Differences in pathogen-sugar interactions between human and bovine milk glycoproteins

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Immune components of breast milk

Why study glycosylation?

Some functions of glycoconjugates include:

1. Alteration of protein structure and function
2. Stabilisation of protein conformation
3. Cell-to-cell communication
4. Cell adhesion and signalling
5. Immune response
Glycans found in milk

Whole fresh milk $\xrightarrow{\text{Spin}}$ Separate the cream from the whey $\xrightarrow{\text{Ultracentrifuge or lower pH and spin}}$ Pellet the caseins to separate from soluble whey proteins

Milk glycans are found attached to:
1) Lipids $\rightarrow$ Glycolipids from the Milk Fat Globule Membrane (MFGM)
2) Proteins $\rightarrow$ Glycoproteins from the whey, casein and MFGM fractions
3) Soluble / unattached $\rightarrow$ Lactose and oligosaccharides from whey

## Differences between human and bovine milk

<table>
<thead>
<tr>
<th>Composition</th>
<th>Human milk</th>
<th>Bovine milk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>87 %</td>
<td>87 %</td>
</tr>
<tr>
<td>Protein</td>
<td>1.3 g/100mL</td>
<td>3.3 g/100mL *</td>
</tr>
<tr>
<td>Fats (total)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saturated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monounsaturated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polyunsaturated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.1 g/100mL</td>
<td>3.9 g/100mL</td>
<td></td>
</tr>
<tr>
<td>1.8 g/100mL</td>
<td>2.5 g/100mL</td>
<td></td>
</tr>
<tr>
<td>1.6 g/100mL</td>
<td>1.0 g/100mL</td>
<td></td>
</tr>
<tr>
<td>0.5 g/100mL</td>
<td>0.1 g/100mL</td>
<td></td>
</tr>
<tr>
<td>Carbohydrates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactose</td>
<td>7 g/100mL</td>
<td>4-5 g/100mL *</td>
</tr>
<tr>
<td>Oligosaccharides</td>
<td>6-23 g/L</td>
<td>Trace – 1.2 g/L*</td>
</tr>
<tr>
<td>Iron</td>
<td>0.07 mg/100mL</td>
<td>0.02 mg/100mL</td>
</tr>
<tr>
<td>Calcium</td>
<td>34 mg/100mL</td>
<td>120 mg/100mL</td>
</tr>
</tbody>
</table>
Human milk oligosaccharides (>200)
- have lactose as the core structure
- 5 monosaccharides: Glc, Gal, GlcNAc, Fuc, NeuAc
- typically 4-6 residues in length, up to 32 residues
- present in high concentrations (up to 23g/L) in human colostrum and between 6-12g/L in mature milk

15 same as human

Bovine milk oligosaccharides (approx. 40) *
- Have lactose or lactosamine as core
- 4 monosaccharides: Glc, Gal, GlcNAc, NeuAc
- typically 3-4 residues in length, up to 7 residues
- present in low concentrations in colostrum (up to 1.2 g/L) and trace amounts in mature milk

Key: Glucose (Glc) • Fucose (Fuc) • Sialic acid (NeuAc) •
Galactose (Gal) • N-acetylglucosamine (GlcNAc) •

Abundant human oligosaccharides

Neutral oligosaccharides
- LNT:
- LNNt:
- 2'-FL:
- 3'-FL:
- LNFP-1:
- LNFP-V:
- LDFH-1:
- LDFT:

Sialylated oligosaccharides
- 3'-SL:
- 6'-SL:
- 3'-S3FL:
- LSTa:
- LSTb:
- LSTc:
- DSLNT:

* bleeds on image
Lewis epitopes in human milk confer protection by binding to pathogens

NB: Mothers who are non-secretors are missing the FUT2 gene and are unable to add fucose residues with an alpha-2 linkage and therefore lack some of these glycans from their milk.
### Distribution of free oligosaccharide classes

<table>
<thead>
<tr>
<th>Oligosaccharides</th>
<th>Human milk (pooled)</th>
<th>Bovine milk (colostrum)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 1000 Da</td>
<td>19.6 %</td>
<td>89.2 %</td>
</tr>
<tr>
<td>&gt; 1000 Da</td>
<td>80.4 %</td>
<td>10.8 %</td>
</tr>
<tr>
<td>% fucosylated</td>
<td>70.1 %</td>
<td>0</td>
</tr>
<tr>
<td>% sialylated</td>
<td>4.8 %</td>
<td>76.3 %</td>
</tr>
<tr>
<td>% neutral</td>
<td>25.1%</td>
<td>23.7 %</td>
</tr>
</tbody>
</table>

Bovine milk contains fewer, less complex oligosaccharides and smaller range of sugar epitopes when compared with human milk.
Human milk oligosaccharides bind to bacteria ……

**GIT pathogens:**
- *Escherichia coli*
  - EPEC, ETEC, EHEC, UPEC strains
- *Helicobacter pylori*
- *Campylobacter jejuni*
- *Vibrio cholerae*
- Norovirus
- *Salmonella* spp.
- *Shigella*

**Oral and respiratory pathogens:**
- *Streptococcus pneumonia*
- *Pseudomonas aeruginosa*
- Influenza virus
- *Streptococcus sanguis*
- *Candida albicans*
- *Aspergillus fumigatus*
- Polyomavirus
Human oligosaccharides bind to *Escherichia coli*

- **UPEC and ETEC** *E. coli* binds to sialylated oligos
- **Low levels of 2′-linked fucose oligosaccharides in milk** lead an increase in diarrhea
- **Stable toxin** binds to H1 and H2 antigens (α1-2 linked fucose)
- **EPEC** *E. coli* binds to fucosylated tetra- and penta-saccharides
- **EHEC** *E. coli* binds to fucose and mannose residues
- **UPEC** *E. coli* binds to neutral oligosaccharides
  - Binding inhibition by 3′-SL, 3′-SLN and sialyl-GP
  - **S-fimbriae of** *E. coli* binds to 3′-SL
  - Sialylated, high and low MW neutral oligos inhibited binding of *E. coli* O119 to Caco-2 cells
  - Glycan of globotriose inhibited *E. coli* JR1 binding

http://www.ecoliblog.com/
Human milk oligosaccharides bind to *Helicobacter pylori*

*H. pylori* is inhibited by 3’-SL. NeuAc-Gal is the minimum binding epitope. In this study bovine 3’-SL was more potent than human 3’-SL.

*H. pylori* infection was reduced in some rhesus monkeys with 3’-SL alone or in combination with two antibiotics.

*H. pylori* binds to the Lewis b and H1 antigens via the BabA adhesin.

*H. pylori* binding to gastrointestinal cells is inhibited by 3’-SL. 3’-SL as a neoglycoprotein is more potent.
Human milk oligosaccharides bind to *Vibrio cholerae* and *Campylobacter jejuni*

Cholera toxin is inhibited by 3’-SL

Neutral oligosaccharides. The classical strain can be inhibited by L-fucose, the El Tor strain by D-mannose

*V. cholerae* binds to the H(O) blood group antigen

High MW neutral and sialylated oligosaccharides inhibit *V. cholerae* binding to Caco-2 cells

*C. jejuni* is inhibited by the H2 antigen and 2’-FL

Low levels of 2’-linked oligosaccharides in milk lead to an increase in diarrhea

http://archive.microbelibrary.org/ASM Only/Details.asp?ID=2734

Human milk oligosaccharides bind to viral pathogens - Norovirus

Secretor and Lewis antigens block the binding of the virus to its host. Binding epitopes not determined.

Norovirus binding investigated using saliva samples and neoglycoproteins

- **VA387**: multiple BGA’s
- **NV**: H and A antigens
- **MOH**: A and B antigens
- **VA207**: Lewis epitopes with an α1-4 linkage

High levels of LDFH-1 in milk decreases the risk of norovirus infection

http://www.noroblog.com/

http://www.cruiselawnews.com/articles/norovirus/
Human milk oligosaccharides bind to oral pathogens

**Streptococcus sanguis**

*S. sanguis* binds to both 3’-SL and 3’-SLN

**Candida albicans**

*C. albicans* binds to a Fuc α1-2 Gal epitope on buccal epithelial cells
Human milk oligosaccharides bind to respiratory pathogens

Neutral oligosaccharides LNT and LNNt, and the internal disaccharide GlcNAc β1-3 Gal bind to:

- **Streptococcus pneumoniae**

Sialyl-oligosaccharides inhibit viral binding by:

- Viral particles bound to 3’-SL and 6’-SLN which were attached to polyacrylic acid carriers.

PA-II lectin binds to Lewis a alone or on LNFP-II and to 3’-FL.

- **Influenza virus**

Neutral oligosaccharides as neoglycoproteins:

- Sialyllactose
- The oligo portion of asialo GM1

Sialyl-oligosaccharides in inhibit viral binding by:

- PA-II lectin binds to Lewis a alone or on LNFP-II and to 3’-FL


http://www.istc.ru/istc/istc.nsf/vaWebPages/InfluenzaEng

Milk glycolipids bind to bacteria as well

<table>
<thead>
<tr>
<th>Glycolipids</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monosialogangliosides</td>
<td></td>
</tr>
<tr>
<td>GM$_1$</td>
<td><img src="image" alt="GM1" /> Cer</td>
</tr>
<tr>
<td>GM$_2$</td>
<td><img src="image" alt="GM2" /> Cer</td>
</tr>
<tr>
<td>GM$_3$</td>
<td><img src="image" alt="GM3" /> Cer</td>
</tr>
<tr>
<td>Disialogangliosides</td>
<td></td>
</tr>
<tr>
<td>GD$_{1a}$</td>
<td><img src="image" alt="GD1a" /> Cer</td>
</tr>
<tr>
<td>GD$_{1b}$</td>
<td><img src="image" alt="GD1b" /> Cer</td>
</tr>
<tr>
<td>GD$_3$</td>
<td><img src="image" alt="GD3" /> Cer</td>
</tr>
<tr>
<td>Trisialoganglioside GT$_{1b}$</td>
<td><img src="image" alt="GT1b" /> Cer</td>
</tr>
</tbody>
</table>

Dominant in mature human milk

Dominant in human colostrum and bovine milk
Milk glycolipids bind to pathogens

- *Salmonella typhimurium* heat labile toxin
- Rotavirus UK strain
- Vacuolating toxin of *H. pylori*
- Inhibited *H. pylori* hemagglutination
- *E. coli* enterotoxin
- ETEC *E. coli*
- *V. cholerae* enterotoxin
- *C. jejuni* enterotoxin
Milk glycolipids bind to pathogens

- **GM₂**
  - Clostridium perfringens delta toxin
  - Rotavirus (all strains)

- **GM₃**
  - E. coli K99 fimbriae
  - NeuGc isoform
  - ETEC E. coli
  - NeuGc isoform
  - H. pylori
  - Rotavirus (SA11 and NCDV strains)
  - EPEC E. coli

- **Cer**
Do sugars in milk competitively bind to these pathogens and remove them?

A) Pathogen or toxin

Adhesins, fimbriae, pili

Pathogen or toxin

Pathogen or toxin

Glycoprotein A

Glycoprotein B

Glycolipid

Cells

B) Pathogen or toxin

Glycoprotein, glycolipid, or free oligosaccharide

Pathogen or toxin

Glycoprotein A

Glycoprotein B

Glycolipid

Cells
Some cow’s milk oligosaccharides bind to bacteria

Evidence also exists for bacterial binding to other neutral and sialylated oligosaccharides from cow’s milk.
Human Milk Oligosaccharides (HMOs) are not digested by infants

- Infants cannot digest HMOs, they arrive intact in the large intestine and appear in the faeces
- HMOs can modulate the establishment of a *Bifidobacteria* environment

*Bifidobacterium longum* bv. *infantis* consumes small oligosaccharides (<1500Da):

- Lacto-N-tetraose, Lacto-N-neohexaose
- Fucosylated Lacto-N-hexaose, Difucosyllacto-N-hexaose
GOS oligosaccharides are added to infant formula

GOS: a non-digestible carbohydrate composed of galactose residues attached to a lactose core

- promotes intestinal *Bifidobacteria*
- are not digested in the gut, used in a 9:1 ratio, 8 g/L
- reduces IgE, IgG1, IgG2, IgG3 which may lead to a reduction in childhood asthma
- decreases risk of atopic dermatitis
- can control infantile jaundice
- not a natural component of human milk
FOS is added to infant formula

Fructose OS / inulin:
• promotes intestinal *Bifidobacteria*
• promotes calcium absorption
• not a natural component of human milk
• reduce oxidative stress by scavenging free radicals
BUT Oligosaccharides are also attached to proteins in milk

*N- and O-linked glycoproteins*
- 6 different monosaccharides: Man, Gal, GalNAc, GlcNAc, Fuc and NeuAc
- can be highly glycosylated, e.g. mucins in milk fat globule membrane
- present in varying concentrations in milk (13-33 g/L)

*Free oligosaccharides*
- 5 different monosaccharides: Glc, Gal, GlcNAc, Fuc, and NeuAc
- typically 4-6 residues in length
- present in high concentrations (up to 23g/L) in human colostrum and between 6-12g/L in mature milk

Glycoproteins offer different binding epitopes compared to free oligosaccharides:
e.g. extended conformations, complex structures, multivalency, protein backbone, variation in composition over time, different solubilities
## Milk proteins / glycoproteins

<table>
<thead>
<tr>
<th>Type of protein</th>
<th>Bovine (g/L)</th>
<th>Human (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Protein</td>
<td>33 – 34.5</td>
<td>8.5 – 13</td>
</tr>
<tr>
<td>Caseins (total)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-casein</td>
<td>15</td>
<td>unknown</td>
</tr>
<tr>
<td>β-casein</td>
<td>8.8</td>
<td>1.3</td>
</tr>
<tr>
<td>κ-casein</td>
<td>4</td>
<td>0.5</td>
</tr>
<tr>
<td>δ-casein</td>
<td>0.7</td>
<td>unknown</td>
</tr>
<tr>
<td>Whey proteins (total)</td>
<td>6.8 (&lt;20 %)</td>
<td>6.1 (&gt;70 %)</td>
</tr>
<tr>
<td>β-lactoglobulin</td>
<td>3.3</td>
<td>0</td>
</tr>
<tr>
<td>α-lactoglobulin</td>
<td>1.3</td>
<td>2.5</td>
</tr>
<tr>
<td>Immunoglobulins</td>
<td>0.8</td>
<td>1.1</td>
</tr>
<tr>
<td>Lactalbumin</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>0.1-0.4</td>
<td>2</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>0.00013</td>
<td>0.15</td>
</tr>
<tr>
<td>MFGM</td>
<td>0.35 – 1.4 (1-4 %)</td>
<td>0.13 – 0.52 (1-4 %)</td>
</tr>
</tbody>
</table>
Glycoproteins compared to free oligosaccharides in milk

Several oligosaccharides inhibit *Helicobacter pylori* binding to gastrointestinal cells

- 3’-SL (3’-sialyllactose) was the strongest inhibitor
- Pre-treatment of gastrointestinal cells with neuraminidase reduced binding
- 3’-SL could detach bacteria bound to gastrointestinal cells
- **However**, when 3’-SL was attached to a neoglycoprotein (20 mol/protein), its inhibitory strength was 1000 x more potent than 3’-SL alone

Glycoconjugate form of 3’-SL was a superior inhibitor of binding
Advantages of neoglycoproteins

In the gut.

Neoglycoproteins have been used to determine binding epitopes:

- *Campylobacter jejuni* binds to the H(2) blood antigen
- Inhibitory activity of the H(2) neoglycoprotein was 3-4 x more potent than the neutral fraction of oligosaccharides alone

In the lungs.

- *Streptococcus pneumoniae* binds to both LSTc and LNnT oligosaccharides
- When rats were given *S. pneumonia* and neoglycoproteins (LSTc-HSA and LNnT-HSA) simultaneously, a 50% reduction in viable bacteria was observed
Comparison of proteins from human and bovine milk

<table>
<thead>
<tr>
<th></th>
<th>Bovine milk</th>
<th>Human milk</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Whey</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Caseins</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- Mucins, Ig-like secretory components
- Lactotransferrin
- Serum albumin
- sIgA (heavy chain)
- Casein subunits
- sIgA (light chain)
- β-lactoglobulin
Mucins: a family of high molecular weight, heavily glycosylated proteins

Milk mucins:  
- mucin-1  
- mucin-15  
- mucin-4: absent from bovine milk  
- mucin-16  
- mucin 20-like protein

MFGM offers a source of mucins as a scaffold with high density O-linked oligosaccharides

Generic structure of a mucin monomer

- oligosaccharide
- cysteine residue
- repeat structure
Human milk sugars on the proteins are more complex than in cow’s milk.
Human milk confers natural defense against infection by clearance of bacteria from the gut by binding to milk mucin sugars.

Can we modify cow’s milk sugars to allow this defense to be mimicked??
Sample preparation for milk glycan profiling

Sample (100 µL milk)

Chloroform/Methanol Precipitation


Remove (glyco)lipids

dry aqueous phase and precipitate

dissolve precipitate

removes free oligosaccharides

10 kDa spin filter

milk proteins

Glycomics

Glycoproteomics
Released glycans for glycoprofiling

Sample prep

Glycoprotein → PVDF membrane blot → Desalt

Release of oligosaccharides
N-linked - enzymatic (PNGase F)

O-linked - chemical (reductive β-elimination)

Analysis

- Capillary LC
- Porous graphitized carbon column
- Ion trap MS (HCT Bruker)
- Analysis in ESI negative polarity mode

Informatics

Probable glycan structures

GlycoSuiteDB by Proteome Systems
Sugars found on human milk glycoproteins

Different milk fractions have different sugar epitopes ……

MS glycoprofile of whole milk vs fat fraction shows significant glycosylation of proteins in the cream and skim fraction.
MS profile of whole milk N- and O- glycosylation over lactation time

The global profile of one person over lactation time looks similar …..

However changes do happen!
Time course of change in isomer ratio occurs in the 5 individuals
What are the different isomers?

Digestion with $\beta$1,4 galactosidase

Cannot be sure whether Le $x$ or Le $a$ as possible steric hindrance by fucose
MS/MS differentiates between the isomers
Bacterial binding to milk fractions

4 major milk fractions
- Lipids
- MFGMP
- Whey proteins (soluble)
- Caseins (insoluble)

3 selected bacteria species
- Escherichia coli
- Salmonella typhimurium
- Lactobacillus rhamnosus

Coat PVDF membrane of 96 well plate with milk fractions
Add fluorescently labelled bacteria
Quantitate the fluorescence

Clara Cheah
Bacterial binding to different fractions over lactation

Bacteria bind differently to fractions from the same volume of human milk
Can we modify cows’ milk to mimic the protective properties of human milk?

• Sialic acid-containing epitopes bind to pathogens
• Fucose-containing epitopes bind to pathogens
• Oligosaccharides on proteins are more potent inhibitors of pathogen binding than oligosaccharides alone
• MFGM contains high density oligosaccharides on mucins
Sialic acid is involved in the differential binding of streptococcal species to milk and salivary glycoproteins

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