSV	Commercial CSE & HP	Prevented virus–host cell interaction and subsequent replication on BHK-21 and gro2C cell line	(Bergefall et al., 2005; Kato et al., 2010; Nahmias & Kibrick, 1964)
HIV-1	Human milk CS	Prevented binding of the HIV co-receptor gp120 to CD4 of host cells	(Newburg et al., 1995)
HRV	Bovine GMP	Prevented infection on Rhesus monkey kidney MA104 cell line	(Inagaki et al., 2014)
Human influenza virus	Bovine GMP	Prevented hemagglutination (HI activity)	(Kawasaki et al., 1993)
EBV	Bovine GMP	Prevented morphological transformation of peripheral lymphocytes	(Dosako et al., 1992)
HIV-1	Bovine α-La-HP	Inhibited viral replication of MT2 cells	(Berkhout et al., 1997)
HSV-1	Bovine α-La-HP	Inhibited viral replication of Vero76 cells	(Oevermann et al., 2003)
HCMV	Methylated α-La and peptic hydrolysates	Viral inhibition on MRC-5 fibroblasts	(Chobert et al., 2007)
HSV-1	Methyl-esterified α-La Tryptic α-La peptides	Viral inhibition on Vero cells	(Sitohy et al., 2007)
H. influenzae type b (Hib) Poliovirus type 1 Diphtheria tetanus acellular pertussis	Nucleotides	Higher antibody response to vaccination in nucleotide supplemented formula fed-infants (not statistically significant to control groups)	(Schaller et al., 2004)
Poliovirus and vaccina	Bovine milk LPO	Reduction in infectivity and cytopathic effect	(Belding et al., 1970)
HIV-1	Bovine milk LPO	Inhibition of viral replication and cytopathic effects on HUT 78 and CEM cell line	(Yamaguchi et al., 1993)
HIV-1	Commercial LPO	Viral growth inhibition on lymphocytes	(Pourtois et al., 1990)
HSV-1	LPO from camel, bovine, and human	Viral growth inhibition on Vero cells	(El-Fakharany et al., 2017)
Human influenza virus	Bovine LPO	Reduced plaque formation MDCK cells	(Sugita et al., 2018)
HIV-1	Lysozyme from chicken egg white and human milk	Viral replication blocked in ACH-2 lymphocytes and U1 monocytes	(Lee-Huang et al., 1999)
HCV	Commercial DHA	Inhibit viral replication	(Leu et al., 2004)
HIV-1	Commercial GML	Viral inhibition and reduced inflammatory response in the simian immunodeficiency virus (SIV)-rhesus macaque model	(Haase et al., 2015; Li et al., 2009)
HIV-1, mumps virus, yellow fever virus and ZIKV	Commercial GML	Reduced binding to TZM-bl cells. Reduced replication and/or toxicity to Vero cell line	(Welch, Xiang, Okeoma, Schlievert, & Stapleton, 2020)
Influenza A and CoV	Commercial GML	Viral disintegration and loss of infectivity on MK, HEp-2 and HELF cell line.	(Hierholzer & Kabara, 1982)
HSV-1 and HSV-2, VSV and visna virus	Commercial GML	Viral inactivation on Vero cells and Sheep fibroblast cultures	(Clarke & May, 2000; Sands et al., 1979; Thormar et al., 1994)

homology to HBGA, with Norovirus having the highest affinity for high-mass α-fucosylated HMOs (Hanisch, Hansman, Morozov, Kunz, & Schroten, 2018). *In vivo* studies on rotavirus (RV) using piglet models revealed that both acidic and neutral fractions significantly reduced infectivity of RV (Hester et al., 2013). Most recently, HMOs were

demonstrated to reduce infectivity of two dominant RV strains in monkey kidney epithelial cells, with significant strain-specific differences. One strain was inhibited most significantly by 2′-FL while a combination of two sialylated HMOs had the greatest effect on the second variant (Laucirica et al., 2017).

However, the protective effects ascribed to HMOs have been unavailable to formula-fed infants until recently. Infant formulas are often supplemented with non-digestible carbohydrates (NDCs) such as galacto-oligosaccharides (GOS) which are enzymatically synthesised from lactose and fructo-oligosaccharides (FOS) which are of plant origin. These NDCs are used to substitute for some HMO functions (Vandenplas et al., 2015) particularly their prebiotic function by selectively stimulating the growth and/or activity of a limited number of beneficial bacteria (Gibson & Roberfroid, 1995). The anti-infective activity of GOS and FOS is less well understood but a limited number of studies indicate they may have some impact on pathogen numbers in the gut (Kittana et al., 2018; Shoaf, Mulvey, Armstrong, & Hutkins, 2006). However, although these oligosaccharides belong to the same chemical class of carbohydrates as HMO, they don't share structural similarities (e.g. their monosaccharide composition and the linkage between them differs) which may suggest that these NDCs don't impart the same biological functions which can be attributed to HMO.

In a new era of infant nutrition and after decades of research in the field, HMOs are now available for a broad range of applications. Two HMOs, 2′-FL and LNnT, have recently been added to infant formula in more than 30 countries and non-infant products are beginning to emerge (Walsh et al., 2020). These HMO-fortified formulas support the developing immune system and are associated with fewer respiratory infections in infants (Goehring et al., 2016; Puccio et al., 2017; Reverri, Devitt, Kajzer, Baggs, & Borschel, 2018). As more HMO structures become available, more HMOs will be added to formulas to more closely mimic the complexity of HMOs in human milk. Infant milk formulas are mainly based on bovine milk which contains lower concentrations of oligosaccharides (~0.03 g/L) and little to no 2′-FL compared to HMOs in human milk (Kunz et al., 2000). At least ten bovine milk oligosaccharides (BMOs) share the same structure as certain HMOs, which could imply common functionalities (Barile et al., 2009; Mariño et al., 2011; Quinn, Joshi, & Hickey, 2020; Robinson, 2019; Silanikove et al., 2010). In this respect, oligosaccharides isolated and purified from the colostrum of Holstein Friesian cows were found to have anti-infective activity against a highly invasive strain of *Camplyobacter jejuni in vitro* when used at similar physiological concentrations to HMOs (Lane et al., 2012). Maldonado-Gomez, Lee, Barile, Lu, and Hutkins (2015) also demonstrated that bovine colostrum oligosaccharides could prevent the adhesion of EPEC *E. coli*, *Cronobacter sakazakii* and *Salmonella enterica* serovar Typhimurium to HEp-2 cell monolayers cultured *in vitro* (Maldonado-Gomez et al., 2015). Both neutral and acidic oligosaccharides isolated from bovine colostrum were also shown to inhibit the adhesion of *S. enterica* IID604 to Caco-2 cells (Urakami et al., 2018). Value may lie in extracting, concentrating and researching these oligosaccharides for the purpose of adding to infant formulae. Indeed, the complexity, variation and numbers of HMOs makes it challenging for all of their associated functions to be duplicated in formulas despite advances in biotechnological production. For this reason, there may be value in supplementing formula with not only HMO such as 2′-FL but also BMO. BMO are largely sialylated which may be attractive in targeting sialic acid-dependent pathogens. However, the addition of BMO to formula also presents challenges. The quantity of oligosaccharides in mature bovine milk as mentioned is low, meaning that extremely efficient enrichment procedures are necessary. Concentrating oligosaccharides from bovine colostrum may be a solution and de Moura Bell et al. (2018) to this end recently developed a novel pilot-scale approach for the recovery of highly pure oligosaccharides, from colostral bovine whey permeate. Another consideration relating to certain bovine sialylated oligosaccharides is that humans lack the ability to synthesize the sialic acid, *N*-glycolylneuraminic acid (Neu5Gc), which is commonly produced in bovine milk (Padler-Karavani & Varki, 2011) and this structure is suggested to play a role in chronic inflammation-mediated diseases (Okerblom & Varki, 2017).

3.2. Glycosaminoglycans

Glycosaminoglycans (GAGs) are highly negatively charged, sulfated linear polysaccharides, formed by repeating disaccharidic units of uronic acid or galactose and one of two amino sugars, *N*-acetylglucosamine or *N*-acetylgalactosamine. GAGs are divided into four main classes: heparin/heparan sulfate (HP/HS), chondroitin sulfate (CS), keratan sulfate, and hyaluronic acid (HA), with HA being the only GAG not covalently bound to a core protein. GAGs exhibit high heterogeneity due to the numerous core proteins and differences in length and diverse enzymatic reactions including *N* and *O*-sulfations of the saccharide chains (Esko, Kimata, & Lindahl, 2009; Vivès, Seffouh, & Lortat-Jacob, 2014). Due to this heterogeneity, GAGs are involved in numerous diverse biological functions including cellular signalling, crosstalk, immunity, development, and pathogenesis (Linhardt & Toida, 2004). The levels of GAGs change throughout lactation in human milk with the peak occurring on day 4 at 3.8 g/L and declining to 0.4 g/L after one month with 73% of this reduction occurring between day 4 and 10 indicating the importance of GAGs in the first two weeks of infant life. GAGs are divided into the four classes, CS and HS/HP dominate and represent 55 and 42% of total GAGs respectively while dermatan sulfate and HA only account for the remaining 3% of total GAGs. The concentration of GAGs is 7 times higher in human milk than bovine milk with dermatan sulfate instead accounting for 40% of total GAGs in bovine milk followed by HS/HP and CS at 30 and 21%, respectively (Coppa et al., 2013, 2011, 2012). Milk GAGs are synthesised in the mammary gland in the form of proteoglycans, with a specific core protein linked with long chains of GAGs synthesised by specific glycosyltransferases. Upon entering the small intestine, the core protein is digested by proteolytic enzymes leaving the remaining GAG chains to reach the large intestine intact. Hence like milk oligosaccharides, GAGs may influence the intestinal microbiota by acting as soluble decoy receptors and through prebiotic properties (Iozzo & Schaefer, 2015).

Microbial pathogens have been shown to have preference for GAG structures, particularly HS, on host cells to aid their initial attachment. Therefore, supplemented GAGs may prevent the targeted adhesion of several pathogenic organisms. HT-29 enterocytes pre-treated with HP, an analog of heparan sulfate, had reduced internalisation of several pathogens including *L. monocytogenes*, *Streptococcus bovis*, *Streptococcus agalactiae*, *Streptococcus pyogenes*, and *S. aureus* 502A (60%-80% inhibition). The HT-29 cell line expresses syndecan-1 at high levels, a proteoglycan decorated predominantly with HS which several pathogenic bacteria target for initial attachment indicating a decoy receptor effect (Henry-Stanley, Hess, Erickson, Garni, & Wells, 2003; Henry-Stanley, Hess, Erlandsen, & Wells, 2005). Hafez, Aboulwafa, Yassien, and Hassouna (2008) examined the antimicrobial action of several GAGs against *S. aureus*, *Staphylococcus epidermidis* and *Staphylococcus saprophyticus* isolates. Pre-incubation of highly sulfated soluble HP and CS-B were the most effective to reduce *S. aureus* adherence to both Vero and Hep-2 cells whereas soluble CS-A and CS-C, less sulfated GAGs, were more effective against *S. epidermidis* and *S. saprophyticus* isolates (Hafez et al., 2008). Coppa et al. (2016) demonstrated a purified GAG complex from human milk, significantly reduced the adhesion of *S. fyris* to Caco-2 cells and *S. fyris* and *E. coli* O119 to Int-407 cells through co-incubation. Rajas et al. (2017) showed that direct interaction of commercial HS and a mixture of HS/CSA–CSC with *Staphylococcus* spp. and *Streptococcus* spp. reduced adhesion (50%) to lung cell lines A549 and MRC5 *in vitro* (Rajas et al., 2017).

GAGs have also been shown to effectively modulate the intestinal cell surface and protect against bacterial colonisation. Pre-treatment of the Vero and Hep-2 cell lines with soluble heparinase, chondroitinase AC and chondroitinase B reduced adherence of *S. aureus*, *S. epidermidis* and

S. saprophyticus isolates (Hafez et al., 2008). Hill et al. (2013) used human milk HA to reduce the adhesion of *S. typhimurium* to HT-29 cells indirectly through treatment of the HT-29 cells with HA for 24 h before bacterial exposure. Higher expression levels of the antimicrobial peptide HβD2 was observed *in vitro* upon exposure of the HT-29 cell line to HA and *in vivo*, from the intestinal mucosa of mice which had been administered HA (Hill et al., 2013). Kim et al. (2017) demonstrated commercial purified HA fragments to protect mice from *Citrobacter rodentium* infection with enhanced epithelial barrier function through increased expression of the tight junction protein ZO-1 observed. A dose-dependent inhibition of *Enterococcus faecalis* adherence to Caco-2 cells was observed by Sava et al., 2009 when porcine HP and HS were added prior to addition of the bacterial inoculum. Burge, Hannah, Eckert, Gunasekaran, and Chaaban (2019) demonstrated pre-treatment of the T84 cell line for 48 h with CS sodium salt led to a 75% reduction in both bacterial adhesion and invasion of the EPEC strain SCB34 (Burge et al., 2019).

GAGs may also work indirectly against bacterial infection by preventing an elaborated immune response and subsequent intestinal damage. In a murine NEC model induced by intraperitoneal dithizone injection and oral administration of *K. pneumoniae*, commercial HA (sodium hyaluronate) supplementation led to a reduction of pro-inflammatory cytokines, tumor necrosis factor-alpha (TNF-α), growth-regulated oncogene-alpha (GRO-α), IL-12p70, and IL-6 (Gunasekaran et al., 2020). Commercial CS supplementation reduced pro-inflammatory cytokine production and attenuated NF-κB activity in THP-1 macrophages exposed to lipopolysaccharide (LPS) (Stabler, Huang, Montell, Vergés, & Kraus, 2017). HP is also regarded as an effective anti-inflammatory agent in both inflammatory bowel disease (IBD) and ulcerative colitis (Mousavi, Moradi, Khorshidahmad, & Motamedi, 2015; Stabler et al., 2017).

HS and other highly sulfated GAGs have been implicated in inhibiting several viral infections to host cells *in vitro* due largely to the fact they are themselves targets of viruses for initial attachment and entry. Baba et al. (1988) demonstrated commercial HP to significantly reduce the adsorption of human immunodeficiency virus (HIV-1) to MT-4 cells and prevent viral destruction of the cells. CS from human milk prevented binding of the HIV co-receptor gp120 to CD4 on host cells, a critical initial step in HIV infection (Newburg, Linhardt, Ampofo, & Yolken, 1995). HP also inhibited the interaction of Herpes Simplex Virus (HSV) envelope glycoproteins B and C to target cell surface HS glycosaminoglycans for initial viral attachment (Laquerre et al., 1998; Spear, Shieh, Herold, WuDunn, & Koshy, 1992). Ghezzi et al. (2017) evaluated the effect of commercial HP when pre-incubated with human neural progenitor (hNPC) and Vero cells for 1 h before subsequent Zika virus (ZIKV) infection. Although HP only slightly inhibited virus replication and infection, it was efficient in preventing activation of the apoptotic cell death molecule caspase-3 and thus preventing virus-induced necrotic death of the hNPC and Vero cells. Commercially available CSE and HP exhibit similar anti-viral activity when incubated with Dengue virus (DENV) on BHK-21 and Vero cells and HSV on gro2C cells. Common carbohydrate structures with distinct sulfation sites between the two structures may explain similar viral inhibitory activity (Bergefall et al., 2005; Kato et al., 2010; Nahmias & Kibrick, 1964). Of particular interest, SARS-associated coronavirus (SARS-CoV) infection of Vero cells was inhibited by 50% when Vero cells were pre-incubated with HP 30 mins before viral addition. The SARS-CoV strain's envelope proteins are thought to be decorated with positively charged amino acids and their intended target of negatively charged sulfate groups on target Vero cell proteoglycans is blocked by the addition of commercial HP (Vicenzi et al., 2004). It is plausible to speculate, that GAGs could act as anti-viral agents against members of the coronavirus family including severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), the causative pathogen of the COVID-19 pandemic. Investigating the potential of GAGs to block COVID-19 virus may be of great interest for future research.

As this section has discussed, GAGs present a wide variety of anti-bacterial and viral activities and their common use by pathogens to gain host entry has given advantageous decoy receptor capability through external supplementation. These features indicate GAGs may provide potential targets in the development of effective antimicrobial and antiviral therapies. Efficient antimicrobial therapies and/or supplementation involving GAGs is dependent on achieving a greater understanding of the host–GAG and pathogen-GAG relationship and their specificity.

4. Proteins

4.1. Immunoglobulins

Immunoglobulins (Igs) represent about 10 to 15% of whey proteins in both human and bovine milk (Golinelli, Del Aguila, Flosi Paschoalin, Silva, & Conte-Junior, 2014; Kulczycki & MacDermott, 1985; Marshall, 2004). They are one of the most abundant bioactive proteins ranging from 19.37 g/L in colostrum to 1.14 g/L in mature milk in humans and 61.4 g/L in colostrum to 0.8 g/L in mature bovine milk (Wheeler, Hodgkinson, Prosser, & Davis, 2007). The major Ig classes are IgA, IgG, and IgM (Korhonen & Marnila, 2009) with IgA being the predominant Ig in human milk and colostrum and IgG being predominant in bovine milk (Butler, Seawright, McGivern, & Gilsdorf, 1986). The main role of immunoglobulins (antibodies) is to agglutinate bacteria, inactivate viruses, neutralize toxins and stimulate an environment for the growth and colonisation of beneficial bacteria (Bojsen et al., 2007; Kvistgaard et al., 2004; McWilliams, 1975; Morrin et al., 2020). In brief, milk Igs give the offspring immunological protection against infections in the gastrointestinal and respiratory tract. Numerous studies (reviewed by Jasion & Burnett, 2015) have been performed demonstrating that Ig preparations derived from human serum as well as bovine colostrum and serum survive digestion and are present in fecal matter. IgG was the predominant Ig in the latter preparations, but often IgA and/or IgM were present in smaller amounts. The glycan chains of milk Igs may protect the Ig protein from digestion by proteolytic enzymes, allowing the intact or only partially digested Ig to reach the intestine (for absorption into the blood-) (O'Riordan, Kane, Joshi, & Hickey, 2014). Cross-species activity between human and bovine immune-related milk proteins has been reported (den Hartog et al., 2014; van Neerven, Knol, Heck, & Savelkoul, 2012), and cows' milk IgG binds to gastrointestinal pathogens that also infect humans, such as *Shigella flexneri*, *Clostridium difficile*, *E. coli*, *Cryptosporidium parvum*, *Helicobacter pylori*, *Streptococcus mutans*, RV (H. Korhonen, Marnila, & Gill, 2000) and human respiratory syncytial virus (den Hartog et al., 2014). Furthermore, a recent report compared the specificity between bovine sIgA and human sIgA isolated from milk to pathogenic and commensal bacteria and concluded that the binding characteristics of both human and bovine sIgA from milk were similar (Hodgkinson et al., 2017). The total amounts of Igs in bovine milk are lower than in human milk (excluding colostrum), and levels are significantly lower or absent in infant formula. This may explain in part the increased incidence of infections in formula-fed infants, compared with breast-fed infants (Dewey, Heinig, & Nommsen-Rivers, 1995) highlighting the benefits that might be associated with supplementing formulas with Igs.

High concentrations of antibodies against specific pathogens may be achieved by immunizing cows with the pathogen or its antigens. On average, 500 g/L of IgG can be obtained from each immunized cow immediately after calving (Kramski et al., 2012). Sears et al. (2017) investigated several commercial hyperimmune bovine colostrum (HBC) products formulated to reduce the risk of ETEC-induced diarrhoea. The products contained high levels of IgG specific for multiple ETEC antigens present in the administered vaccines. Antimicrobial activity was measured using *in vitro* novel functional assays. The IgG present in the products greatly reduced ETEC motility in soft agar and promoted complement-mediated lysis. In a study by Sponseller et al., 2015, a cow was repeatedly immunised with recombinant mutants of toxins A and B

of *C. difficile*, and the resultant HBC was examined in *C. difficile* infected gnotobiotic piglets with resultant diarrhoea. Control piglets received non-immune colostrum and developed moderate to severe diarrhoea and colitis. In contrast, HBC-treated piglets had mild or no diarrhoea and mild or no colitis. A study by Hu et al. (2015) demonstrated that bovine antibody-based oral immunotherapy had a significant clearance effect on intragastric *H. pylori* in a group of 30 antibody treated adults. Ramezanalizadeh et al. (2017) went on to demonstrate *in vitro* that a HBC reduced the attachment and growth of *S. mutans* and *S. sobrinus* by 69% and 43% respectively, using a microtitre plate method.

In relation to viruses, den Hartog et al. (2014) investigated whether bovine milk-derived IgG could modulate immune responses against human respiratory syncytial virus (RSV). The group investigated the specificity and functional relevance of bovine IgG against RSV and other human respiratory pathogens, the ability of bovine IgG to bind to human Fc receptors, and the induction of effector functions in human myeloid cells. Bovine IgG was found to recognise human RSV, influenza haemagglutinin and *Haemophilus influenzae*. Four independent studies (two in Bangladesh and two in Europe) describe rotavirus-diarrhoea treatment with hyperimmune colostrum products. Three of the studies showed significant clinical effects including a reduction in duration of diarrhoea (Mitra et al., 1995) and stool frequency (Mitra et al., 1995; Sarker et al., 1998) the duration of RV excretion (Hilpert et al., 1987) and need for oral rehydration solution (ORS) (Sarker et al., 1998). In the fourth study, a trend was observed towards a shorter duration and decreased stool frequencies in the actively treated group, but this was not significant compared to the control group (Ylitalo, Uhari, Rasi, Pudas, & Leppaluoto, 1998). Considering the studies described above, there may be merit in the use of immune milks and Ig concentrates as dietary ingredients. Such ingredients would most probably leave the normal gut microflora intact and provide a strategy to prevent infections (Morrin et al., 2020). As a result of the progress made in the separation and isolation of Igs from bovine colostrum and cheese whey, Ig products may be feasible (reviewed by Feeney, Morrin, Joshi, & Hickey, 2018). Recently, Billakanti, Fee, Naik, and Carbonell (2014) employed a hexamer peptide (HWRGWV) affinity matrix for the isolation of bovine Igs from various dairy streams (skim milk, acid whey, and colostrum). Bound Igs were recovered with a purity of $>85\%$ in a single step. Methods such as this hold promise as an alternative to conventional proteinA/G chromatography for direct capture of Igs from streams containing relatively high Ig concentrations such as colostrum, transgenic, or hyperimmune milk.

4.2. Glycomacropeptide

Glycomacropeptide (GMP), also termed caseinomacropeptide, is a bioactive milk peptide released from κ-casein via enzymatic digestion physiologically or chymosin digestion during the cheese-making process (Eigel et al., 1984). After milk ingestion, GMP is also generated by pepsin hydrolysis in the adult human gastrointestinal (GI) tract (Yvon, Beucher, Guilloteau, Le Huerou-Luron, & Corring, 1994). GMP encompasses 20–25% of the total protein found in whey protein isolate and whey protein concentrate derived from cheese whey. GMP is rich in branched-chain amino acids (leucine, isoleucine, valine), contains only one residue of methionine and interestingly, has no aromatic amino acids (phenylalanine, tryptophan, and tyrosine) or cysteine associated with its structure (Mercier, Brignon, & Ribadeau-Dumas, 1973; Thomä-Worringer, Sørensen, & López-Fandiño, 2006). Due to large commercial availability, bovine GMP is one of the most researched and best characterized milk proteins.

GMP is a heavily sialylated glycoprotein, which alongside other carbohydrates, largely dictate the antibacterial activity of the peptide to be one of a decoy receptor effect (Wang, Brand-Miller, McVeagh, & Petocz, 2001). Direct interaction between casein whey GMP and the cholera toxin (CT) prevented the binding of CT to its target receptor ganglioside GM1 found on the CHO-K1 cell line. Enzymatic digestion of GMP using sialidase and pepsin, reduced the inhibition of CT binding to GM1. Thus, the direct inhibitory effect of GMP towards CT could be attributed to the presence of sialic acid and the peptide sequence (Kawasaki et al., 1992). In another study, treatment of bovine GMP with *N*-acetylneuraminindase and β-galactosidase also led to reduced binding with the CT B-subunit, with sialic acid (NeuAc) being the more important sugar in conferring activity (Oh, Worobo, Kim, Rheem, & Kim, 2000). Bovine GMP also directly binds to *S. enteritidis*, *Morganella morganii* and *E. coli*. De-sialylation of GMP eradicated binding to *E. coli* O157 and partially to *S. enteritidis*. Additionally, periodate oxidation of GMP eliminated binding to both *E. coli* O157 and *S. enteritidis*. The study also demonstrated a reduction in adhesion of *E. coli* 0157 to intestinal Caco-2 cells when co-incubated with GMP (Nakajima et al., 2005). Kim et al. (2016) isolated *N*-acetylneuraminic acid from GMP and examined its antibacterial potential against *H. pylori* and *H. felis* both *in vitro* and *in vivo*. *In vitro* studies revealed that *N*-acetylneuraminic acid reduced the growth of *H. pylori* in a dose-dependent manner through direct interaction. *H. pylori* colonisation was also prevented by GMP supplementation in *H. pylori* challenged murine subjects (Kim et al., 2016).

Caseinglycomacropeptide was found to reduce the adhesion of verotoxigenic (VTEC) strains and EPEC *E. coli* strains to HT-29 cells through the decoy receptor mechanism (Rhoades et al., 2005). Feeney et al. (2017) later also demonstrated a reduction in adhesion of a selected enterohemorrhagic (EHEC) *E. coli* (*E. coli* 12,900 O157:H7) and EPEC (*E. coli* O125:H32 and O111:H2) to HT-29 and Caco-2 cell monolayers through direct interaction between commercially available GMP and the bacterial strains. This was confirmed to be a decoy receptor effect as pre-incubation of GMP with either of the two selected cell lines did not give a similar reduction in adhesion of the strains. However, pre-incubation of the Caco-2 cell line with GMP led to delayed translocation of EPEC through the tight junctions indicating GMP may also indirectly combat infection by strengthening intestinal integrity (Feeney et al., 2017). Brück, Kelleher, Gibson, Graverholt, and Lönnerdal (2006) examined several different digests of bovine GMP to prevent adhesion of *S. flexneri*, *S. typhimurium* and EPEC *E. coli* to Caco-2 cells via direct binding of the milk component to the pathogens. Pepsin/pancreatin as well as pepsin-digested GMP were most effective for EPEC. *S. typhimurium* was more inhibited by pepsin/pancreatin-digested GMP while *S. flexneri* was most affected by undigested or pepsin digested GMP. GMP's ability to retain a decoy receptor effect after digestion was hypothesised to be due to its sialic acid substructure which remains largely intact after digestion (Brück et al., 2006).

Several studies also indicate the protective role GMP may have against pathogens *in vivo*. Mice challenged with CT and enterotoxins LT1 and LT2 of *E. coli* had reduced diarrhoea when given GMP in a dose-dependent manner (Isoda, Kawasaki, Tanimoto, Dosako, & Idota, 1999). Rong et al. (2015) demonstrated *E. coli* K88 challenged piglets supplemented with commercial GMP to have no increase of enterobacteria in intestinal contents. Parameters for intestinal injury (shortened villi and deepening crypts) and disrupted intestinal barrier function (permeability measured by D-lactate and diamine oxidases) were reduced in the GMP-supplemented group (Rong et al., 2015). Gusatavo-Hermes et al. (2013) also investigated the protective effects of commercial GMP in *E. coli* K88 challenged weaning piglets. Initial *in vitro* studies revealed GMP to prevent attachment of ETEC K88 to ileal mucosa tissues via decoy receptor activity with *E. coli* fimbriae confirmed to bind GMP. The subsequent *in vivo* study allied this result, with ETEC K88 colonisation of the ileum prevented in GMP-treated groups (Gustavo Hermes et al., 2013).

Only a limited number of studies exist on the anti-viral activity of GMP. Bovine GMP has been found to prevent human RV infection of the Rhesus monkey kidney MA104 cell line *in vitro*. The study determined the antiviral activity of GMP to be due to decoy receptor activity and not through interaction with the Rhesus monkey kidney cell line MA104. Loss of anti-HRV activity was observed after GMP had been de-glycosylated via *O*-glycosidase treatment and not through de-

sialylation, indicating glycans other than sialic acid were responsible for GMP's anti-HRV activity (Inagaki et al., 2014). Kawasaki et al. (1993) demonstrated that bovine GMP prevents hemagglutination (HI activity) by four strains of human influenza virus by direct binding. Sialidase treatment of GMP led to loss of inhibition of the four strains, indicating again that sialic acid majorly contributes to GMP's antiviral function. Morphological transformation of peripheral lymphocytes by Epstein–Barr virus (EBV) was also prevented by bovine GMP (Dosako, Kusano, Deya, & Idota, 1992). GMP is a promising milk component for anti-viral activity due to its high concentration of attached sialic acid, a known target of many viruses (Wasik, Barnard, & Parrish, 2016). However, at least 13 genetic variants of bovine kappa-casein have been identified which have different post-translational modifications and vary in their level of phosphorylation and glycosylation (Thomä-Worringer et al., 2006). This factor should be considered going forward in any studies targeted at investigating GMP's anti-bacterial or anti-viral effects.

The addition of GMP to infant formula has also raised concern due to its high threonine content (12–13 threonine residues) which increases the occurrence of hyperthreoninaemia in infants fed formula containing GMP versus breastfed infants (Rigo et al., 2001). However, more recent studies suggest this increased occurrence of hyperthreoninaemia is due to differences in threonine metabolism among the infants tested (Sandström, Lönnerdal, Graverholt, & Hernell, 2008). Taking this into consideration, future studies are required to increase our knowledge of the biological and structural functions of GMP while also focusing on the safety of its inclusion as a food ingredient.

4.3. α-lactalbumin

α-lactalbumin (α-La) is the predominate protein in human milk constituting 22% of total protein and accounting for 36% of whey proteins. Similarly, in bovine milk, α-La is the most abundant protein constituent after β-lactoglobulin accounting for 3.5% of total protein and approximately 17% of whey proteins (Kamau, Cheison, Chen, Liu, & Lu, 2010). α-La through numerous studies is known to play both a biochemical role and nutritional role in early infant development. In mammary epithelial cells, α-La is one of two regulatory components of lactose synthase, a complex responsible for the biosynthesis of lactose (Brew & Hill, 1975). α-La initiates the conversion of galactose into *N*-acetylglucosamine, via binding of α-La to β1,4-galactosyltransferase (GT-1). The production of *N*-acetylglucosamine leads to the subsequent synthesis of lactose from UDP-galactose and glucose in the Golgi complex (Davis, Harris, Lien, Pramuk, & Trabulsi, 2008; Grobler, Wang, Pike, & Brew, 1994). α-La also provides a rich source of bioactive peptides and numerous essential amino acids including tryptophan, lysine, cysteine, leucine, isoleucine, valine and sulfur-containing amino acids all of which are vital for early infant nutrition (Krissansen, 2007; Lönnerdal & Lien, 2003). Also, peptides derived from hydrolysed α-La play an important role in infant pathogenic defence. Sharing a 72% homology with human milk α-La amino acid sequences, bovine α-La may confer benefits (Lien et al., 2004).

No intact α-La has been found in infant stool, indicating the protein is fully digested during transit through the gastrointestinal tract. α-La is partially digested in the upper GI tract releasing several peptides with numerous bioactivities. A portion of the undigested α-La may also reach the large intestine where it may also exert beneficial effects on the gut microbiota in the defence against pathogens (Lönnerdal, 2014). Bactericidal peptides generated from proteolytic digestion of bovine α-La, using trypsin and chymotrypsin, demonstrate growth inhibition of several Gram-negative bacteria through direct interaction. The growth of *E. coli*, *K. pneumoniae*, *S. aureus*, *Staphylococcus epidermis*, streptococci, and *Bacillus subtilis* was inhibited to varying degrees when co-incubated with the α-La peptide digests (Pellegrini, Thomas, Bramaz, Hunziker, & von Fellenberg, 1999). Digested α-La also was shown to reduce the metabolic activity of *E. coli* down to 21% within 6 h of co-incubation (Pihlanto-Leppälä et al., 1999).

A folding variant of α-La, termed HAMLET (human alpha-lactalbumin made lethal to tumor cells), has demonstrated bactericidal activities against numerous bacterial organisms (Håkansson et al., 2000). Purified from milk casein, the active complex in a molten globule like state paired with a C18:1 (oleic) fatty acid, has antibacterial activity against several pathogens including *H. influenzae*, EPEC *E. coli* and *S. typhimurium* and *Streptococci* species (Håkansson et al., 2000; Marks, Clementi, & Hakansson, 2012; Peso Echarri et al., 2012). Amongst the streptococcal species, namely S. *pneumoniae*, S. *pyogenes* and *S. agalactiae*, the HAMLET complex was shown to induce direct bactericidal activity via contact of the complex to the bacterial membrane, with subsequent membrane depolarization and disruption (Alamiri, Riesbeck, & Hakansson, 2019; Clementi, Marks, Duffey, & Hakansson, 2012; Marks et al., 2012; Marks, Clementi, & Hakansson, 2013; Meikle, Mossberg, Mitra, Hakansson, & Niederweis, 2019). This antimicrobial activity was reliant on sodium dependent calcium transport and kinase activity. HAMLET administered with specific antibiotics has also resulted in the reversal of antibiotic resistance of several antibiotic resistant strains of *S. pneumoniae*, *Mycobacterium tuberculosis*, *S. aureus*, and *A. baumannii* (Alamiri et al., 2019; Clementi et al., 2012).

Bovine α-La also may combat pathogens indirectly through modulation of the mucosal surface. Administration of α-La to rats led to increased mucin and bicarbonate secretion via increased prostaglandin synthesis and therefore enhanced the protective mucin layer (Ushida, Shimokawa, Matsumoto, Toida, & Hayasawa, 2003). α-La may also work indirectly against pathogens through enhancing the innate immune response. A tripeptide GLF (glycylleucyl-phenylalanine) derived from residues of both human and bovine milk α-La counteracted *K. pneumoniae* infection indirectly via enhanced phagocytosis of both human and murine macrophages (Migliore-Samour et al., 1992). GLF has also been shown to bind specific sites on human phagocytic blood cells namely neutrophils and monocytes leading to subsequent stimulation of superoxide anion production by neutrophils, increased monocyte-macrophage adherence, chemotaxis and phagocytosis of human senescent red blood cells (Gattegno, Migliore-Samour, Saffar, & Jolles, 1988; Jaziri et al., 1992; Kayser & Meisel, 1996; Migliore-Samour et al., 1992; Rusu, Drouin, Pouliot, Gauthier, & Poubelle, 2009). Formula fed infant monkeys supplemented with bovine α-La demonstrate enhanced ability to counteract EPEC *E. coli* infection with reduced diarrhea observed. A microbiota profile similar to breast-fed infant monkeys was also achieved with supplementation of α-La (Brück et al., 2003). Fermentation of infant faecal cultures in a two-stage compound continuous culture model supplemented with α-La significantly decreased the levels of inoculated EPEC *E. coli* and S. *Typhimurium* (Brück, Graverholt, & Gibson, 2003).

Bovine α-La and its peptide digests also have demonstrated heightened antiviral activity when chemically modified with 3-hydroxyphthalic (HP) anhydride. When co-incubated with HIV-1 the modified α-La-HP effectively inhibited replication of the virus in MT2 cells (Berkhout et al., 1997). Oevermann, Engels, Thomas, and Pellegrini (2003) demonstrated bovine α-La-HP to effectively inhibit HSV-1 replication in Vero76 cells. The modified protein was more potent when co-incubated with HSV-1 on the Vero76 cell line and when also applied after the cell line had been infected with the virus. Higher concentrations of the modified α-La-HP were required when incubated with the cell line before viral infection. This indicates α-La-HP inhibition may be more effective with direct interaction to the virus and not though modulation of the cell line. Methylated α-La and peptic hydrolysates also possessed an anti-cytomegaloviral activity in MRC-5 fibroblasts when co-incubated with the virus (Chobert, Sitohy, Billaudel, Dalgalarrondo, & Haertle, 2007). Sitohy, Billaudel, Haertle, & Chobert, 2007 demonstrated the anti-viral activity of methyl-esterified α-La or the derived tryptic peptides against HSV-1 when co-incubated on Vero cells.

Bovine α-La has benefits to the infant regarding growth, plasma amino acids, safety and tolerability. However, potential new

applications such as antimicrobial therapy deserve attention. The potential antibacterial and antiviral effects of α-La bioactive peptides released during digestion in the infant may enhance gastrointestinal and immune function and also warrant further investigation. The choice of technology to use in the production of α-La is guided by the expected purity levels, amounts needed, and subsequent processing. There is no single method that produces 100% pure α-La. It is available commercially as α-La–enriched whey protein concentrates obtained by filtration methods, with α-La constituting approximately 45% of total protein. Highly purified α-La, accounting for more than 93% of total protein, is obtained by ion exchange methods (reviewed by Layman, Lönnerdal, & Fernstrom, 2018).

4.4. Osteopontin

Osteopontin (OPN), previously named Eta-1 (early T-lymphocyte activation-1) or SSP1 (secreted phosphoprotein 1) is a negatively charged, highly phosphorylated and glycosylated protein (Schack, Lange et al., 2009). OPN belongs to the SIBLING protein family of *N*-linked glycoproteins that have affinity towards cell surface integrins and the CD44 receptor. OPNs are involved in biomineralization, tissue remodelling, and immune regulation (Kahles, Findeisen, & Bruemmer, 2014). OPNa, the full-length isoform of OPN, is found at high concentrations (138 mg/mL) in human milk, indicating it may be of importance in the development of the infant (Schack, Lange et al., 2009). Although OPN is found at much lower concentrations in bovine milk (18 mg/mL), the proteins share a 61% sequence homology and both contain similar integrin-binding, proteolytic and PTM sites. Interestingly, the level of OPN remains low in human colostrum until around day 3 of lactation where an increase in levels is observed thereafter. Levels of OPN remain high past 1 year post-lactation, preserving half of the maximum level observed (Nagatomo et al., 2004).

A role for OPN in the defence against pathogenic infection has been shown in several studies. OPN-null murine subjects were associated with increased *M. tuberculosis* bacillus Calmette-Guerin (BCG) growth in macrophages compared to their wild-type counterparts (Nau et al., 1999). Bovine OPN has shown to directly bind to both *S. aureus* and *S. agalactiae* serotypes and also the $\alpha_x\beta_2$ integrin receptor of monocytes, opsonizing the bacteria-OPN complex for phagocytosis (Schack, Stapulionis et al., 2009). Supplementation with OPN is also known to influence the intestinal gene expression profile of monkeys bringing it closer to that of their breast-fed counterparts. In a study performed by Donovan et al. (2014), jejunum gene expression of monkeys fed standard IF, IF supplemented with bovine OPN (125 mg/L) or breast milk from 0 to 3 months was examined. The number of genes differing between breast-fed (BF) and OPN supplemented formula-fed (FF) groups was lower (217 genes) compared to the non-supplemented OPN FF group (1214 genes). The OPN supplemented group displayed a differential gene expression in genes involved in cell survival, proliferation, movement and communication closer to that of the BF group (Donovan et al., 2014). Occludin expression, an essential protein in the regulation of tight junction integrity, was also modulated by OPN. OPN deficiency led to a reduced expression of the phosphorylated form of occludin in the colonic mucosa of the murine subjects indicating OPN may be important for efficient tight junction formation (Woo, Lee, Park, Go, & Kim, 2019). Intestinal epithelial cell proliferation and differentiation are critical in the defense against pathogens. Therefore, the modulation of gene expression in the intestinal epithelium by OPN described here may indicate a reduced susceptibility to pathogenic invasion and infection.

OPN may also contribute to pathogenic defence by altering immune responses. For instance, OPN is known to induce a Th1 cytokine response which is required for pathogenic defense (Ashkar et al., 2000). OPN-Knock-out (KO) mice are known to be susceptible to bacterial and viral infections. Immune markers were measured in mouse pups nursed by wild-type or OPN-KO dams and subsequently administered the *E. coli* LPS, serotype O111:B4. Plasma OPN and TNF-α were significantly up-regulated in both control and OPN-deficient groups after LPS injection, but a considerably higher increase in plasma OPN and TNF-α was observed in the pups nursed by OPN-KO dams. These observations indicate that milk OPN reduced inflammation resulting from LPS administration. Similarly, plasma IFN-γ was dramatically enhanced in response to the LPS challenge in pups nursed by both WT and OPN-KO dams, but the fact that the pups nursed by WT dams showed a significantly larger increase in IFN-γ suggests that milk OPN may contribute to resistance to LPS administration (bacterial infection) by altering immune responses (Jiang & Lönnerdal, 2020). Similarly, a lower inflammatory score and less neutrophil infiltration were observed in OPN-fed dextran sulfate sodium-treated mice (Kanwar et al., 2016), and bovine OPN supplemented (30 g/L) formula has also led to a reduced incidence of diarrohoea in piglets injected with LPS (Ren et al., 2019). Moreover, OPN may improve immunity by altering the gut microbiota. Compared to ETEC-infected piglets fed regular algae, ETEC-infected piglets fed OPN-enriched algae showed decreased α-diversity, altered microbiota composition and short-chain fatty acid profiles (Wang, Smith, Adams, Tran, Dilger, & Donovan, 2019).

Recently, Liu, Jiang, and Lönnerdal (2019) demonstrated that a commercial bovine lactoferrin-OPN complex was more resistant to *in vitro* digestion when compared to lactoferrin (LF) or OPN alone. Higher binding and uptake of the LF-OPN in Caco-2 cells was observed compared to individual components alongside a greater promotion of proliferation and differentiation of the intestinal epithelial cells. Antibacterial activity of OPN alone prevented EPEC growth but not adhesion to the Caco-2 cells whereas LF and the LF-OPN complex inhibited both growth and adhesion of EPEC to cells (Liu et al., 2019).

OPN is minimally digested *in vitro* and the presence of OPN in both the intestine and plasma alludes to the theory that OPN may also be involved in systemic functions as witnessed with other milk bioactive components (da Silva et al., 2009; Donovan & Comstock, 2016). Fully understanding OPNs functional properties in detail is of critical importance for its development as an IF ingredient. Processes for isolation of OPN from bovine milk have been disclosed. OPN is isolated from bovine whey using anion exchange technology and the final product contains ~78% protein of which 95% is OPN (Kvistgaard, Matulka, Dolan, & Ramanujam, 2014).

4.5. Lactoperoxidase

Lactoperoxidase (LPO) is a mammalian heme-containing glycoprotein from the family of peroxidase enzymes (PODs). The peroxidase family convert halides and pseudohalides into oxidants and hypohalous and hypothiocyanous acids which serve as potent antibacterial and antiviral chemical oxidants (Petrides & Nauseef, 2000; Wever, Kast, Kasinoedin, & Boelens, 1982). LPO is found in milk, saliva and tears, among other secretions and milk LPO plays a crucial role in protecting the lactating mammary gland and the intestinal tract of the newborn infant from pathogenic microorganisms (Naidu, 2000). Human milk LPO (hLPO) is made up from a single polypeptide chain containing 632 amino acid residues, with a molecular weight of 80 kDa while bovine LPO (bLPO) is made up of 612 amino acid residues with a molecular weight of 78 kDa (Cals, Mailliart, Brignon, Anglade, & Dumas, 1991; Paul & Ohlsson, 1985). LPO is the second most abundant enzyme in bovine milk (30 mg/L) with peroxidase activity 20 times higher than that of hLPO (1.2–19.4 units/mL LPO). hLPO and bLPO share 85% sequence identity and also share similar physicochemical properties (Sharma et al., 2013). Due to the high homology between hLPO and bLPO, bLPO is used for both scientific research and industrial applications due to the wide availability of bovine milk and the following section refers mainly to studies using bLPO. The anti-infective effect of milk LPO is attributed to the oxidation of thiocyanate (SCN^-) ions catalysed by LPO in the presence of H_2O_2, known commonly as the lactoperoxidase/thiocyanate/hydrogen peroxide (LPO) system. The end product, hypothiocyanite (OSCN-), is responsible for the

aforementioned antimicrobial and antiviral activity as discussed below (Pruitt, 2020).

The anti-microbial action of the LPO system has been observed only through direct interaction with the pathogen itself leading to agitation of the membrane, interference with intracellular metabolic functions and/or membrane-associated biosynthesis. The difference in composition and structural diversity in the cell wall and membranes of bacterial pathogens has also been shown to determine their susceptibility to the LPO system. Marshall and Reiter (1980) demonstrated commercially purchased OSCN- had bactericidal activity against *E. coli* with a greater amino acid and K+ leakage and overall destruction of the bacterial membrane observed than that of the *S. lactis* membrane in which treatment with the anion only had a bacteriostatic effect (Marshall & Reiter, 1980). The oxidation of sulphydryl groups of *E. coli* into sulphenyl derivatives prevents bacterial respiration leading to a bactericidal effect (Thomas & Aune, 1978). However, Shin, Hayasawa, and Lonnerdal (2001) also reported bLPO may be bactericidal through the inactivation of sulfhydryl groups of dehydrogenases associated with the cytoplasmic membrane of *E. coli* (Shin et al., 2001). Cell membrane permeability is also an important factor in determining susceptibility of *Salmonella* spp. to bactericidal or bacteriostatic effects of the bLPO system, alongside the growth phase of the bacteria (Purdy, Tenovuo, Pruitt, & White, 1983). Growth inhibition of *Bacillus cereus* by bLPO was shown to be directly proportional to the number of $OSCN^-$ anions present and inhibition of *B. cereus* was associated with a reduced extracellular release of collagenase activity, a virulence factor of the species (Tenovuo, Makinen, & Sievers, 1985). Inhibition of glycolysis by synthetic LPO systems have been reported specifically targeting the glyceraldehyde 3-phosphate dehydrogenase of streptococcal spp. (Carlsson, Iwami, & Yamada, 1983). Oxidation of hexokinase, aldolase and glucose-6-phosphate dehydrogenase has also been observed which may inhibit bacterial glycolysis (Adamson & Pruitt, 1981; Hawkins, 2009; Mickelson, 1977). Reduction of various pathogens has been observed in milks which has been attributed to the LPO system including VTEC *E. coli*, *P. fluorescens*, *C. jejuni*, *S. aureus*, *L. monocytogenes* and *Brucella melitensis* (Seifu, Buys, & Donkin, 2005; Wolfson & Sumner, 1993). Injection of LPO after Lactoferrin (Lf) from camel colostrum milk led to the significant clearance of *Acinetobacter baumannii* in lung and blood cultures in an *A. baumannii* pneumonia mouse model. The LPO/Lf combination also led to significantly enhanced concentrations of IL-4 and IL-10 which prevented damage caused by the inflammatory response to infection (Mahdi et al., 2018). Additional information on the antimicrobial role of the LPO system is comprehensively reviewed by Sarr, Toth, Gingerich, and Rada (2018).

The LPO system, namely its product OSCN-, demonstrates a wide spectrum of virucidal activity against both DNA and RNA viruses. The bLPO system has antiviral properties with halides (iodide, bromide) against poliovirus and vaccina virus when co-incubated. LPO used in combination with glucose oxidase and in the presence of sodium iodide inactivated several cell-free and cell-associated HIV-1 isolates (Belding, Klebanoff, & Ray, 1970). When bLPO was co-incubated with HIV-1, loss of viral replication was observed as well as inhibition of cytopathic effects on infected T-cell HUT 78 and CEM cell lines (Yamaguchi, Semmel, Stanislawski, Strosberg, & Stanislawski, 1993). An OSCN-generating system consisting of glucose–glucose-oxidase, thiocyanate and lactoperoxidase also effectively inhibited the growth of HIV when co-incubated before being inoculated onto lymphocytes. Viral growth was measured via quantitating the specific p24 viral capsid protein either in the culture cells or in the supernatant. Significantly lower levels of p24 was observed in groups treated with the LPO mixture (Pourtois et al., 1990). HSV-1 growth was also inhibited on Vero cells when pre-incubated with LPO from either camel, bovine, and human origin with bLPO showing the highest anti-viral activity (El-Fakharany, Uversky, & Redwan, 2017). OSCN- produced by the bLPO system also has antiviral activity against four influenza viruses (IFV) of both type A and B. The exposure of viral particles to OSCN- before viral adsorption to MDCK cells significantly reduced the plaque formation of IFV compared to pre-exposure of OSCN- to MDCK cells or the exposure of MDCK cells following virus adsorption to OSCN-. Therefore, it could be concluded OSCN- anti-IFV activity was associated with attachment to the viral envelope and not due to viral adsorption on host cells (Sugita et al., 2018). Although the LPO/H2O2/SCN- system is only detailed in a limited number of studies assessing antiviral activities, early observations indicate a beneficial use of the LPO system in the fight against viral infection. The industrial source of lactoperoxidase is derived with well-defined purification methods from bovine milk. Methods based on ion exchange chromatography are preferred for a large-scale production but have lower purification fold compared to affinity chromatography methods (Urtasun et al., 2017). A method using ion exchange resins (CM-cellulose) developed by Borzouee, Mofid, Varshosaz, and Samsam Shariat (2016) obtained a LPO preparation with a 10.26% yield and purification fold of 59.13. Uğuz and Ozdemir (2005) described a method using Amberlite CG 50H+ ion exchange resin and double gel filtration using Sephadex G-50 and Sephadex G-100 that obtained a LPO formulation with a 28% yield and purification fold of 11.5. Although, differences in structure, physicochemical properties and chemical activity between human and bovine LPO are small, again slight differences in post-translational modifications on the proteins should be considered during isolation and when assessing its anti-infective potential.

4.6. MFGM

The milk fat globule membrane (MFGM) is a trilayer consisting of proteins and phospholipids which surrounds the lipid droplets that are secreted by the lactating mammary gland, ensuring they remain dispersed throughout the milk rather than aggregating (Dewettinck et al., 2008). MFGM is enriched with glycerophospholipids, sphingolipids, cholesterol, and proteins, some of which are glycosylated, and are known to exert numerous biological roles (Lee et al., 2018). The proteome of the human MFGM is complex with several hundred identified proteins including mucin 1 (MUC1), xanthine dehydrogenase/oxidase (XDH/XO), CD36, PAS-6 and PAS-7, adipophilin (ADPH), and butyrophilin (BTN) (Fontecha et al., 2020; Lee et al., 2018; Liao, Alvarado, Phinney, & Lonnerdal, 2011; Lu et al., 2016). Bovine MFGM-rich fractions contain approximately the same number of proteins (Affolter, Grass, Vanrobaeys, Casado, & Kussmann, 2010). The MFGM polar lipids (phospho- and sphingolipids) account for 60–70% of total milk polar lipids and their concentration (about 20 mg/100 mL) is comparable between human milk fat and bovine milk fat (Cilla, Diego Quintaes, Barbera, & Alegria, 2016; Fontecha et al., 2020; Garcia et al., 2012; Russo et al., 2013; Zou et al., 2012). The proteins, polar lipid metabolites, and gangliosides in MFGM have been extensively studied *in vitro* for their anti-adhesive (Bu et al., 2007; Fuller, Kuhlenschmidt, Kuhlenschmidt, Jiménez-Flores, & Donovan, 2013; Kvistgaard et al., 2004; B. Liu, Yu, Chen, Kling, & Newburg, 2012; Martin, Hancock, Salisbury, & Harrison, 2004; Novakovic et al., 2015; Otnaess, Laegreid, & Ertresvag, 1983; Ross, Lane, Kilcoyne, Joshi, & Hickey, 2015) and antibacterial properties (Hester et al., 2013; Idota & Kawakami, 1995).

The proteins of MGFM in particular have been shown to contribute to defence against pathogenic bacterial and viral infection in the GI tract. MUC1 purified from human milk MGFM inhibited the invasion of *S. typhimurium* in Caco-2 and FHs74 cells *in vitro* at concentrations found similar to those in human milk (150 μg/L) (B. Liu et al., 2012). MUC1 has also been observed to bind the intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) of dendritic cells, a known target receptor of pathogens. MUC1, therefore may block the interaction of pathogens with dendritic cells. Saeland et al. (2009) demonstrated the interaction of human milk MUC1 with DC-SIGN to prevent the transmission of HIV from dendritic cells to CD4+ T cells *in vitro* (Saeland et al., 2009). BTN2A1, butyrophilin is also a ligand for DC-SIGN of monocytes and dendritic cells and may also contribute to pathogenic defence and inhibition of viral transmission (Malcherek et al., 2007).

Another MFGM protein, lactadherin when pre-incubated with rotavirus-infected cells prevented the virus's ability to replicate (Yolken et al., 1992). XOR is known to reduce nitrite to nitric oxide and peroxynitrite in the GI tract and is associated with strong antimicrobial properties. Stevens et al. (2000) observed a reduction in the growth rates of *E. coli* and *Salmonella enteritides* with increasing milk XOR activity *in vitro* (Stevens et al., 2000).

Indeed, the anti-infective properties of MFGM are well documented and many recent comprehensive reviews exist examining its role in detail (Douëllou, Montel, & Thevenot Sergentet, 2017; Fontecha et al., 2020; Ross et al., 2015). We also refer the reader to a recent review of double-blind, randomized, controlled trials exploring the effects of MFGM supplementation to the diet of infants or children on infections compared with no supplementation (Hernell, Lönnerdal, & Timby, 2020). Commercial bovine sources of MFGM have recently come to the market as novel food ingredients and have been added to various products, including infant formula. Dairy-based ingredients containing MFGM fragments including minor lipids fall in two categories, namely MFGM-enriched ingredients and phospholipid extracts. MFGM-enriched ingredients are obtained by a combination of physical processes, whereas most of the phospholipid extracts are obtained by solvent extraction from MFGM-enriched fractions. The commercial processes to produce MFGM-enriched ingredients were recently reviewed by Fontecha et al. (2020). Going forward, better characterization of the minor MFGM proteins along with monitoring the factors influencing variability between the different production methods should be considered.

4.7. Lactoferrin

Lactoferrin (Lf) is an iron-binding glycoprotein and consists of a single polypeptide chain. It is the second most abundant protein in human milk, with concentrations ranging from 6 g/L in early milk to 2 g/L in mature milk (Rai et al., 2014). In bovine milk the concentrations are much lower and range from 0.8 g/L in early milk to 0.1 g/L in mature milk (Sánchez, Aranda, Pérez, & Calvo, 1988). There is a high degree of homology in protein sequence (77%) between human Lf and bovine Lf (Manzoni et al., 2010; Nguyen et al., 2016). For this reason, bovine lactoferrin has been used in numerous *in vitro* and *in vivo* studies. The anti-infective properties of Lf are well documented and many recent comprehensive reviews exist examining this role in detail (Berlutti et al., 2011; Redwan, Uversky, El-Fakharany, & Al-Mehdar, 2014; Telang, 2018; Ward & Conneely, 2004). Lf is both bactericidal and bacteriostatic in that it restricts the growth of several pathogens and kills many others. In human milk, the iron-free form of Lf is the most common form and it has been shown to kill *P. aeruginosa*, *Vibrio cholera*, *S. pneumoniae*, *S. mutans*, *E. coli*, and *C. albicans* (Arnold, Brewer, & Gauthier, 1980; Lönnerdal, Erdmann, Thakkar, Sauser, & Destaillats, 2017). The bacteriostatic effects of Lf result, in part, from its ability to withhold iron from bacteria that require it for growth. It also exhibits antiviral, antifungal, and antiprotozoan activities that are likely distinct from its ability to chelate iron (Lönnerdal et al., 2017). Lf proteolysis results in the release of peptides which also display antibacterial activities (Bruni et al., 2016). For instance, lactoferricin, a peptide derivative of Lf, is reported to exhibit numerous biological activities in common with those of Lf (Walzem, Dillard, & German, 2002). These preclinical findings are supported by the discovery that administration of bovine Lf to very low-birth-weight infants protects against late-onset sepsis and necrotizing enterocolitis (NEC) resulting from a variety of infections (Manzoni et al., 2012, 2014; Pammi & Abrams, 2015). Recently, Donovan (2016) reviewed the role of LF in gastrointestinal and immune development and function from a preclinical perspective. Many of the trials performed have used the piglet as a model for the human infant and have shown that bovine LF is well tolerated and retains bioactivity within the gut. We also refer the reader to a recent review by Embleton and Berrington (2020), on clinical trials performed with Lf in newborns and a review by Superti (2020) on bovine Lf. The fact that Lf is available in large quantities adds to its growing applications. Indeed, many companies have recently ramped up production of Lf in response to demands for the ingredient in new applications outside of infant nutrition, including adult immunity.

5. Fats

Lipids in milk are allocated in groups according to their solubility in apolar and organic solvents insoluble in water, being classified as neutral lipids: triglycerides (TAG), diglycerides (DAG) and monoglycerides (MAG), polar lipids: phospholipids and glycolipids, and miscellaneous lipids: sterols, carotenoids and vitamins (Parodi, 2004; Visentainer et al., 2018). In human milk, the lipids are present as fat globules, mainly constituted of TAG surrounded by a structural membrane composed of phospholipids, cholesterol, proteins and glycoproteins (Gallier et al., 2015; Visentainer et al., 2018). Human milk lipids constitute the largest fraction of the total energy intake during infancy, providing an average of 44% energy supply, consisting 98% (m/m) of neutral lipids (TAG, DAG and MAG) (Jensen, 1999). Hence, the fatty acid composition of these constituents defines the nutritional and physicochemical properties of human milk fat (Koletzko et al., 2001). The majority of fatty acids (FA) in human milk are saturated fatty acids (SFA) (German & Dillard, 2010), followed by mono-unsaturated fatty acids (MUFA) and about 20% of the FA in human milk are omega (n)-3 or n-6 polyunsaturated fatty acids (PUFA). These include longer chain PUFA (carbon chain-length $>$ 20, LCPUFA) of the n-6 and n-3 family, such as arachidonic acid (C20:4n-6; ARA) and docosahexaenoic acid (C22:6n-3; DHA), as well as their respective C18 precursors linoleic acid (C18:2n-6; LA) and alpha-linolenic acid (C18:3n-3; LA) that can be converted to LCPUFA after ingestion (Salem, Wegher, Mena, & Uauy, 1996). Given that the anti-infective effects of MFGM are discussed above and well documented in the literature, the proceeding section will focus only on the anti-infective properties of PUFAs. Namely, two specific fatty acids will be discussed due to a number of anti-infective studies observed in the literature. DHA, a fatty acid presently added to infant formulae and glycerol monolaurate (GML), a fatty acid formed from glycerol and lauric acid, will be discussed. Infants that are fed formulas that are fortified with PUFAs are known to have lower incidences of upper respiratory infections and allergies, bronchitis, nasal congestion, cough, diarrhea that requires medical attention, atopic dermatitis, eczema, and contact dermatitis (Foiles et al., 2016; Lapillonne, Pastor, Zhuang, & Scalabrin, 2014; Pastor, Soler, Mitmesser, Ferguson, & Lifschitz, 2006).

5.1. Docosahexaenoic acid (DHA)

Docosahexaenoic acid (DHA) is a major omega-3 PUFA (n-3 PUFAs) and is classed as an essential omega-3 FA in human nutrition (Fao, 1995). DHA is a key ingredient for optimal cognitive function, development and overall health in early infant life, founded through numerous studies. Despite the importance of DHA, the FA is not synthesised by the human body and is instead acquired through diet (Richard, Lewis, & Field, 2016). Breastmilk is an external source of DHA for infants during the first months of life provided the breastfeeding mother achieves a daily minimum DHA intake of 200 mg for a daily supply of 100 mg DHA/day to the infant (Koletzko et al., 2014).

A limited number of studies exist detailing the mechanistic action of DHA antimicrobial activity but do indicate DHA to directly affect the bacterial cell membrane. Correia et al. (2012) demonstrated the inhibitory effect of commercial purified DHA on *H. pylori* growth, both *in vitro* and *in vivo*. Incubation of DHA with *H. pylori* reduced viable CFU counts of *H. pylori* in a dose-dependent manner (Correia et al., 2012). Scanning electron microscopy of the *H. pylori* strains incubated with DHA demonstrated a change in morphology from the normal bacillary shape of *H. pylori* to a coccoid shape. A coccoid *H. pylori* morphology is associated with loss or reduced cell viability and may indicate DHA

interference with the bacterial cell wall peptidoglycan (Chaput, Labigne, & Boneca, 2007). Infected murine subjects exhibited reduced colonisation of *H. pylori* when supplemented with DHA and displayed significantly lower levels of serum PGE2 and other immune cells associated with inflammation of the gastric mucosa (Correia et al., 2012). DHA was also shown to inhibit the growth of *A. baumannii* via incorporation into the *A. baumannii* cell membrane impacting membrane permeability and integrity (Jiang et al., 2019). A synergistic action between human milk purified lysozyme and commercial DHA also inhibits the metabolic activity of *P. aeruginosa*. DHA was shown to enhance lysozyme mediated disruption of the bacterial cell membrane (as previously discussed in the lysozyme section of this review) subsequently allowing a greater accumulation of DHA into the bacterial cell membrane and eventual cell death of *P. aeruginosa* (Martinez et al., 2009). DHA is known to possess both *in vitro* and *in vivo* antimicrobial activity against several *Burkholderia cepacian* strains. Incubation of commercial DHA with *B. cenocepacia* K56-2, a prevalent cystic fibrosis strain, led to bacteriostatic or bactericidal effects *in vitro* determined by the concentration used. A mode of action was not identified *in vitro* with no direct correlation observed between DHA and bacterial cell surface hydrophobic properties. Supplementation of a single dose of DHA in the *Galleria mellonella* caterpillar model of *B. cenocepacia* infection led to reduced bacterial counts and a higher survival rate. Administration of DHA was associated with increased expression of the antimicrobial protein gallerimycin in the *in vivo* larvae model (Mil-Homens, Bernardes, & Fialho, 2012).

DHA (commercially sought) has antimicrobial activity against several oral pathogens when co-incubated *in vitro* including *S. mutans*, *C. albicans*, *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum*, and *Porphyromonas gingivalis* albeit no mode of action explained (Huang & Ebersole, 2010). Bioconverted commercial DHA inhibits growth of Gram-positive pathogens *in vitro* including *B. subtilis*, *L. monocytogenes*, *S. aureus* and also Gram-negative bacteria specifically *Enterobacter aerogenes*, *E. coli O157:H7*, *P. aeruginosa*, *S. enteritidis* and *S. typhimurium* (S. Y. Shin, Bajpai, Kim, & Kang, 2007). DHA has also exhibited anti-hepatitis C virus (HCV) activity in a HCV subgenomic RNA replication model (Leu, Lin, & Hsu, 2004). Although DHA has been observed as important for the development of neurological and cognitive function of FF infants, a limited number of studies on DHAs antibacterial and antiviral activities exist although positive results have been evident to date. Further studies on the anti-infective potential of DHA and the mechanisms underpinning its action will guide its use as an ingredient to prevent infection. Also strengthening its use, it is well tolerated and widely available commercially. Indeed, a number of recent studies have focused on enhancing the DHA content in dairy products through nutritional manipulation in cows (reviewed by Nguyen, Malau-Aduli, Cavalieri, Malau-Aduli, & Nichols, 2019; Huang et al., 2020).

5.2. Glycerol monolaurate

Fatty acids (FAs) and monoglycerides (MGs) contain both bacteriostatic and bactericidal properties against a wide range of bacteria, divulged through numerous studies (Kabara, Swieczkowski, Conley, & Truant, 1972). In particular, glycerol monolaurate (GML) (2,3-dihydroxypropyl dodecanoate) is a 12-carbon lauric acid glycerol ester commonly used as an emulsifier and preservative in the food and cosmetic industry. GML has been shown to have potent antimicrobial and antiviral activity, however, clinical studies have not been established. Recently, Schlievert et al., 2019 determined the concentration of GML in whole pasteurised human milk to be 3000 μg/mL compared to 150 μg/mL found in bovine milk and null in infant formula. Hence, GML may have a protective function against infectious agents in the gastrointestinal tract of breast-fed infants and may have potential benefits for formula fed infants. The following sections will further describe the antimicrobial and antiviral activity of GML.

Early studies indicated synthetic GML to have both antibacterial (Kabara et al., 1972) and antiviral activity (Hierholzer & Kabara, 1982) and due to the wide availability of synthetic GML, the following section refers mainly to studies using this form unless otherwise stated. GML has direct growth inhibition against *E. coli*, *S. aureus* and *B. subtilis*. The antibacterial activity of GML against the three microorganisms was greater when given synergistically with nisin, a commonly used antimicrobial in food products (Zhang, Wei, Cui, Zhao, & Feng, 2009). GML disrupted and prevented biofilm formation of two Gram-positive bacteria *S. aureus* and *E. faecalis* biofilms, when co-incubated. GML in combination with the antibiotic gentamicin sulfate presented a greater bactericidal effect against *S. aureus* biofilms, with all detectable elimination of biofilm bacteria observed when used in combination (Hess, Henry-Stanley, & Wells, 2014). The authors alluded that due to GML being a lipid surface-acting agent, it can disrupt the lipid material of biofilms which is otherwise thought to prevent the diffusion of antibiotics (Hess, Henry-Stanley, & Wells, 2015).

The exact mechanism by which synthetic GML exerts this direct bactericidal activity is not certain. One hypothesis involves the disruption of permeability of the cell membrane. Monoglycerides are non-ionic and form micelles, which may enable them to penetrate the lipid membrane of bacteria and disrupt cell permeability. Indeed, GML has shown antibacterial activity with all Gram-positive bacteria but not entirely with Gram-negative bacteria including *Enterobacteriaceae* and *P. aeruginosa*, due to the presence of the intact LPS (Schlievert & Peterson, 2012). Some Gram-negative microbes with lipo-oligosaccharide such as *Neisseria*, are susceptible to killing by GML (Schlievert, Deringer, Kim, Projan, & Novick, 1992). GML is also thought to interfere with membrane signal transduction. Inhibition of *S. aureus* growth correlated to the inhibition of β-lactamase induction and disruption to the production of *S. aureus* exoproteins, including TSST-1 and α-toxin (Projan, Brown-Skrobot, Schlievert, Vandenesch, & Novick, 1994; Ruzin & Novick, 2000; Schlievert et al., 1992). GML suppresses vancomycin resistance of *Enterococcus faecalis*, via inhibition of signal transduction in the VanS-VanR pathway and subsequently preventing activation of the vanA promoter by vancomycin (Ruzin & Novick, 1998). The exact mechanism of inhibition of signal transduction may occur due to direct binding to transmembrane receptors on bacterial surfaces and/or modulation of bacterial membrane structure and/or their receptors.

Recently, anti-infective capabilities of GML isolated from milk samples has been observed. Stark differences in the levels of GML in human and bovine milk were observed by Schlievert et al., 2019 and antimicrobial activity of the two milks were compared with an additional commercial infant formula. Human milk GML exerted a greater bactericidal effect against *S. aureus*, *B. subtilis*, *C. perfringens* and *E. coli* than bovine milk or IF tested. Loss of antibacterial activity was noted against *S. aureus* when GML and other components >10,000 molecular weight were precipitated from human milk via ethanol extraction. Removal of GML from bovine milk resulted in loss of bactericidal activity against *S. aureus*. Antibacterial activity of the milks was reinstated after GML was subsequently added back in. Human milk also demonstrated anti-inflammatory activity inhibiting IL-8 production by the superantigen toxic shock syndrome toxin-1 (TSST1) on human squamous epithelial cells (HSECs) when co-incubated with TSST-1. Bovine and IF milk did not demonstrate the same anti-inflammatory effect, suggesting that the increased level of GML in human milk may also be involved in anti-inflammatory activities against pathogens.

GML has also attracted much attention in recent studies for potent antiviral activity against a range of diverse viruses. GML administered both daily and before vaginal challenge, was shown to be effective against viral load in the simian immunodeficiency virus (SIV)-rhesus macaque model of HIV-1 transmission to women. Aside from direct inhibition of the virus, GML also was revealed to act indirectly on epithelial cells, as pre-treatment with GML led to a reduction in cytokines (MIP-3 alpha and IL-8 production) which enable infection via recruitment of innate and adaptive immune cells to the portal entry to

subsequently disrupt barrier integrity (Haase et al., 2015; Li et al., 2009). Welch, Xiang, Okeoma, Schlievert, & Stapleton, 2020 recently exposed GML anti-viral activity not to be dependent on cellular binding but instead, related to direct interference with the virus and modulatory changes to the viral envelope. GML reduced HIV-1 cell binding to TZM-bl cells by 35% when co-incubated together with the cell line, however, the chief inhibition by GML occurred at viral entry following CD4 binding but before CXCR4 interactions. The authors hypothesised GML alters conformational changes in the HIV gp120 trimer structure required for coreceptor binding and subsequently preventing HIV-1 replication. GML was also co-incubated with three other enveloped viruses including mumps virus, yellow fever virus and ZIKV in corresponding cell lines. GML demonstrated anti-viral activity against all three viruses at noncytotoxic concentrations demonstrating GML to have broad inhibitory activity against a number of viruses. However, GML had no inhibitory effect when examined with non-enveloped viruses including enterovirus 68 (EV68), hepatitis A virus (HAV) and adenovirus (AdV) confirming again GML's mode of action to associate with direct viral envelope interference. Electron microscopy images of influenza A and coronavirus (CoV) co-incubated with a GML mixture on primary rhesus monkey kidney (MK) cells, a human laryngeal epidermoid carcinoma cell line (HEp-2), and a human embrvonic lung diploid fibroblast cell strain (HELF) also revealed disintegration of the viral envelope (Hierholzer & Kabara, 1982). Other studies also correlate this mechanism of action with anti-viral activity of commercial monoglycerides observed with enveloped viruses HSV-1 and HSV-2, vesicular stomatitis virus (VSV) and visna virus but no activity observed with nonenveloped poliovirus or rhinovirus (Clarke & May, 2000; Sands, Auperin, & Snipes, 1979; Thormar, Isaacs, Kim, & Brown, 1994). GML is associated with potent antimicrobial and antiviral properties and may serve as a key ingredient in human breast milk in the formation of a beneficial microbiota, in pathogenic deterrence and in immune maturation in the infant gastrointestinal tract. Due to differences in concentrations observed in bovine and infant formulae products, GML may be an important ingredient for future addition.

6. Future perspectives

A healthy intestinal microbiome is essential for homeostasis in the gut and in overall health; however, the uncontrolled excessive growth of certain bacterial populations leads to a variety of harmful conditions. Aside from the many anti-infective activities associated with the milk components described in this review, many of these milk components can also influence microbial colonisation in other ways. Breastmilk is a rich source of prebiotics for beneficial microorganisms to flourish in the infant intestinal tract (O'Callaghan & van Sinderen, 2016). The International Scientific Association for Probiotics and Prebiotics (ISAPP) in December 2017 expanded the concept of prebiotic to "a substrate that is selectively utilized by host microorganisms conferring a health benefit (Gibson et al., 2017). Studies have found that breast-fed infants accumulate a higher occurrence of bifidobacteria residing in their gut in contrast to their formula-fed peer (Bäckhed et al., 2015). This differences in the microbiome between the two groups has been correlated with formula-fed infants having a higher incidence of infection, allergy and disease (Arslanoglu, Moro, & Boehm, 2007; Isaacs et al., 2010; Wang, Downing, Petocz, Brand-Miller, & Bryden, 2007). Many of the human and bovine milk bioactive factors discussed in this review possess prebiotic activity. Milk oligosaccharides, lactoferrin, lysozyme, α-La, immunoglobulins etc., have each shown to positively influence the growth of bifidobacterial species commonly dominating the infant gut (Kavanaugh et al., 2015; Minami, Odamaki, Hashikura, Abe, & Xiao, 2016; Morrin et al., 2020; Oda, Wakabayashi, Yamauchi, & Abe, 2014; Wernimont, Northington, Kullen, Yao, & Bettler, 2015). It is possible that bioactive factors in bovine milk may provide natural and sustainable approaches to control infectious bacterial and viral disease by both suppressing pathogenic microorganisms while also strengthening intestinal homeostasis through increasing numbers of health-promoting microorganisms.

Preceding supplementation of bioactive factors in infant formula is a critical examination through numerous clinical trials of their safety and tolerability. The level of in-depth examination required for each ingredient is dependent on the nature and structural comparison to their human milk counter-part. Components synthesised by biofermentation such as human milk oligosaccharides can be produced identical to those found in human milk. For bioactive components extracted from bovine milk, additional scrutiny is required regarding structural homology and post-translational modifications. Post translational modifications (PTMs) of proteins can be critical for bioactivity (Appella & Anderson, 2000; Müller, 2018). Protein glycosylation is one of the most commonly occurring post-translational modifications, and directly affects protein structure, function and recognition. Protein glycosylation is a particularly pertinent PTM for both human and bovine milk components with its influence in many cases dictating activities such as pathogen binding and immune development. For proteins with limited PTM such as α-La and which shares 72% homology with human milk α-La, less scrutiny is required as α-La bioactivity is not dependent on PTM. For glycoproteins such as lactoferrin and immunoglobulins, both their anti-bacterial and prebiotic bioactivities have been directly associated with their glycan structures (Morrin et al., 2020; O'Riordan, Kilcoyne, Joshi, & Hickey, 2017). These PTMs will vary considerably between human and bovine sources and thus, their bioactive functions may differ (Nwosu et al., 2012). These glycan structures may also change considerably throughout lactation as reported in lectin array studies of immunoglobulins, Lf and MGFM and thus depending on at which lactation stage they are generated from, bioactivity may also vary (S. Feeney et al., 2019; O'Riordan et al., 2014; Ross et al., 2016). In-depth analysis is required including *in vitro* studies, animal models and clinical trials to assess similarity in function and suitability for supplementation regarding both the immediate and long-term effects of these milk bioactives (Lönnerdal, 2012).

Nutritional requirements for an infant's growth and development change remarkably within the first year of life reflected through the compositional changes observed in human breast milk throughout lactation (Lönnerdal, 1986). Variations in milk composition occur throughout lactation including decreased protein concentration and changes in both the protein utilisation and protein/energy ratio. The requirement for reduced intake of specific nutrients and the increased need of others are reflected in the breast milk produced and together, all reflect the shifting growth rate and developing physiological requirements of the infant (Koletzko & Dokoupil, 2015; Lönnerdal, Forsum, & Hambraeus, 1976). Complement compositional changes to infant formula may be more beneficial to the formula-fed infant's physiological and immunological development to reflect their breast-fed peer. Thus far, clinical research in infants fed formulas with a caloric density similar to HM show a growth pattern similar to breast fed infants (Marriage, Buck, Goehring, Oliver, & Williams, 2015). Also included in compositional changes is the modifications taking place to the structure and complexity of certain nutrients such as milk oligosaccharides, which play a vital role in infant development and protection as mentioned in this review (Samuel et al., 2019). The composition of human milk not only changes throughout lactation but also varies with pre-/post-feed, time of the day, between mothers, environment and populations (J. Jiang et al., 2016; Mizuno et al., 2009; Pundir et al., 2017). Genetic factors and physiology also play a role with variations in composition observed by infant sex as well as maternal lifestyle, diet and nutritional status (Bzikowska-Jura et al., 2018). Maternal dietary habits have been shown to influence mainly the lipid fraction of milk and non-nutritive milk components such as hormones, steroids, growth and satiety factors (Leghi et al., 2020; Mäkelä, Linderborg, Niinikoski, Yang, & Lagström, 2013). The apparent synergy between milk factors such as the relationship observed between α-lactalbumin, β-lactoglobulin and Lf against pathogenic attack may suggest that supplementation of specific

milk bioactives when combined are more beneficial than when used alone and may reflect the heterogenous breast milk environment (Taha et al., 2010).

Another consideration is that apart from immunoglobulins, breastmilk also contains many other immune bioactives such as growth factors, cytokines, and immunity factors which have not been included in this review. Limited data exists in relation to their anti-infective potential as a supplement in infant formula. For example, Mank, Naninck, Limpens, van Toledo, van Goudoever, and van den Akker (2020) recently reviewed such bioactive factors which have been studied as supplement to enteral nutrition in randomized controlled trials. Six studies investigating the effect of a recombinant hormone or growth factor as an enteral supplement in order to prevent prematurity-related complications were reviewed. Four of these studies tried to establish an effect of providing recombinant human erythropoietin (rhEPO) and/or recombinant human granulocyte colony-stimulating factor (rhG-CSF). The authors came to the conclusion that it was difficult to draw conclusions to date on their addition to formula and that additional studies are required to provide more conclusive results. Nevertheless, such components and those present in low concentrations in milk warrant further investigation and characterisation in terms of their anti-infective properties. Milk components which can be associated with clearly defined anti-infective effects against relevant pathogens have enormous potential as functional food ingredients. By understanding their structure and functions, it is tempting to suggest that in the future not only infant formula will benefit but many novel foods and beverages will be available based on their potential to reduce the risk of infection. Before this scenario can be realized, economical and rapid enrichment and purification procedures must be put in place.

7. Conclusion

Human milk not only supplies the nutritional needs of the newborn and reduces the risk of infection by conferring protection at the intestinal surface, but additionally shapes the infant's gut microbiota and instructs immunomodulation. Further suitable animal studies and well controlled human clinical trials will be required to further validate if the bovine milk counterparts described in this review can impart the same functions. Never has the need for novel anti-infective agents been so great with the emergence of the novel human coronavirus SARS-CoV-2 which has caused a worldwide pandemic of respiratory disease (COVID-19). SARS-CoV-2 utilises spike (S) glycoprotein to bind to the cell membrane protein angiotensin-converting enzyme 2 (ACE2) to enter human cells (Shang et al., 2020). This knowledge could contribute to the identification of decoy ligands able to specifically target ACE2 or coronavirus spike proteins to prevent viral infection. Looking to milk for a solution, may prove to be a promising approach.

Ethics statements

This is a review article. It has not involved any human subjects and animal experiments.

CRediT authorship contribution statement

Sinead T. Morrin: Writing - original draft, Writing - review & editing. **Rachael H. Buck:** Writing - review & editing. **Michael Farrow:** Writing - review & editing. **Rita M. Hickey:** Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: [Sinead T. Morrin, Rachael H. Buck and Michael Farrow are employees of Abbott Laboratories Ireland and USA.].

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