Sugar-pathogen interactions:

Breast milk as the first line of defence against infections

Wai Yuen CHEAH

BBiotech (Hons) The University of Queensland

This thesis is presented for the degree of

Doctor of Philosophy

Department of Chemistry and Biomolecular Sciences

Faculty of Science

Macquarie University, Sydney

14th September 2014

Table of Contents

Abstract 9

Statement of candidate 12

Acknowledgements 13

Publication and conference proceedings 15

Presentations and awards 17

Abbreviations 18

Symbol nomenclature for representation of glycan structure 21

Chapter 1. Introduction 22

1.1 Breastfeeding confers protection against disease 22

1.1.1 Diarrhoeal diseases and their prevention by breastfeeding 24

1.2 Mechanisms of gastrointestinal infection 25

1.3 The composition of milk 30

1.4 Milk oligosaccharides 34

1.4.1 Synthesis and structural features of human milk oligosaccharides 34

1.4.2 A comparison of human and bovine milk oligosaccharides 35

1.4.3 Evidence that milk oligosaccharides may inhibit pathogen adherence to host cells 38

1.5 Milk glycoproteins 45

1.5.1 Synthesis and structural features of glycans on human milk glycoproteins 45

1.5.2 A comparison of human and bovine milk glycoproteins 47

1.5.3 Evidence that glycans on milk glycoproteins may influence pathogen adhesion 48

1.6 Milk glycolipids 57

1.6.1 Synthesis and structural features of glycans on human milk glycolipids 57

1.6.2 A comparison of human and bovine milk glycolipids 59

1.6.3 Evidence that glycans on milk glycolipids may influence pathogen adhesion 59

1.7 Intestinal microflora in infants 64

1.8 Chapter summary and project aims 64

Chapter 2. General materials and methods 66

2.1 Bacterial strains and growth conditions 66

2.2 Fluorescent labelling of whole bacterial cells 66

2.3 Protein quantification and SDS-PAGE 67

2.4 Release of *N*- and *O*-linked glycans 67

2.5 Mass spectrometry 69

Chapter 3. Measuring sugar-pathogen interactions 73

Rationale 73

3.1 Introduction 74

3.2 Materials and methods 80

3.2.1 Glycan microarray 80

3.2.1.1 Preparation of glycan microarray 80

3.2.1.2 Culturing, harvesting and fluorescent labelling of whole bacterial cells 81

3.2.1.3 Application of CFDA-SE labelled bacterial cells to glycan microarray 81

3.2.1.4 Fluorescent image acquisition and data processing 82

3.2.2 Polyvinylidene difluoride (PVDF) plate assay 82

3.2.2.1 Human milk protein preparation 82

3.2.2.2 PVDF plate assay of bacterial adhesion to milk proteins 83

3.2.2.3 Fluorescence measurement 84

3.2.2.4 Inhibition of bacterial binding with *N*-acetylneuraminic acid (sialic acid) 84

3.2.2.5 Removal of sialic acid residues on total human milk proteins with neuraminidase 85

3.2.2.6 Data analysis of released *N*- and *O*-linked glycans by mass spectrometry 85
3.2.2.7 Monosaccharide analysis 85
3.3 Results 86
3.3.1 Glycan microarray analysis of CFDA-SE labelled bacteria 86
3.3.1.1 *Escherichia coli* O111:NM (ATCC 43887) 86
3.3.1.2 *Salmonella typhimurium* (Group B) clinical isolate 86
3.3.1.3 *Lactobacillus rhamnosus* (ATCC 53103) 87
3.3.1.4 Variations in glycan microarray results 89
3.3.2 PVDF plate assay 90
3.3.2.1 Comparing protein precipitation methods 90
3.3.2.2 Comparison of binding efficiencies of fluorescently-labelled gastrointestinal tract- and oral-associated bacterial to total human milk proteins 92
3.3.2.3 Sialic acid involvement in the binding of bacteria to human milk proteins 93
3.3.2.4 Validation that sialic acid is involved in binding of bacteria to human milk proteins 95
3.3.2.5 Confirmation of desialylation of human milk glycoproteins by neuraminidase 96
3.3.3 Monosaccharide analysis of released *N*- and *O*-linked glycans from human milk glycoproteins 105
3.4 Discussion 108

Chapter 4. Binding of human gastrointestinal bacteria to bovine and human milk proteins 115

Rationale 115
4.1 Introduction 116
4.2 Materials and methods 117
4.2.1 Milk samples 117

4.2.2 Methanol/chloroform/water precipitation for isolation of total milk proteins 118
4.2.3 Preparation of skim milk 118
4.2.4 Fractionation of skim milk using the Microflow MF10 system 118
4.2.5 Separation of whey and casein from skim milk by high-speed centrifugation 119
4.2.6 Fast protein liquid chromatography (FPLC) gel filtration of milk whey 120
4.2.7 Bacterial strains and growth conditions 120
4.2.8 PVDF plate assay 120
4.2.8.1 Inhibition of bacterial binding with *N*-acetylneuraminic acid (sialic acid) 121
4.2.8.2 Neuraminidase treatment of bovine and human milk proteins 121
4.3 Results 121
4.3.1 Skim milk fractionation by molecular size electrophoresis 121
4.3.2 Milk protein separation by centrifugation 123
4.3.3 Fractionation of milk whey proteins by size exclusion on the ÄKTA FPLC system 126
4.3.4 Bacterial binding assays 128
4.3.4.1 Bacterial binding to total proteins of bovine and human milk 128
4.3.4.2 Bacterial binding to major protein fractions of bovine and human milk 129
4.3.4.3 Bacterial binding to fractions of bovine and human milk whey 130
4.3.4.4 Inhibition of bacterial binding to milk fractions by free sialic acid 132
4.3.4.5 Binding of bacteria to bovine and human milk whey proteins and casein following neuraminidase treatment 134
4.4 Discussion 135

Chapter 5. Involvement of *N*-linked glycans from milk whey proteins in inhibition of pathogen adhesion and invasion of the gut 141

Rationale 141
5.1 Introduction 142

5.2 Materials and methods 143
5.2.1 Milk sample preparation for whey protein extraction 143
5.2.2 Preparative release of *N*-linked glycans from bovine and human milk whey proteins 143
5.2.3 Bacterial species 144
5.2.4 HT-29 cell culture 144
5.2.5 Bacterial binding and invasion assays 145
5.2.5.1 PVDF plate assay for measuring bacterial interaction with glycoproteins of bovine and human milk and the inhibition of binding by *N*-linked glycans from whey 145
5.2.5.2 Cell-based assay for measuring bacterial adherence and invasion of HT-29 cells 145
5.2.6 Mass spectrometric analysis of *N*-linked glycans released from bovine and human milk whey proteins 147
5.2.7 Monosaccharide analysis of released *N*-linked glycans from bovine and human milk whey proteins 147
5.2.8 Membrane preparation of HT-29 cells prior to glycan analysis 147
5.2.9 PNG*ase* F treatment of HT-29 cell membrane proteins to release *N*-linked glycans for mass spectrometry analysis 148
5.3 Results 148
5.3.1 Inhibition of bacterial binding to milk fractions by *N*-linked glycans from bovine and human milk whey proteins 148
5.3.2 Comparison of the ability of human gastrointestinal bacteria to adhere and invade HT-29 cells 152
5.3.3 Inhibition of bacterial adhesion and invasion of HT-29 human gastrointestinal cells by pre-incubation with *N*-linked glycans from bovine and human milk whey proteins 153

5.3.4 Monosaccharide analysis of released *N*-linked glycans from bovine and human milk whey proteins 155
5.3.5 Mass spectrometric analysis of *N*-linked glycans released from bovine and human milk whey proteins and HT-29 cell membrane proteins 156
5.4 Discussion 164

Chapter 6. Summary and future directions 169
6.1 Development of methods for assessing bacterial binding to conjugated milk glycans 169
6.2 Sialic acid involvement in bacterial binding to human milk proteins 170
6.3 Comparison of the bacterial binding of bovine and human milk proteins 171
6.4 Glycan involvement in bacterial adherence to bovine and human milk proteins 172
6.5 Glycan involvement in bacterial adherence to human gastrointestinal cells 172
6.6 Comparison of the *N*-linked glycan structures on human and bovine milk whey and human gastrointestinal cell membrane proteins 173
6.7 Conclusions 174
6.8 Future directions 174

References 176

Appendices 212
Appendix A: Statutory Declaration 212
Appendix B: Publication I 214
Appendix C: Conference proceeding I 228
Appendix D: Conference proceeding II 229
Appendix E: Conference proceeding III 230
Appendix F: Conference proceeding IV 231

Appendix G: Conference proceeding V 232

Supplementary Data 233

Table S1 233

Table S2 239

Table S3 243

Figure S1 261

Table S4 262

Abstract

Bacterial adhesion to host cell surfaces is the first step leading to bacterial infection and involves adhesins on the bacterial cell surface binding to host cell receptors. Milk glycans, carried on glycoproteins and glycolipids, and as free oligosaccharides, are thought to offer protection to infants by binding to pathogenic bacteria with subsequent clearing of the harmful microorganisms from the gastrointestinal tract.

The quantitative interaction between milk glycoproteins and fluorescently-labelled human gastrointestinal-colonising bacteria were studied using glass slide glycan microarray technology and a new 96 well microtitre plate based assay. The latter assay more closely resembled the complex binding of total milk proteins to the human gastrointestinal bacteria, *Escherichia coli*, *Salmonella typhimurium*, *Campylobacter jejuni* and *Lactobacillus rhamnosus*, and demonstrated that the bacteria bound differentially to the milk proteins and that the adhesion was glycan-dependent. Reduced binding was observed for all four gastrointestinal bacterial strains when sialic acid was removed from the milk glycoproteins, suggesting that bacterial binding was at least partially sialic acid specific. The binding to total human milk proteins by the oral streptococci species that are encountered at the initial stage of digestion in the mouth was shown to also involve sialic acid in *Streptococcus gordonii* adhesion but *Streptococcus mutans* appeared to recognise different binding epitopes.

Bovine and human milk protein fractions were compared for their capacity to bind to human gastrointestinal-colonising bacterial strains. Although there was more protein in bovine milk, volume equivalents of each type of milk protein bound the bacteria differentially with the pathogenic *S. typhimurium* and the commensal bacterium *L. rhamnosus* having a higher binding affinity to total human milk proteins as compared to total bovine milk proteins. All the human gastrointestinal bacteria bound more strongly to the whey proteins than to caseins, with involvement of a number of different whey glycoproteins (e.g. immunoglobulins,

lactoferrin, β-lactoglobulin) in bacterial adhesion. Again, sialic acid was shown to be involved in the bacterial binding to both bovine and human milk glycoprotein fractions.

As further indication of the importance of glycans as receptors for bacterial adhesion to gastrointestinal cells and for the role of milk glycoproteins in competitively inhibiting this binding, *N*-linked glycans released from both human and bovine milk whey proteins were shown to significantly inhibit all three pathogens, *C. jejuni*, *E. coli and S. typhimurium,* from binding to human gastrointestinal epithelial cells. Interestingly, *N*-linked glycans released from human milk whey proteins were more effective at inhibiting the binding of *C. jejuni* and *L. rhamnosus* to the intestinal cells than bovine milk whey protein *N*-linked glycans, whereas milk glycans from both mammalian species inhibited *E. coli* and *S. typhimurium* adhesion to approximately the same extent. The human milk *N*-linked glycans were also significantly more effective in inhibiting invasion of *C. jejuni* into the cells than the bovine milk *N*-linked glycans.

This study showed predominately sialylated, core fucosylated structures in the released *N*- and *O*-linked glycans of both human and bovine milk proteins. There was a significant difference in the *N*-glycosylation specific structures between the secreted milks and human cell surface glycoproteins with a high abundance of sialylated structures, well described bacterial lectin receptors, seen across all three sources. Both the human milk glycoproteins and the human epithelial cells carried Lewis epitopes, known receptors for bacterial adhesion.

In summary, we have shown that the bacterial interactions with cell surface (epithelial cells) and secreted glycoproteins in both human and milk are glycan-dependent. The data in this study repeatedly emphasises the anti-adhesive and anti-invasive properties of human (and to a lesser extent bovine) milk protein glycans to prevent enteric bacterial infections in the gastrointestinal tract of breastfed infants. It also provides further insights into the possibility

of using *N*-linked human milk glycan structures to mimic this innate immune protection of breastfed infants and improve the formulation of breast milk substitutes.

Statement of candidate

I certify that the work in this thesis entitled "Sugar-pathogen interactions: Breast milk as the first line of defence against infections" has not previously been submitted for a degree nor has it been submitted as part of the requirements for a degree to any other university or institution other than Macquarie University. I also certify that the thesis is an original piece of research and it has been written by me. Any help and assistance that I have received in my research work and the preparation of the thesis itself have been appropriately acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

Wai Yuen Cheah (41653270)

30th September 2013

Acknowledgements

I would like to thank many people who have helped me reach the completion of my thesis. Foremost, this thesis would not be possible without the financial support of the International Macquarie University Research Excellence (iMQRES) Scholarship and Macquarie University Postgraduate Research Fund (PGRF). National and International conferences were made possible by generous support from the respective organisers: Australasian Proteomics Society, Glycom, Human Proteome Organisation and International Milk Genomics Consortium. This work was facilitated by access to the Australian Proteome Analysis Facility (APAF) established under the Australian Government's National Collaborative Research.

I would like to thank my supervisors, Professor Nicolle Packer and Professor Helena Nevalainen for continuous support, guidance and detailed review of work undertaken during my four years in their laboratories. Additionally, thanks to my mentors, Dr. Daniel Kolarich, Dr. Jasmine Grinyer, Dr. Liisa Kautto, Dr. Maja Christiansen and Dr. Robyn Peterson for their valuable suggestions and advice. I would also like to acknowledge my reviewers, Professor Ian Paulsen, A/Prof. Joanne Jamie, A/Prof. Mark Molloy, A/Prof. Robert Willows and Professor Shoba Ranganathan, as well as my anonymous thesis examiners for their constructive feedback on the work described in this thesis. Sincere thanks to Dr. Angela Sun, Dr. Arun Everest-Dass, Georgina Giannikopoulos, Dr. Jasmine Grinyer, Katherine Wongtrakul-kish, Dr. Matthew McKay, Merrina Anugraham, Dr. Robyn Peterson and Dr. Wendy Noble for providing insightful comments and thesis proofreading.

I am grateful to Professor Michael Jennings (Institute for Glycomics) and Professor Hazel Mitchell (University of New South Wales) for giving me the opportunity to work with many brilliant researchers and students in their laboratories, including Dr. Christopher Day, Jose Burgos-Portugal, Karina Huinao, Dr. Lauren Hartley, Dr. Nadeem Kaakoush and Natalia Casta, who have assisted me and helped to broaden the value of this thesis. I would also like

to acknowledge every past and present colleagues of the Glyco@MQ group for their stimulating discussions and free food during Friday lab meetings. To the staff and students at APAF F7B-400, Biomarker Discovery laboratory E8C-326, Brown laboratory E8A-308, EDGE laboratory E8C-320, Karuso laboratory F7B-214, Microscopy Unit E8A-15 and MUCAB laboratory F7B-311, thank you for helping me along the way and for making the lab a warm and inviting place to work in. Anne Micallef, Catherine Wong, Jane Yang and Maria Hyland thank you for taking care of various aspects of the administration of my research.

I am thankful to the “Cashew Gang” and “Makan buddies”, including Uncle and Aunty MS Wong, Uncle Lo, Aunty Mag, Aunty Stephanie, Jacquelene, Jean, Angelica, Chin Chin, Cindy, Corrine, Denise, Elizabeth, Jacinta, Jujiao, Leo, Linda, Rayson, Regina, Sarina, Shilen, Suja and Yixin for excellent company and keeping me sane along this journey. Finally, to my parents, relatives and friends back in Singapore, Malaysia and Australia, thank you for your endless love, understanding and encouragement.

Publication and conference proceedings

Publication I (Appendix B)

Peterson R, *Cheah WY, Grinyer J, Packer N. 2013. Glycoconjugates in human milk: protecting infants from disease. *Glycobiology.* [Epub ahead of print]. doi:10.1093/glycob/cwt072.

* The research and summary tables prepared by the candidate for the literature review (Chapter 1 of this thesis) were used for the publication "Glycoconjugates in human milk: protecting infants from disease". The candidate also contributed to the writing of approximately 40 % of the text of this publication.

Conference proceeding I (Appendix C)

Everest-Dass A, Cheah WY, Nguyen-Khuong T, Kautto L, Petersen R, Lisacek F, Kolarich D, Campbell MP, *Packer NH. 2013. Enabling glycoanalysis with informatics: discovery of glycans involved in infection. In proceeding of: GLYCO 22 XXII International Symposium on Glycoconjugates. *Glycoconj J.* 30:281–461. doi 10.1007/s10719-013-9474-x.

*Oral presentation by Packer NH.

Conference proceeding II (Appendix D)

Grinyer J, Cheah WY, Kolarich D, *Packer N. 2011. Differences in pathogen-sugar interactions between human and bovine milk. In proceeding of: 8th International Symposium Milk Genomics and Human Health.

*Oral presentation by Packer N.

Conference proceeding III (Appendix E)

*Grinyer J, Cheah WY, Kolarich D, Packer N. 2011. Protein-sugar interactions between secreted fluids and pathogens as a protective mechanism. In proceeding of: The First International Conference on the Glycobiology of Human Milk Oligosaccharides. Volume 3.

doi:10.3945/an.112.002006.

*Oral presentation by Grinyer J.

Conference proceeding IV (Appendix F)

*Kolarich D, Jensen PH, Cheah WY, Grinyer J, Packer N. 2011. Glycomics of human breast milk: an antimicrobial defence mechanism. GLYCO 21 XXI International Symposium on Glycoconjugates. *Glycoconj J.* 28:197-369.

*Poster presentation by Kolarich D.

Conference proceeding V (Appendix G)

*Packer N, Kautto L, Grinyer J, Nguyen-Khuong T, Cheah WY, Dass AVE, Andersen MT, Willcox M, Kolarich D. 2011. Body fluids: sweet protection against infection? In proceeding of: GLYCO 21 XXI International Symposium on Glycoconjugates. *Glycoconj J.* 28:197-369.

*Poster presentation by Packer NH.

Presentations and awards

*Cheah WY, Godlewski M, Kolarich D, Packer N, Grinyer J. 2011. Sialic acid is involved in the differential binding of streptococcal species to milk and saliva glycoproteins. Oral and poster presentations in the 8th International Symposium on Milk Genomics and Human Health, Melbourne, Australia.

*Awarded student travel award from the International Milk Genomics Consortium.

*Cheah WY, Kolarich D, Kautto L, Grinyer J, Packer N. 2010. Does milk protect infant from bacterial infection? Poster presentation in the 15th Lorne Proteomics Symposium, Victoria, Australia.

*Awarded student poster prize.

*Cheah WY, Kolarich D, Kautto L, Grinyer J, Packer N. 2010. Milk glycoproteins and infant protection against pathogens. Poster presentation in the 9th Annual World Congress of the Human Proteome, Sydney, Australia.

*Awarded student travel award from the Human Proteome Organisation.

*Cheah WY, Wongtrakul-kish K, Kolarich D, Grinyer J, Packer N. 2011. Role of sialic acid in innate immune protection provided by mammalian milk. Poster presentation in the 16th Lorne Proteomics Symposium, Victoria, Australia.

*Awarded student poster prize

*Cheah WY, Wongtrakul-kish K, Kolarich D, Grinyer J, Packer N. 2011. Role of sialic acid in innate immune protection provided by mammalian milk. Poster presentation in The First International Conference on the Glycobiology of Human Milk Oligosaccharides, Copenhagen, Denmark.

*Awarded student travel award from Glycom A/S.

Abbreviations

2’-FL	2'-fucosyllactose
3’-FL	3'-fucosyllactose
3’-SL	3'-sialyllactose
6’-SL	6'-sialyllactose
3’-SLN	3'-sialyllactosamine
6’-SLN	6'-sialyllactosamine
α_{s1}-CN	Alpha s1-casein
α_{s2}-CN	Alpha s2-casein
α-LA	Alpha-lactalbumin
ATCC	American Type Culture Collection
EACA	ε-aminocaproic acid
Asn	Asparagine
β-CN	Beta-casein
β-LG	Beta-lactoglobulin
BSSL	Bile salt-stimulated lipase
bLF	Bovine lactoferrin
BMOs	Bovine milk oligosaccharides
BSA	Bovine serum albumin
BHI	Brain-heart infusion broth
BFP	Bundle-forming pili
BTN	Butyrophilin
CE	Capillary electrophoresis
CBPs	Carbohydrate binding proteins
CFDA-SE	Carboxyfluorescein diacetate *N*-succinimidyl ester
Cer	Ceramide
CD4+	Cluster of differentiation 4
CFG	Consortium for Functional Glycomics
CMV	Cytomegalovirus
DC-SIGN	Dendritic cell-specific ICAM 3-grabbing non-intergrin
DNA	Deoxyribonucleic acid
DSLNT	Di-sialyllacto-*N*-tetraose
DSL	Di-sialyllactose
$GD_{1\alpha}$, GD_{1a}, GD_{1b}, GD_2, GD_3	Di-sialylogangliosides
DTT	Dithiothreitol
Dol-P	Dolichol phosphate
Dol-P-P-GlcNAc	Dolichol pyrophosphate *N*-acetylglucosamine
ESI	Electron spray ionisation
ER	Endoplasmic reticulum
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
EV71	Enterovirus 71
ELISA	Enzyme-linked immunosorbent assay
EDTA	Ethylenediaminetetraacetic acid
EICs	Extracted ion chromatograms
FPLC	Fast protein liquid chromatography
FITC	Fluorescein isothiocyanate
FU	Fluorescence unit
Fuc	Fucose
FUT2	Fucosyltransferase 2
FUT3	Fucosyltransferase 3

γ-CN	Gamma-casein
Gal	Galactose
Gb_4	Globotetraosylceramide
Gb_3	Globotriaosylceramide
Glc	Glucose
Glcase	Glucosidase
GlcCer	Glucosylceramide
GlcA	Glucuronic acid
GP	Glycoprotein
GCC	Graphitised carbon column
HA	Haemagglutination
LT	Heat-labile enterotoxin
STa	Heat-stable enterotoxin of *Escherichia coli*
HS	Heparan sulphate
HSV	Herpes simplex virus
HMW	High molecular weight
HPAEC-PAD	High-performance anion-exchange chromatography with pulsed amperometric detection
HPLC	High-performance liquid chromatography
HBA	Horse blood agar
HEL	Human embryonic lung
HIV	Human immunodeficiency virus
hLF	Human lactoferrin
HMOs	Human milk oligosaccharides
HA	Hyaluronic acid
IdoA	Iduronic acid
Ig	Immunoglobulin
ISTD	Internal standard
IAA	Iodoacetamide
pI	Isoelectric point
κ-CN	Kappa-casein
LNB	Lacto-*N*-biose
LNFP	Lacto-*N*-fucopentaose
LNnT	Lacto-*N*-*neo*tetraose
LNT	Lacto-*N*-tetraose
LF	Lactoferrin
LacCer	Lactosylceramide
Le	Lewis-type structure
LC	Liquid chromatography
Man	Mannose
MS	Mass spectrometry
MFGM	Milk fat globule membrane
MW	Molecular weight
GM_1, GM_2 and GM_3	Mono-sialogangliosides
MOPs	3-(*N*-morpholino) propanesulfonic acid
MUC	Mucin
MOI	Multiplicity of infection
GalNAc	*N*-acetylgalactosamine
GlcNAc	*N*-acetylglucosamine
NeuAc	*N*-acetylneuraminic acid
LacNAc	*N*-acetyllactosamine
NeuGc	*N*-glycolylneuraminic acid

GA_1, GA_2	Neutral glycolipid
NMWL	Nominal molecular weight limit
NM	Non-motile
NSP4	Non-structural protein 4
NV	Norovirus
ND	Not detected
NMR	Nuclear magnetic resonance
PNA	Peanut agglutinin
GP_{1c}	Penta-sialylloganglioside
PNG*ase* F	Peptide-*N*-glycosidase F
%	Percentage
PMT	Photomultiplier tube
PBS	Phosphate buffered saline
PVDF	Polyvinylidene difluoride
PVP	Polyvinylpyrrolidone
PGC-LC-ESI-MS	Porous graphitised carbon-liquid chromatography-electrospray ionisation-mass spectrometry
Pro	Proline
RFU	Relative fluorescence unit
RSV	Respiratory syncytial virus
rpm	Revolutions per minute
RNA	Ribonucleic acid
RT	Room temperature
RV	Rotavirus
S-HA	Saliva-coated hydroxyapatite
SAG	Salivary agglutinin
sIgA	Secretory immunoglobulin A
Ser	Serine
Stx1 and Stx2	Shiga toxins
SLe	Sialyl Lewis-type structure
LST	Sialyllacto-*N*-tetraose
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SPE	Solid phase extraction
sHCM	Soluble human colonic mucin
Sp	Spacer
spp.	Species
SEM	Standard error of the mean
S/No.	Structure number
SPR	Surface plasmon resonance
$GQ_{1b\alpha}$, GQ_{1b}, GQ_{1c}	Tetrasialylogangliosides
Thr	Threonine
TCA	Trichloroacetic acid
$GT_{1a\alpha}$, $GT_{1b,}$ GT_{1c}, GT_2, GT_3	Trisialylogangliosides
T3D	Type 3 Dearing
ΔUA	Unsaturated hexuronic acid
UDP	Uridine diphosphate
VacA	Vacuolating toxin
WHO	World Health Organisation

Symbol nomenclature for representation of glycan structure

All monosaccharide types (sugars) assigned in this thesis are represented by specific symbol shapes, as outlined by the Consortium for Functional Glycomics (CFG; Varki *et al.* 2009b).

Monosaccharide	Abbreviation	Symbol
Deoxyhexose		
Fucose	Fuc	
Hexose		
Galactose	Gal	
Glucose	Glc	
Mannose	Man	
***N*-Acetylhexosamine**		
N-Acetylgalactosamine	GalNAc	
N-Acetylglucosamine	GlcNAc	
Acidic sugar		
Glucuronic acid	GlcA	
Iduronic acid	IdoA	
N-Acetylneuraminic acid	NeuAc	
N-Glycolylneuraminic acid	NeuGc	

The complete set of symbol nomenclature is available from: http://www.functionalglycomics.org/static/consortium/Nomenclature.shtml. GlycoWorkbench (https://code.google.com/p/glycoworkbench/) was used to draw and annotate the structures (Copper *et al.* 2001, 2003).

Chapter 1. Introduction

The first step in infection often involves the binding of a pathogen to specific cell surface receptors, which are commonly glycan (sugar) structures attached to epithelial cell membranes (Kelly and Younson 2000; Sakarya and Öncu 2003). There is strong evidence that breastfeeding reduces the risk of gastrointestinal tract infection and diarrhoea in infants and young children (Fisk *et al.* 2011; Grulee *et al.* 1934; Mwiru *et al.* 2011; Oddy 2001; Tarrant *et al.* 2012; WHO, 2000). It has been hypothesised that some of the protective effects of human milk against gastrointestinal pathogens could be attributable to milk glycans, in the form of free oligosaccharides and glycoconjugates (glycoproteins and glycolipids), which may act as decoys for pathogen attachment (Moran *et al.* 2011; Morrow *et al.* 2005; Newburg 1996, 1997, 1999, 2009; Newburg *et al.* 1995, 2004, 2005). The work presented in this thesis investigated the potential of human and bovine milk glycans to protect against pathogen adherence to the gastrointestinal tract, with a particular focus on the conjugated glycans of milk glycoproteins. This chapter provides a comprehensive background on the reported health benefits of breastfeeding, the compositions of human and bovine milk and their glycan moieties, and research that has implicated glycan involvement in pathogen attachment and inhibition.

1.1 Breastfeeding confers protection against disease

A newborn does not have a mature immune system and is unable to make its own antibodies to protect against enteric pathogens (Miller 1966). Hence, infants are highly susceptible to infection immediately after birth (Guarner and Malagelada 2003; Pawson 1995; Walker 2000). The health benefits of breastfeeding have been reported since the early 1930s, where a significant reduction of seven-fold, two-fold and four-fold in mortality rates caused by gastrointestinal infection, respiratory infection and other diseases, respectively, was observed for breastfed infants as compared to non-breastfed infants that were either partially breastfed

or formula-fed (Grulee *et al.* 1934). Research has since shown that prolonged and exclusive breastfeeding also reduces the risk of other diseases in infants, including otitis media infections and coeliac disease (Table 1.1). Hence from these multiple studies, the World Health Organisation (WHO) recommends exclusive breastfeeding for the first six months of age for infants to provide them maximum protection from illness, and continued breastfeeding with complementary foods for infants up to two years of age or beyond (WHO 2011).

Table 1.1. Breastfeeding confers significant protection against many infant infections and childhood diseases.

Diseases	**Reference**
Infant infections	
Gastrointestinal infection	Duijts *et al.* 2009; Feachem and Koblinsky 1984; Howie *et al.* 1990
Pneumonia	Boccolini *et al.* 2011; Céser *et al.* 1999
Otitis media infection	Aniansson *et al.* 1994; Chantry *et al.* 2006; Duffy *et al.* 1997; Duncan *et al.* 1993
Respiratory infection	Cushing *et al.* 1998; Downham 1976; Pisacane *et al.* 1994; Stepans *et al.* 2006
Urinary tract infection	Hanson 2004; Mårild *et al.* 1990; Pisacane *et al.* 1990, 1992
Childhood diseases	
Acute lung disease	Klein *et al.* 2008
Atopic disease	Iyengar and Walker 2012; Saarinen and Kajosaari 1995
Cancer	Bener *et al.* 2001; Davis *et al.* 1988; Grufferman *et al.* 1998; Martin *et al.* 2005; Shu *et al.* 1999
Cardiovascular disease	Bergström *et al.* 1995; Owen *et al.* 2011
Cholera	Clemens *et al.* 1990; Qureshi *et al.* 2006

Diseases	Reference
Coeliac disease	Fälth-Magnusson *et al.* 1996; Palma *et al.* 2012; Peters *et al.* 2001
Crohn's disease	Koletzko *et al.* 1989; Moffatt *et al.* 2009
Insulin-dependent diabetes mellitus	Alves *et al.* 2012; Fort *et al.* 1986, Gouveri *et al.* 2011
Meningitis	Silfverdal *et al.* 1997, 1999, 2002
Necrotising enterocolitis	Kelley 2012; Lucas and Cole 1990
Sudden infant death syndrome	Chapman 2011; Ford *et al.* 1993; Hauck *et al.* 2011; Young *et al.* 2012

1.1.1 Diarrhoeal diseases and their prevention by breastfeeding

Diarrhoea is the most common symptom seen in infectious diseases associated with the gastrointestinal tract in infants and children, and can range from mild to deadly (Elliott and Dalby-Payne 2004; Nataro and Kaper 1998; Scallan *et al.* 2005). It is defined by the WHO as the result of three or more watery or loose bowel movements in a 24 hour period (WHO 2009). Three clinical types of diarrhoea have been described: 1) acute watery diarrhoea, which lasts less than 14 days; 2) acute bloody diarrhoea or dysentery that contains blood in the faeces; and 3) persistent diarrhoea, which continues beyond 14 days. In some cases, severe dehydration and electrolyte loss could lead to infant death. Acute diarrhoeal diseases remain the second leading cause of morbidity and mortality in children less than five years of age worldwide, after pneumonia (Figure 1.1).

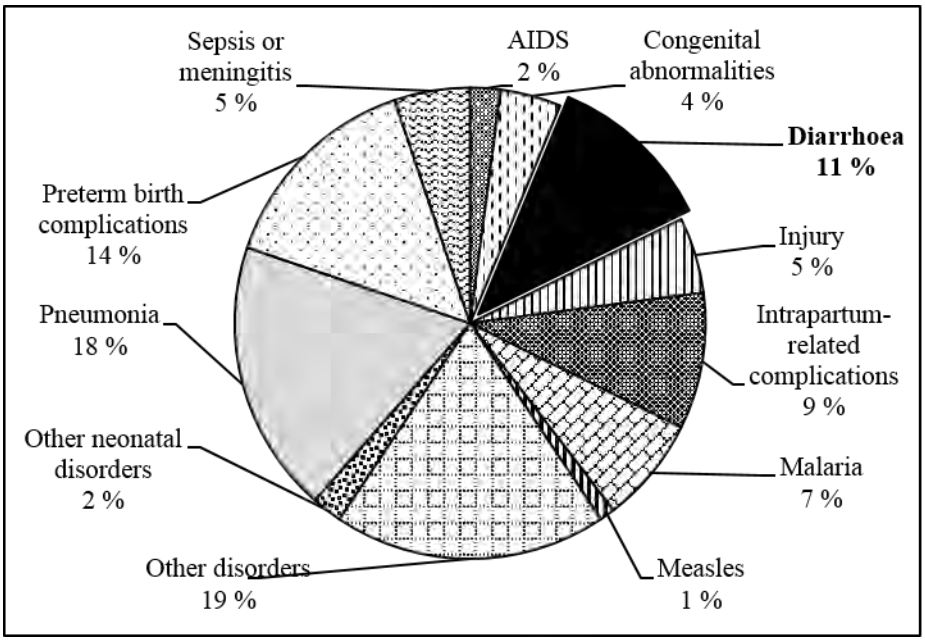


Fig. 1.1. Diarrhoea remains one of the leading causes of death in children under five years of age. Figure taken from Liu L *et al.* 2012.

A WHO collaborative study on the Role of Breastfeeding on the Prevention of Infant Mortality (2000) found that infants who were breastfed for the first six months of life had a six-fold reduced mortality risk due to diarrhoea compared to infants that were not breastfed. Infants who were breastfed from six to eleven months of age had a two-fold reduced mortality risk due to diarrhoea. These WHO results are consistent with several more recent studies (Arifeen *et al.* 2001; Fischer Walker *et al.* 2012; Kramer *et al.* 2001; Lamberti *et al.* 2011; Morrow and Rangel 2004; O'Reilly *et al.* 2012; Strand *et al.* 2012; Ubesie *et al.* 2012), indicating that breastfeeding is the most effective and practical solution for protecting infants against diarrhoea and preventing mortality.

1.2 Mechanisms of gastrointestinal infection

Common gastrointestinal pathogens known to cause infectious diarrhoea in infants are listed in Table 1.2. The leading causes of diarrhoeal disease in children under five years of age in developing countries, as established by the WHO (1990), are bacterial pathogens such as

enterotoxigenic *Escherichia coli* (ETEC; 15 %), *Campylobacter jejuni* (12.5 %), *Shigella* (10 %), *Vibrio cholerae* (7.5 %), enteropathogenic *E. coli* (EPEC; 2.5 %) and *Salmonella* (2.5 %), as well as viruses such as rotavirus (20 %) and parasites such as *Cryptosporidium* (10 %).

There are several steps involved in pathogen infection of the gastrointestinal tract. Firstly, the pathogen must attach to the surface of epithelial cells. Bacteria utilise adhesins (lectins) to facilitate their initial attachment to host cell receptors, which are typically glycan moieties of membrane glycoproteins or glycolipids (Borén *et al.* 1993; Mahdavi *et al.* 2002; Young *et al.* 2007). For instance, the EPEC utilise bundle-forming pili (BFP) to mediate adherence to the apical surface of intestinal epithelial cells (Cleary *et al.* 2004; Girón *et al.* 2002). This initial binding can then activate the secretion of effector proteins, which stimulate the pro-inflammatory pathway and destroy the microvilli by triggering an actin cytoskeleton rearrangement (Caron *et al.* 2006). Some gastrointestinal bacteria, such as *Campylobacter* and *Salmonella* also produce flagella that enable invasion of the host epithelial cells (Ledeboer *et al.* 2006). The bacteria acquire nutrients from the host cells and form micro-colonies, sometimes inducing host cell lysis (Srikanth and McCormick 2008). The infection causes fluid secretion and electrolyte loss in watery and bloody diarrhoea (Tesh and O'Brien 1991).

Table 1.2. Major bacterial, viral and parasitic pathogens that cause gastrointestinal infections in children.

Pathogens	Characteristics	Transmission routes	Infection sites	Types of diarrhoea
Bacteria				
Campylobacter jejuni	Gram-negative, curved, helical-shaped, non-spore forming, microaerophilic	Food borne, waterborne	Small and large intestines	Acute watery diarrhoea, dysentery
Clostridium perfringens (previously known as *Clostridium welchii*)	Gram-positive, rod-shaped, spore-forming, anaerobic	Foodborne	Large intestine	Watery diarrhoea
EPEC	Gram-negative, rod-shaped	Foodborne, waterborne	Small and large intestines	Acute watery or persistent diarrhoea
ETEC	Gram-negative, rod-shaped	Foodborne, waterborne	Small and large intestines	Dysentery
Helicobacter pylori (previously known as *Campylobacter pyloridis*)	Gram-negative, microaerophilic	Faecal-oral, foodborne, waterborne	Stomach	Self-limiting diarrhoea
Salmonella spp.	Gram-negative, rod-shaped, non-spore-forming	Faecal-oral, foodborne, waterborne	Small and large intestines	Acute watery diarrhoea
Shigella spp.	Gram-negative, rod-shaped, non-spore forming, non-motile	Faecal-oral, foodborne, waterborne	Small and large intestines	Acute watery diarrhoea, dysentery, persistent

Pathogens	Characteristics	Transmission routes	Infection sites	Types of diarrhoea
				diarrhoea
Vibrio cholerae	Gram-negative, comma-shaped, facultatively anaerobic	Foodborne, waterborne	Small intestine	Acute watery diarrhoea
Yersinia enterocolitica	Gram-negative coccobacillus-shaped	Foodborne, waterborne	Small intestine	Severe watery diarrhoea but not bloody
Viruses				
Calicivirus	Positive-sense, single stranded RNA, not enveloped	Contaminated surfaces, foodborne, waterborne	Stomach and intestine	Acute gastroenteritis
Rotavirus	Double-stranded RNA	Faecal-oral, foodborne, waterborne	Small intestine	Acute watery diarrhoea
Protozoan				
Cryptosporidium parvum	Completes its life cycle within a single host, resulting in cyst stages	Foodborne, waterborne	Small intestine	Acute watery diarrhoea, persistent diarrhoea
Giardia lamblia	Anaerobic flagellated, life cycle alternates between an actively swimming trophozoite and an infective, resistant cyst	Faecal-oral, foodborne, waterborne	Small intestine	Acute and chronic diarrhoea

Pathogens	Characteristics	Transmission routes	Infection sites	Types of diarrhoea
Worms				
Trichuris trichiura	Roundworm, eggs deposited from faeces to the soil	Faecal-oral	Large intestine	Dysentery

Abbreviations: EPEC, enteropathogenic *Escherichia coli*; ETEC, enterotoxigenic *E. coli*; RNA, ribonucleic acid; spp., species.

Data compiled from WHO (1990, 2009).

1.3 The composition of milk

Human milk is often described as an exceptionally complex biological fluid that is unique from another species' milk (Hamosh 1998; Kunz and Lönnerdal 1993; Kunz and Rudloff 1993; Kunz *et al.* 1999a, 1999b, 2000; López Álvarez 2007; Newburg *et al.* 2004; Picciano 2001; Séverin and Wenshui 2005). Part of the uniqueness of human milk is signified by its superior ability to protect human infants from disease (Section 1.1). Infant formulas based on cow's milk can provide nutritional benefit, but anti-pathogenic properties equivalent to human milk are yet to be obtained. The following comparison and analysis of the contents of human and bovine milk reveal the differences between the two forms of milk and components which may play significant roles in disease prevention.

Table 1.3 is a collation of data on the contents of human and bovine milk based on numerous publications from past research. It was noted when compiling this data that some variations are evident in the exact proportions of each component from either milk source, probably due to variations amongst individuals, different stages of lactation and methods of analysis; hence, figures from publications that are representative of the majority of research data are shown. The lactose content in human milk (70 g/l) compared to bovine milk (48 g/l) is noticeably higher. The role of lactose is mainly nutritional, and generally not associated with anti-pathogenic properties. It is also clearly evident that bovine milk contains substantially more total proteins (33 g/l) than human milk (8.9 g/l), which can be attributed to a very high level of casein (26 g/l) in bovine milk, whereas the proteins of human milk contain little casein (3 g/l) and are whey-predominant. There is a similar abundance of total whey proteins in human milk (6.4 g/l) and bovine milk (7 g/l), although the components of the whey vary, with higher levels of alpha-lactalbumin (α-LA), lactoferrin (LF), lysozyme and immunoglobulin A (IgA) in human milk, whilst beta-lactoglobulin (β-LG) is only found in bovine milk. The two milks have a similar lipid content, but free milk oligosaccharides are substantially more abundant in human milk (5 - 15 g/l) than bovine milk (0.05 g/l; Table 1.3).

Table 1.3. Composition of human and bovine milk.

Main fractions	Human milk (g/l)	Bovine milk (g/l)
Lactose	70[1]	48[1]
Free oligosaccharides	5 - 15[2]	0.05[3]
Fucosylated	(50 - 80 % of total HMOs)[2,4]	(1 % of total BMOs)[5]
Sialylated	(10 - 20 % of total HMOs)[2,4]	(70 % of total BMOs)[5]
Total proteins	8.9[6]	33[6]
Casein	2.5[6]	26[6]
α_{s1}-CN	Trace[7]	10.3[7]
α_{s2}-CN	Trace[7]	2.7[7]
β-CN	2.1[7]	8.5[7]
κ-CN	0.4[7]	3.2[7]
γ-CN	Not detected[7]	1.4[7]
Whey	6.4[6]	7[6]
α-LA	2.5[6]	1.2[6]
β-LG	Not detected[6]	3.0[6]
LF	1.7[6]	Trace[6]
Serum albumin	0.5[6]	3.0[6]
Lysozyme	0.5[6]	Trace[6]
IgA	1.0[6]	0.03[6]
IgG	0.03[6]	0.60[6]
IgM	0.02[6]	0.03[6]
Others	Trace[6]	Trace[6]
Total lipids	41[1]	37[1]
Phospholipids	0.2 - 0.5[8]	0.07 - 0.4[9]
Monoacylglyerol	Not detected[8]	0.04 - 0.1[9]
Triacylglyerol	40 - 40.6[8]	35.9 - 36.3[9]
Free fatty acid	0.04[8]	0.04 - 0.1[9]
Cholesterol	0.1 - 0.5[8]	0.1[9]
1,2-Diacylglycerol	0.004[8]	Not detected[9]
Cholesteryl esters	Trace[8]	0.07[9]
Sterols	0.1 - 0.3[10]	0.07 - 0.1[10]
Sterol esters	Trace[10]	Trace[10]

Main fractions	Human milk (g/l)	Bovine milk (g/l)
Glycolipids	0.41[10]	0.37[10]

Abbreviations: α_{s1}-CN, alpha s1-casein; α_{s2}-CN, alpha s2-casein; α-LA, alpha-lactalbumin; β-CN, beta-casein; β-LG, beta-lactoglobulin; BMOs, bovine milk oligosaccharides; γ-CN, gamma-casein; HMOs, human milk oligosaccharides; Ig, immunoglobulin; κ-CN, kappa-casein; LF, lactoferrin.

[1]Data compiled from Hale and Hartmann 2007. [2]Data compiled from Bao *et al.* 2007; Chaturvedi *et al.* 2001a; Coppa *et al.* 1999; Davidson *et al.* 2004; Gabrielli *et al.* 2011; Kunz *et al.* 1999a; Newburg *et al.* 2000. [3]Data compiled from Gopal and Gill 2000. [4]Data complied from Kobata 2010; Ninonuevo *et al.* 2006; Wu *et al.* 2010, 2011. [5]Data compiled from Tao N *et al.* 2008, 2009. [6]Data compiled from Räihä 1985; Wal 1998. [7]Data compiled from Crittenden RG and Bennett LE 2005. [8]Data compiled from Rodriguez-Palmero *et al.* 1999. [9]Data compiled from Jensen 2002. [10]Data compiled from Jensen *et al.* 1980.

Lysozyme is known as an anti-bacterial agent in human milk that helps to protect the infants against bacterial infections (Lönnerdal 2003). It is believed to cause the lysis of certain potentially damaging Gram-positive bacteria and a few Gram-negative bacteria in the gastrointestinal tract of breast-fed infants, thereby favouring the establishment of beneficial intestinal microflora and improving the immunity of infants. Other components of human milk that are known to have bactericidal activity include alpha-lactalbumin (α-LA; Lönnerdal and Lien 2003), lactoferrin (LF; Lönnerdal 2003) and secretory immunoglobulin A (sIgA; Glass *et al.* 1983; Groer *et al.* 2004), all of which are present in higher quantities in human milk than bovine milk (Table 1.3). Immunomodulatory agents not featured in Table 1.3, such as maternal-derived macrophages, neutrophils, lymphocytes, nucleic acids, cytokines and antioxidants have also been identified as unique to human milk and contribute to the developing immune system of infants (Ebrahim 1998; Garofalo 2010; Kulkarni *et al.* 1994).

In addition to the above factors, there is reportedly another more subtle form of disease prevention in the form of the milk glycans, present both as free oligosacharrides, which are at

much higher abundance in human milk (5 - 15 g/l) than bovine milk (0.5 g/l; Table 1.3), and as attached glycans of the glycoproteins and glycolipids. The majority of the glycans is minimally digested and remain largely intact in infant faeces (Chaturvedi *et al.* 2001b; Engfer *et al.* 2000; Gnoth *et al.* 2000). Thus, milk glycans may help limit the binding of pathogens to the infant gastrointestinal tract by mimicking host epitopes on epithelial cell surfaces (Kunz and Rudloff 1993; Zopf and Roth 1996), binding to the pathogens and flushing them from the body (Figure 1.2; and Figure 1, Publication I, Appendix B).

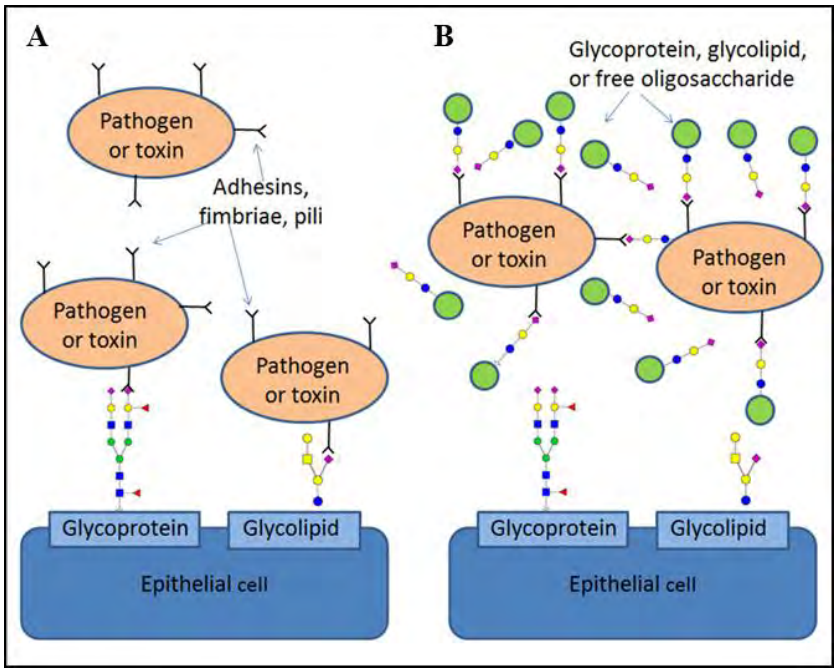


Fig. 1.2. A) A pathogen can have various glycan-recognising receptors on its surface (such as adhesins, fimbriae or pili), which bind to specific glycans attached to glycoproteins and glycolipids in epithelial cell membranes of the gastrointestinal tract. Once bound, the pathogens can cause infection. B) Glycans from milk glycoproteins, glycolipids or free oligosaccharides may have identical glycan epitopes to those recognised by the pathogen. Hence, the milk glycans could act as decoys to limit epithelial cell adhesion and reduce the risk of infection.

The potential role of milk glycans for the prevention of pathogen attachment to the infant gastrointestinal tract was the focus of the work carried out for this thesis. Hence, the following sections provide a more detailed description of the synthesis and structural features

of human and bovine milk oligosaccharides, glycoproteins and glycolipids, and review the extent of research to date that has specifically investigated the role that the glycan moieties of these molecules may play in preventing pathogen attachment to an infant's gastrointestinal tract.

1.4 Milk oligosaccharides

1.4.1 Synthesis and structural features of human milk oligosaccharides

The synthesis of milk oligosaccharides depends on the expression of glycosyltransferases and the presence of sugar nucleotides in lactating mammary glands (Bode 2009, 2012). In humans, the building blocks of milk oligosaccharides consist of five monosaccharides: D-glucose (Glc), D-galactose (Gal), *N*-acetyl-D-glucosamine (GlcNAc), L-fucose (Fuc) and sialic acid (*N*-acetylneuraminic acid, NeuAc). Human milk oligosaccharides (HMOs) carry a lactose unit (Galβ1-4Glc) at their reducing end, which can be elongated by the addition of fucose, sialic acid, or by a β1-3-linked lacto-*N*-biose (LNB, Galβ1-3GlcNAc, type 1 chain) or β1-4-linked *N*-acetyllactosamine (LacNAc; Galβ1-4GlcNAc, type 2 chain). Further modifications include additional fucose and sialic acid residues via α1-2/3/4 and α2-3/6 linkages, respectively (Amano *et al.* 2009; Kobata 2010).

There is considerable variation in the types of HMOs produced by different mothers, depending on their ability to produce the enzymes fucosyltransferase 2 (FUT2, encoded by the secretor gene) and fucosyltransferase 3 (FUT3, encoded by the Lewis gene). Milk of "secretor" mothers contains α1-2-linked fucosylated HMOs, whilst mothers who are Lewis positive produce HMOs in which fucose is added to the sub-terminal GlcNAc (Chaturvedi *et al.* 2001a; Johnson and Watkins 1992; Johnson *et al.* 1992; Kumazaki and Yoshida 1984; Stahl *et al.* 2001; Thurl *et al.* 1997, 2010; Viverge *et al.* 1990a, 1990b). Figure 1.3 (and Figure 2, Publication I, Appendix B) shows the blood group H antigen and Lewis structures that can occur on HMOs; these structures can also be features of the attached glycans

adorning human milk glycoproteins (discussed further in Section 1.5).

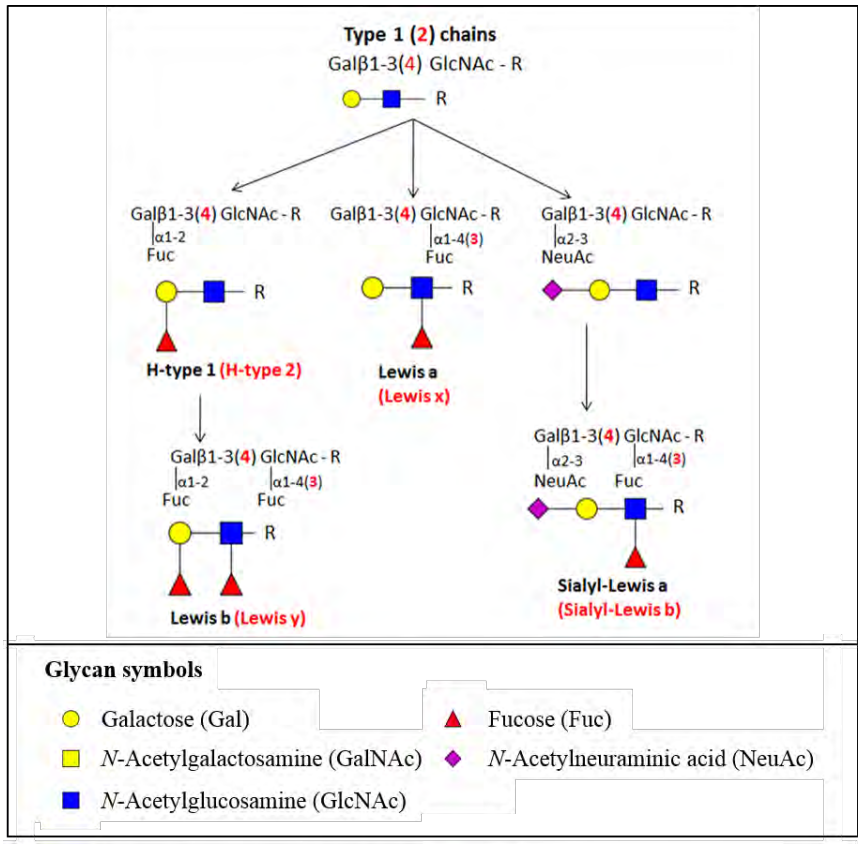


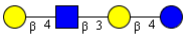
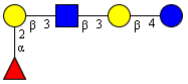
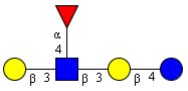
Fig. 1.3. The blood group H antigen and Lewis structures found in glycan-containing molecules (free oligosaccharides and glycoconjugates) in human milk. Type 1 chains are characterised by Galβ1-3GlcNAc, whereas type 2 chains have a Galβ1-4GlcNAc linkage (shown in red font). Note: Those in the population classed as secretors have a functional FUT2 gene and fucose residues can be added to glycans via an α1-2 linkage, whereas non-secretors do not have glycans containing fucose residues linked by an α1-2 linkage.

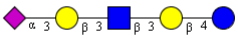
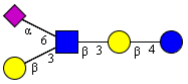
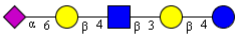
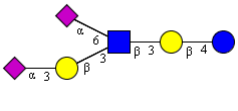
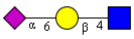
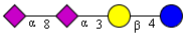
1.4.2 A comparison of human and bovine milk oligosaccharides

A very high concentration of free oligosaccharides is evident in human milk from the beginning of the lactation period (22 - 24 g/l in colostrum). Although the concentration of the HMOs decreases in mature milk (5 - 12 g/l), levels remain consistently and substantially higher than the concentration of milk oligosaccharides in bovine milk (0.05 g/l; Table 1.3) or bovine colostrum (1 g/l; Ninonuevo *et al.* 2006). There are more structural varieties of HMOs than bovine milk oligosaccharides (BMOs), with over 200 HMOs (Ninonuevo *et al.* 2006)

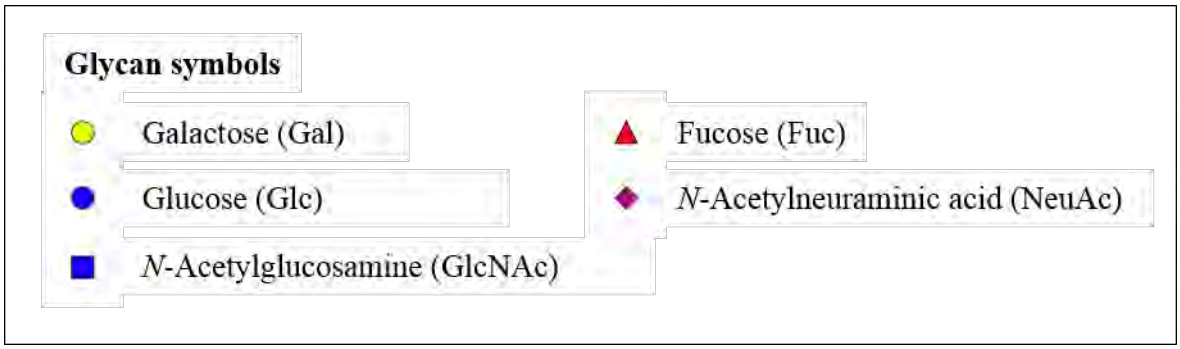
and only 40 BMOs (Tao N *et al.* 2008) reported. The BMOs are generally shorter and less branched than HMOs (Urashima *et al.* 2013). Fucosylated HMOs are more abundant (50 - 80 %) than sialylated HMOs (10 - 20 %), while BMOs contain high sialylation (70 %) and a low level of fucosylation (1 %; Table 1.3). Some studies report no fucosylated BMOs (Tao *et al.* 2009), others report very low levels (Saito *et al.* 1987; Sundekilde *et al.* 2012). Another form of sialic acid, *N*-glycolylneuraminic acid (NeuGc), is also present in bovine milk, but has not been observed in human milk. Table 1.4 shows the main neutral and acidic oligosaccharides present in human and bovine milk.

Table 1.4. Main neutral and acidic oligosaccharides available in human and bovine milk.

Type of oligosaccharides	**Structures**	**Availability in human or bovine milk**
Neutral oligosaccharides		
2'-fucosyllactose, 2'-FL Fucα1-2Galβ1-4Glc		Human
3'-fucosyllactose, 3'-FL Fucα1-3Galβ1-4Glc		Human
Lacto-*N*-tetraose, LNT Galβ1-3GlcNAcβ1-3Galβ1-4Glc		Human
Lacto-*N*-*neo*tetraose, LNnT Galβ1-4GlcNAcβ1-3Galβ1-4Glc		Human
Lacto-*N*-fucopentaose I, LNFP I Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc		Human
Lacto-*N*-fucopentaose II, LNFP II Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc		Human

Type of oligosaccharides	Structures	Availability in human or bovine milk
N-acetyllactosamine Galβ1-4GlcNAc		Bovine
N-acetylgalactosaminylglucose GlcNAcβ1-4Glc		Bovine
Acidic oligosaccharides		
3'-sialyllactose, 3'-SL NeuAcα2-3Galβ1-4Glc		Human
6'-sialyllactose, 6'-SL NeuAcα2-6Galβ1-4Glc		Human and Bovine
Sialyllacto-*N*-tetraose a, LSTa NeuAcα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc		Human
Sialyllacto-*N*-tetraose b, LSTb Galβ1-3(NeuAcα2-6)GlcNAcβ1-3Galβ1-4Glc		Human
Sialyllacto-*N*-tetraose c, LSTc NeuAcα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glc		Human
Disialyllacto-*N*-tetraose, DSLNT NeuAcα2-3Galβ1-3(NeuAcα2)-6GlcNAcβ1-3Galβ1-4Glc		Human
3'-sialyllactosamine, 3'-SLN NeuAcα2-3Galβ1-4GlcNAc		Bovine
6'-sialyllactosamine, 6'-SLN NeuAcα2-6Galβ1-4GlcNAc		Bovine
Disialyllactose, DSL NeuAcα2-8NeuAcβ2-3Galβ1-4Glc		Bovine

Data obtained from Gopal and Gill (2000).



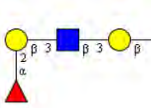
1.4.3 Evidence that milk oligosaccharides may inhibit pathogen adherence to host cells

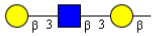
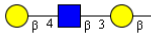
Milk oligosaccharides have been proposed to promote the health of the newborn in several ways, including by acting as prebiotics to increase the growth of beneficial gut microorganisms, such as lactobactobacilli and bifidobacteria (Chichlowski *et al.* 2011; Penders *et al.* 2005; Yu *et al.* 2013), and by competitively binding to selectins to reduce inflammation (Bode *et al.* 2004a, 2004b). The specific structural characteristics of HMOs that inhibit the binding of pathogens or their toxins to epithelial cells have also been well studied *in vivo* and *in vitro*, wherein HMOs containing fucose and/or sialic acid residues at their termini are particularly important in preventing pathogen adhesion and infection (Table 1.5). For example, neutral HMOs in human colostrum containing GlcNAc and fucose residues were able to inhibit the binding of *E. coli* to uroepithelial cells (Coppa *et al.* 1990), and α1,2-linked fucosylated HMOs were capable of inhibiting the binding of heat-stable enterotoxin (STa) of *E. coli* to T84 intestinal cells (Crane *et al.* 1994), whereas human milk 3'-sialyllactose (3'-SL) prevented *H. pylori* colonisation (Mysore *et al.* 1999).

As shown in Table 1.5, the effect of HMOs on multiple pathogens, ranging from bacteria, viruses and fungi have been well studied, but there are limited comparative studies on the effect of HMOs and BMOs or BMOs alone on pathogen binding. This could be due to the low abundance of oligosaccharides in bovine milk (Table 1.3). However, neutral and acidic free oligosaccharides from bovine colostrum have shown potential as anti-adhesive agents in

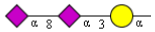
reducing a non-toxigenic strain of EHEC O157:H7 binding to and invasion of HT-29 intestinal cells (Lane *et al.* 2012a). The acidic oligosaccharides in bovine colostrum also inhibited the binding of *H. pylori* to various gastrointestinal epithelial cells, where the inhibition was proven to be more effective by 3'-SL than 6'-SL (Simon *et al.* 1997). Table 1.5 summarises evidence from past research that HMOs and BMOs bind to human pathogens and their toxins, and thus may inhibit pathogen binding to host cells.

Table 1.5. Free oligosaccharides in human and bovine milk recognised by a diverse range of pathogens and/or bacterial toxins.

Microbial recognition epitopes		Targets	Proposed protective mechanisms	Reference
In human milk				
α1-2 linked fucosyloligosaccharides		Stable toxin of *Escherichia coli*	Inhibited heat-stable enterotoxin *E. coli* binding to human colonic T84 cells and suckling mice	Crane *et al.* 1994; Newburg *et al.* 1990, 2004
		Norovirus (NV)	May act as decoy receptors to inhibit NV binding	Jiang *et al.* 2004
2'-FL (Fucα1-2Galβ1-4Glc)		*Campylobacter jejuni*	Inhibited binding of *C. jejuni* to intestinal cells and reduced risk of diarrhoeal infections observed in infants	Morrow *et al.* 2004; Ruiz-Palacios *et al.* 2003
α1-2Gal-containing fucosyloligosaccharides		*Candida albicans*	Inhibited binding of *C. albicans* to human buccal epithelial cells	Brassart *et al.* 1991
LNFP II containing α1-2 linked fucosyloligosaccharides		Not known	Reduced respiratory and gastrointestinal illness observed in infants	Stepans *et al.* 2006
Secretor and Lewis antigens		NV	May act as decoy receptors to inhibit binding of specific NV strains	Jiang *et al.* 2004

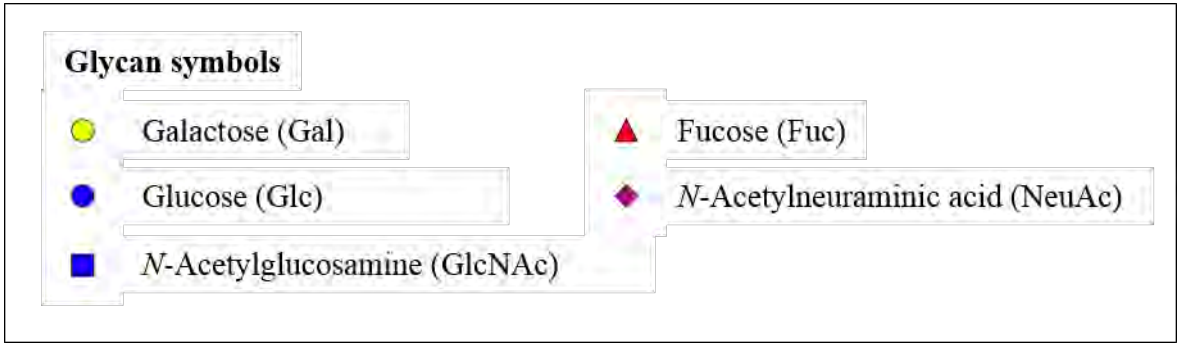
Microbial recognition epitopes		Targets	Proposed protective mechanisms	Reference
containing α1-2 linked fucose				
LNT (Galβ1-3GlcNAcβ1-3Galβ1-4Glc)		*Streptococcus pneumoniae* and *Haemophilus influenzae*	Inhibited binding of *S. pneumoniae* and *H. influenzae* to human pharyngeal or buccal epithelial cells	Andersson *et al.* 1986
LNnT (Galβ1-4GlcNAcβ1-3Galβ1-4Glc)				
Neutral tri-tetrasaccharides containing GlcNAc and Fuc residues	Not known	*E. coli*	Inhibited binding of *E. coli* to uroepithelial cells but sialyloligosaccharides had no effect	Coppa *et al.* 1990
3'-SL (NeuAcα2-3Galβ1-4Glc)		Cholera toxin	Inhibited cholera toxin inducing fluid accumulation	Idota *et al.* 1995
		Helicobacter pylori	Inhibited *H. pylori* colonisation in Rhesus monkeys	Mysore *et al.* 1999
3'-SL		Reovirus strain	3'-SL inhibited the binding of T3D to HeLa cells	Iskarpatyoti *et al.* 2012

Microbial recognition epitopes		Targets	Proposed protective mechanisms	Reference
		type 3 Dearing (T3D)		
and 6’-SL (NeuAcα2-6Galβ1-4Glc)			3’-SL and 6’-SL inhibited T3D-mediated HA to bovine erythrocytes	
		rs1HA3 strain	3’-SL and 6’-SL inhibited HA-mediated by rs1HA3	
3'-SL and 6'-SL		Human rotavirus (RV) OSU strain	Inhibited 125I-radiolabelled human RV OSU strain binding to and infectivity/replication in MA-104 cells	Hester *et al.* 2013
LNnT, 2’-FL and 3'-SL and 6'-SL			Decreased NSP4 replication in acute RV-infected piglets	
Free oligosaccharides	Not known	*Vibrio cholerae*	Inhibited hemagglutination of classical *V. cholerae* Inaba or Ogawa strains (by L-fucose) and El Tor *V. cholerae* (by D-mannose) to human erythrocytes but glycolipids had no effect	Holmgren *et al.* 1983
Oligosaccharide fractions containing fucosylated tetra- and pentasaccharides	Not known	Enteropathogenic *E. coli* (EPEC)	Inhibited EPEC to HEp-2 cells	Cravioto *et al.* 1991

Microbial recognition epitopes		Targets	Proposed protective mechanisms	Reference
In bovine milk				
3'-SL in bovine colostrum			3'-SL inhibited *H. pylori* binding to gastrointestinal epithelial cells more effective than 6'-SL	Simon *et al.* 1997
3'-SL		EHEC O157:H7	A mixture of acidic BMOs reduced EHEC O157:H7 binding to, invasion of and translocation of HT-29 cells	Lane *et al.* 2012a, 2012b
DSL (NeuAcα2-8NeuAcα2-3Galβ1-4Glc)				
and 6'-SLN (NeuAcα2-6Galβ1-4GlcNAc) of bovine colostrum				
In human and bovine milk				
A mixture of neutral HMOs and acidic BMOs	Not known	*Neisseria meningitidis*	Neutral HMOs inhibited *N. meningitidis* binding to bovine thyroglobulin, but acidic BMOs completely inhibited *N. meningitidis* binding to bovine thyroglobulin and partially to human salivary agglutinin	Hakkarainen *et al.* 2005

Abbreviations: 6'-SLN, 6'-sialyllactosamine; 3'-SL, 3'-sialyllactose; 6'-SL, 6'-sialyllactose; BMOs, bovine milk oligosaccharides; DSL,

disialyllactose; EHEC, enterohaemorrhagic *Escherichia coli*; EPEC, enteropathogenic *E. coli*; 2'-FL, 2'-fucosyllactose; HA, haemagglutination; HMOs, human milk oligosaccharides; LNFP II, lacto-*N*-fucopentaose II; LNnT, lacto-*N*-*neo*tetraose; LNT, lacto-*N*-tetraose; NSP4, non-structural protein 4; NV, norovirus; RV, rotavirus; T3D, Type 3 Dearing..



1.5 Milk glycoproteins

1.5.1 Synthesis and structural features of glycans on human milk glycoproteins

Milk glycoproteins are synthesised in the mammary gland by the attachment of *N*- and *O*-linked glycans to nascent proteins in the endoplasmic reticulum (ER), and further modifications by various glycosyltransferases in the compartments in the Golgi apparatus (Scheiffele and Füllekrug 2000). In the first step in the formation of *N*-linked glycans, GlcNAc-1-P from uridine diphosphate (UDP)-GlcNAc in the cytoplasm is added to dolichol phosphate (Dol-P) on the cytoplasmic face of the ER membrane to generate dolichol pyrophosphate *N*-acetylglucosamine (Dol-P-P-GlcNAc). During the early processing steps, mannose (Man) residues are sequentially added, one at a time by mannosyltransferases. Once five mannose residues are attached to the structure, Dol-P-P-GlcNAc is extended to Dol-P-P-$GlcNAc_2Man_5$, where the precursor glycan located in the cytosol gets "flipped" so that the glycan structure is exposed inside the ER lumen. More mannose and glucose residues are then added to form a mature *N*-linked glycan precursor ($Glc_3Man_9GlcNAc_2$). After the mature precursor is detached from its Dol-PP anchor and transferred to an asparagine (Asn)-X-serine (Ser)/threonine (Thr) sequon of nascent protein in the ER, a series of processing reactions occurs. Glucosidases (Glcase) I and II in the lumen of ER remove three glucose residues, while the ER chaperone proteins, calnexin and calreticulin, as well as α-glucosyltransferase, regulate the folding and quality control of the newly synthesised glycoproteins (Varki *et al.* 2009a; Williams 2006). Finally, the *N*-linked glycoprotein is transported to the Golgi, where further processing of the glycan structure continues by trimming outer mannose residues of the glycoprotein by mannosidase until $Man_5GlcNAc_2Asn$ is generated for diversification of *N*-linked glycans (Potapenko *et al.* 2010).

A typical core unit of an *N*-linked glycoprotein consists of two GlcNAc and three mannose residues covalently attached to an asparagine residue of a polypeptide chain, with a consensus sequence Asn-X-Ser/Thr, where X can be any amino acid except proline (Pro; Figure 1.4). However, the complexity can be extended by attachment of other monosaccharides to the antennal region of its tri-mannosyl chitobiose core, with the resultant *N*-linked oligosaccharides having "high mannose-type", "complex-type" or "hybrid-type" structures (Figure 1.4).

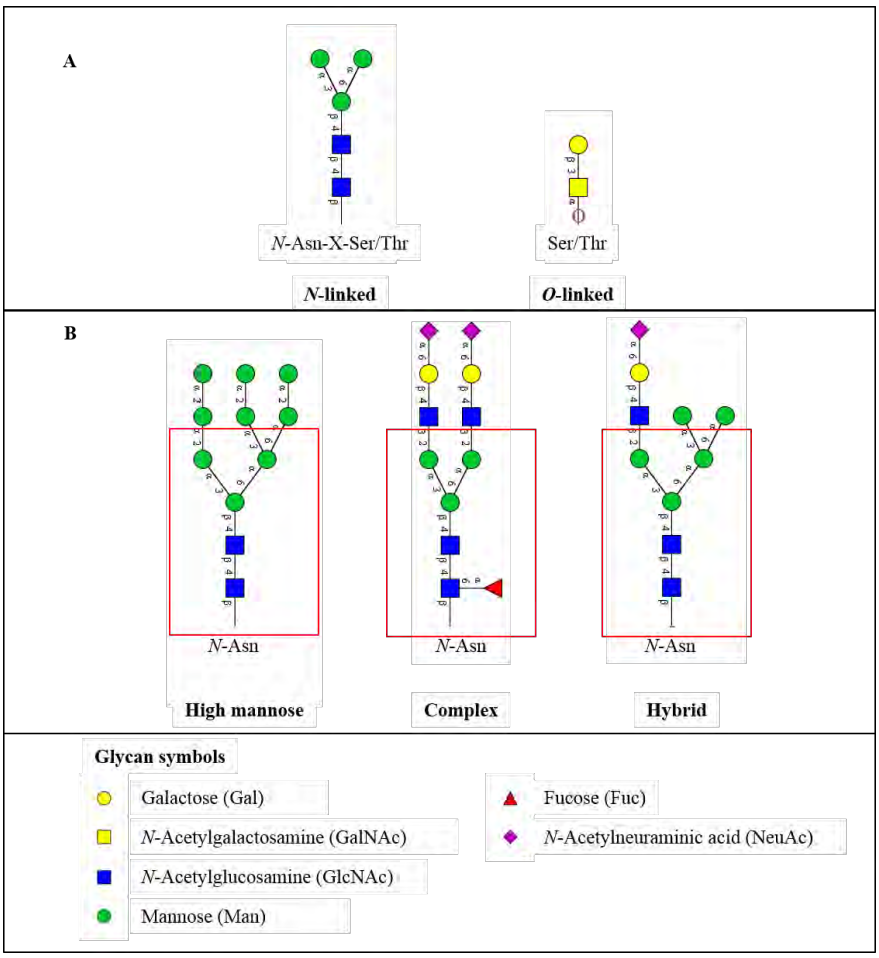


Fig. 1.4. (A) The two main types of protein glycosylation are either *N*- or *O*-linked. (B) A glycoprotein can contain three major classes of *N*-linked glycans. Examples are of a high mannose *N*-linked glycan containing just mannose residues attached to the tri-mannosyl chitobiose core (in red box), a complex *N*-linked glycan including other monosaccharides, and a hybrid *N*-linked glycan with one arm containing a high mannose-type structure and the other arm a complex-type structure, attached to the core. Abbreviations: Asn, asparagine; Ser, serine; Thr, threonine. Figure taken from Potapenko *et al.* 2010.

Synthesis of *O*-linked glycoproteins does not require the Dol-PP anchor. Instead *O*-linked glycans are formed by an entirely separate process, which occurs mostly in the Golgi complex. A single GalNAc residue is directly transferred onto the hydroxyl oxygen of a serine or threonine residue by a specific glycosyltransferase. This GalNAc can be extended into a variety of core structures (Figure 1.5). Compared to *N*-linked glycans, the oligosaccharide chain on *O*-linked glycans is usually shorter and there is not a defined amino acid consensus sequence for the attachment of the GalNAc (Brockhausen *et al.* 2009; Moran *et al.* 2011). The subsequent monosaccharides that are attached are dependent on enzymes present in different cell types. At present, there are at least eight known *O*-GalNAc-linked core structures (Figure 1.5), but only cores 1 - 4 have been described in humans (Moran *et al.* 2011; Morelle and Michalski 2005). Each of these core structures can be extended and further modified by either fucose or sialic acid residues (Potapenko *et al.* 2010).

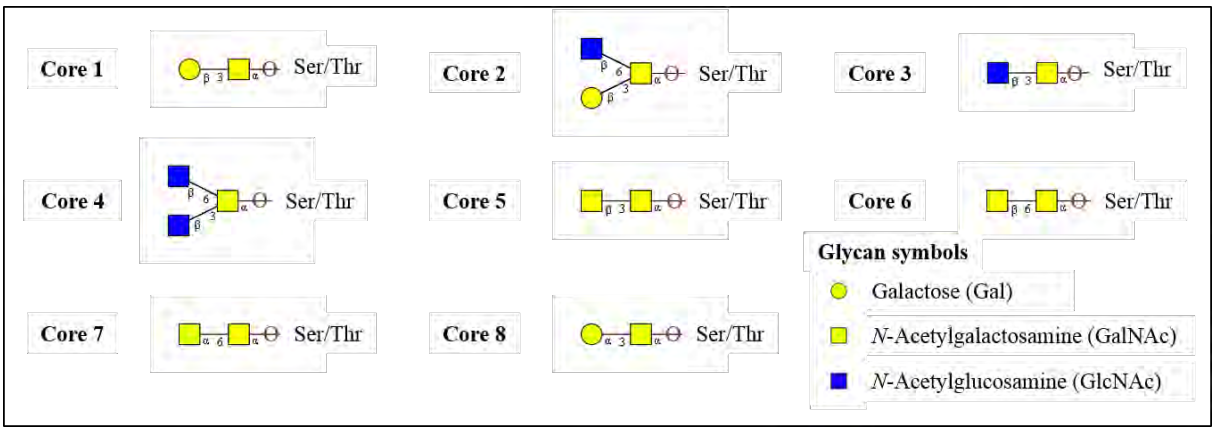


Fig. 1.5. There are eight main core *O*-linked glycan structures in vertebrates, of which cores 1 - 4 have been described in humans (Moran *et al.* 2011; Morelle and Michalski 2005).

1.5.2 A comparison of human and bovine milk glycoproteins

Over 70 % of human milk proteins have been reported as glycosylated (An *et al.* 2009; Apweiler *et al.* 1999). The major glycoproteins in human whey milk are α-lactalbumin, lactoferrin and sIgA, while κ-casein representing approximately 15 % of total casein is the only glycosylated form of casein (Table 1.3). Although the proteins in human milk fat globule membrane (MFGM) represent less than 5 % of the total proteins in human milk (Cavaletto *et al.* 2008), some proteins such as bile salt-stimulated lipase (BSSL), butyrophilin (BTN), lactadherin, mucins (MUC1, MUC4, MUC5B, MUC16) and xanthine oxidase appear to be glycosylated.

The whey to casein protein ratios vary significantly between human and bovine milk (Table 1.3), where human milk contains proportionally more whey proteins (72 % of total human proteins) and bovine milk is more abundant in casein (79 % of total bovine proteins). Although the total protein content of bovine milk (33 g/l) is much higher than that of human milk (8.9 g/l; Table 1.3), the total amount of whey proteins is similar between the two species (6.4 g/l and 7 g/l in human and bovine milk respectively; Table 1.3), where the components of the whey vary slightly. The two major whey proteins of bovine milk are β-lactoglobulin and α-lactalbumin, of which only α-lactalbumin is known to be glycosylated. In contrast to the substantial amounts of lactoferrin and sIgA in human milk whey, these glycoproteins are present only in trace amounts in bovine milk (Heine *et al.* 1991; Kunz and Rudloff 1993; Lönnerdal and Lien EL 2003). The glycoprotein IgG is the dominant immunoglobulin in bovine colostrum and milk, whereas sIgA is the major immunoglobulin in human colostrum and milk (Table 1.3). The bovine casein fraction is composed of α_{s1}- (40 % of total bovine casein), α_{s2}- (10 % of total bovine casein), β- (33 % of total bovine casein), κ- (12 % of total

bovine casein) and γ- (5 % of total bovine casein) components, of which only κ-casein is glycosylated, and is present at levels of 3.2 g/l, compared to 0.4 g/l of κ-casein in human milk (Table 1.3; Crittenden RG and Bennett LE 2005).

The glycoproteins of human and bovine milk are not only present in different proportions, they also appear to carry significantly different glycan structures. For example, the glycosylation of MFGM proteins in human milk has been found to be remarkably different to that of bovine MFGM proteins with several differences in terms of their glycan epitopes (Wilson *et al.* 2008). The *N*-linked glycoproteins in human MFGM had terminal fucose-containing epitopes, such as the Lewis and blood group H epitopes (Figure 1.3) that are absent in bovine MFGM. In the analysis of *O*-linked glycans, the glycoproteins in bovine MFGM contained mostly core 1-type structures (Galβ1-3GalNAc), whereas core 2-type structures (Galβ1-3Glcβ1-6GalNAc) were mostly found on human MFGM glycoproteins. These findings agree with Nwosu *et al.* (2012), who reported that human milk contains more complex structures and an elevated level of protein-bound fucose than bovine milk.

1.5.3 Evidence that glycans on milk glycoproteins may influence pathogen adhesion

There is emerging evidence to suggest that the glycan moieties of milk glycoproteins may play a similar role as milk oligosaccharides in preventing pathogen adhesion to epithelial surfaces by virtue of their ability to mimic the pathogen receptors of the potential host cells. Compared to free oligosaccharides, the milk glycoproteins may offer glycan epitopes with more diverse and complex structures that are extended in different conformations and confer multivalency. These conjugated milk glycans have been specifically implicated as having a greater potential role in the protective mechanism against pathogen adhesion to epithelial surfaces than milk oligosaccharides (Crane *et al.* 1994; Morrow *et al.* 2005; Newburg *et al.* 1990, 2005; Ruiz-Palacios *et al.* 2003; Zivkovic *et al.* 2011), yet comparatively little research has been devoted to identifying the specific glycan moieties of the glycoproteins that are integral to the interactions. Table 1.6 (and Table 1, Publication I, Appendix B) summarises the research that has shown that human milk glycoproteins affect the binding of pathogens to potential host cells; if determined, the specific glycan epitopes critical to the interactions are shown. Very few studies have been carried out to investigate the ability of bovine milk glycoproteins to inhibit pathogen binding; these studies are discussed in the text below.

Table 1.6. Human milk glycoproteins that may inhibit the adhesion of pathogens and/or their toxins to human epithelial cells. Glycan epitope implicated in pathogen or toxin adhesion/ inhibition is specified if known.

Glycoprotein (binding epitope if known)	**Target**	**Experimental evidence**	**Reference**
sIgA High mannose glycans	Type 1 fimbriated *Escherichia coli*	Inhibited binding to HT-29 (human epithelial colorectal adenocarcinoma) cells	Wold *et al.* 1990
sIgA Fucose-containing glycans	Enteropathogenic *E. coli* (membrane bound EPEC adherence factor)	Inhibited binding to HEp-2 (human epithelial laryngeal carcinoma) cells	Cravioto *et al.* 1991
sIgA Fucose-containing glycans	*Helicobacter pylori*	Inhibited binding to mucosa cells from human gastric epithelial tissue	Falk *et al.* 1993
sIgA Sialic acid-containing glycans	S-fimbriated *E. coli*	Inhibited binding to human buccal epithelial cells	Schroten *et al.* 1998
sIgA Glycans (undefined)	Toxin A of *Clostridium difficile*	Inhibited binding to hamster intestinal brush border membranes	Dallas and Rolfe 1998
sIgA	Heat-labile enterotoxin of *E. coli*	Infected children fed human milk containing high levels of sIgA remained asymptomatic	Cruz *et al.* 1988

Glycoprotein (binding epitope if known)	**Target**	**Experimental evidence**	**Reference**
Lactoferrin	Enterotoxigenic *E. coli* (ETEC)	Inhibited ETEC induced haemagglutination of human erythrocytes *in vitro*	Giugliano *et al.* 1995
Lactoferrin	EPEC	Inhibited binding to HeLa (human epithelial cervical adenocarcinoma) cells	de Araújo and Giugliano 2001
Lactoferrin	*Shigella flexneri*	Inhibited binding to HeLa (human epithelial cervical adenocarcinoma) cells	Gomez *et al.* 2003
Lactoferrin	*Salmonella typhimurium*	Inhibited binding to HeLa (human epithelial cervical adenocarcinoma) cells	Bessler *et al.* 2006
Lactoferrin *N*-linked glycans	*Listeria monocytogenes*, *Salmonella enterica*	Inhibited adhesion to human Caco-2 (human epithelial colorectal adenocarcinoma) cells	Barboza *et al.* 2012
Lactoferrin Terminal fucose	*Salmonella enterica* (Typhimurium)	Inhibited adhesion to human Caco-2 (human epithelial colorectal adenocarcinoma) cells	Barboza *et al.* 2012
Lactoferrin Terminal fucose	*Salmonella enterica* (Heidelberg)	Inhibited adhesion to human Caco-2 (human epithelial colorectal adenocarcinoma) cells	Barboza *et al.* 2012

Glycoprotein (binding epitope if known)	**Target**	**Experimental evidence**	**Reference**
Lactoferrin	Cytomegalovirus (CMV) and human immunodeficiency virus (HIV-1)	Blocked human CMV invasion of human fetal lung fibroblasts and inhibited HIV-1 induced cytopathic effect on human MT4 T-cells	Harmsen *et al.* 1995
Lactoferrin	Rotavirus (RV)	Inhibited rotavirus haemagglutination and binding in human erythrocytes and HT-29 (human epithelial colorectal adenocarcinoma) cells	Superti *et al.* 1997
Lactoferrin	Respiratory syncytial virus (RSV) and CMV	Inhibited RSV invasion of HEp-2 cells and CMV invasion of HEL (human embryonic lung) cells	Portelli *et al.* 1998
Lactoferrin	Poliovirus	Inhibited infection of Vero (African green monkey kidney) cells	Marchetti *et al.* 1999
Lactoferrin	HIV-1	Inhibited virus-cell fusion and entry into human MT4 T-cells	Swart *et al.* 1996
Lactoferrin	Herpes simplex virus 1 and 2 (HSV-1 and HSV-2)	Inhibited cell-to-cell spread of HSV-1 and HSV-2 in Vero (African green monkey kidney) cells	Jenssen *et al.* 2008

Glycoprotein (binding epitope if known)	**Target**	**Experimental evidence**	**Reference**
κ-casein GlcNAcβ1-3Gal-	*Streptococcus pneumonia* and *Haemophilus influenzae*	Inhibited binding to human respiratory tract epithelial cells	Aniansson *et al.* 1990
κ-casein Sialic acid-containing glycans	*Streptococcus mutans* GS-5	Inhibited binding to saliva-coated hydroxyapatite	Vacca-Smith *et al.* 1994
Mucin Sialic acid motif (NeuAcα2-3)	S-fimbriated *E. coli*	Inhibited binding to buccal epithelial cells	Schroten *et al.* 1992a, 1992b, 1993
Mucin MUC 1 and MUC 4	*S. enterica* (Typhimurium)	Inhibited invasion of Caco-2 (human epithelial colorectal adenocarcinoma) cells and human fetal small intestine cells (FHs 74 Int)	Liu B *et al.* 2012
Mucin Sialic acid-containing glycans	RV	Inhibited infection of MA-104 (African green monkey kidney) cells, virus replication in tissue, and gastroenteritis in mice	Yolken *et al.* 1992
Mucin Secretor and Lewis epitopes (fucosyl side chains)	Norovirus (NV)	Blocked the adhesion of recombinant norovirus-like particles to saliva	Jiang *et al.* 2004

Glycoprotein (binding epitope if known)	**Target**	**Experimental evidence**	**Reference**
MUC1 Lewis X Galβ1-4(Fucα1-3)GlcNAc	HIV (gp120 envelope protein)	Blocked interaction with dendritic cells, and transmission from dendritic cells to CD4+ T cells	Saeland *et al.* 2009
Bile salt-stimulated lipase Glycan involvement unknown	*Giardia lamblia*	Induced swelling and lysis of trophozoites	Gillin *et al.* 1983a, 1983b
Bile salt-stimulated lipase α1-2-linked fucose	NV	Inhibited the attachment of recombinant norovirus-like particles to saliva	Ruvoën-Clouet *et al.* 2006
Bile salt-stimulated lipase Lewis X Galβ1-4(Fucα1-3)GlcNAc	HIV-1	Bound to DC-SIGN on dendritic cells. Prevents transfer of HIV-1 transfer to CD4+ T cells	Naarding *et al.* 2005, 2006
Bile salt-stimulated lipase	*Streptococcus mutans*	Inhibited binding to saliva- and gp340 (salivary agglutinin)-coated hydroxyapatite	Danielsson Niemi *et al.* 2009
Lactadherin Sialic acid-containing glycans	RV	Inhibited infection of MA-104 (African green monkey kidney) cells, virus replication in tissue, and gastroenteritis in mice	Yolken *et al.* 1992

Glycoprotein (binding epitope if known)	Target	Experimental evidence	Reference
Lactadherin	RV	Decreased incidence of severe diarrhoea in infected infants fed with human milk containing high levels of lactadherin	Newburg *et al.* 1998
Lactadherin	RV	Pre-incubation prevented rotavirus infection of Caco-2 (human epithelial colorectal adenocarcinoma) cells	Kvistgaard *et al.* 2004

Abbreviations: CD4+, cluster of differentiation 4; CMV, cytomegalovirus; EPEC, enteropathogenic *Escherichia coli*; ETEC, enterotoxigenic *E. coli*; DC-SIGN, dendritic cell-specific ICAM 3-grabbing non-intergrin; gp, glycoprotein; HSV, herpes simplex virus; HEL, human embryonic lung cells; HIV, human immunodeficiency virus; κ-CN, kappa-casein; NV, norovirus; RSV, respiratory syncytial virus; RV, rotavirus; sIgA, secretory immunoglobulin A.

As shown in Table 1.6, the main human milk glycoproteins that have been implicated in inhibiting pathogen adhesion to host cells are sIgA, lactoferrin, κ-casein, and glycoproteins from the MFGM (mucins, BSSL and lactadherin). The role that the glycan moieties of these glycoproteins may play in inhibiting pathogen adhesion is discussed below. Comparative studies on the corresponding bovine milk glycoproteins are included where available.

Secretory IgA (sIgA)

The antibody sIgA is known to pass through the digestive tract and bind to a range of pathogens via numerous glycan structures that adorn the sIgA protein backbone. Examples include fucosylated epitopes that bind to *Helicobacter pylori* and prevented adhesion to gastrointestinal mucosa cells (Falk *et al.* 1993), high mannose structures which bind to type 1 fimbriae of *Escherichia coli* and prevented *E. coli* adhesion to human colonic epithelial cells (Wold *et al.* 1990) and sialylated receptors containing Neuα2-3Gal antigens that bind to S-fimbriated of *E. coli* thereby reducing adhesion to buccal epithelial cells (Schroten *et al.* 1998). The protective value of sIgA has also been reported by Cruz *et al.* (1988), in which children infected with the toxin-producing *E. coli* remained asymptomatic when fed breast milk containing high levels of sIgA, whereas infants receiving breast milk with significantly lower levels of sIgA developed gastroenteritis.

Lactoferrin (LF)

Human milk lactoferrin (hLF) is also adorned with sialylated and fucosylated *N*-linked glycans, and has been shown to inhibit the binding of *Salmonella typhimurium* (Bessler *et al.* 2006), *Shigella flexneri* (Gomez *et al.* 2003) and EPEC to human colon epithelial cells (de Araújo and Giugliano 2001) and inhibited ETEC-induced haemagglutination of human erythrocytes *in vitro* (Giugliano *et al.* 1995), although the specific glycan moieties involved were not determined. In a more recent study, terminal fucose residues of hLF were identified as critical to the inhibition of *Salmonella enterica* Typhimurium adhesion to human colon epithelial cells (Barboza *et al.* 2012).

The potential for the glycan moieties of bovine milk lactoferrin (bLF) for pathogen inhibition has not been reported. However, it is known that the glycosylation of bLF is substantially different to hLF, with bLF carrying predominantly high mannose structures (Man_{5-9}; Hurley and Rejman 1993; Hurley *et al.* 1993), and hLF carrying highly sialylated and fucosylated complex or hybrid glycans (Barboza *et al.* 2012; Lönnerdal 2003; Picariello *et al.* 2008). It seems quite possible that the different glycosylation of hLF and bLF may affect the ability of

the glycoprotein to act as an effective decoy for pathogen attachment, but this is yet to be experimentally investigated.

Kappa-casein (κ-CN)

The only glycosylated form of casein in human milk is κ-CN, which has numerous *O*-glycosylation sites in the C-terminal portion of the molecule (Fiat *et al.* 1980). Human milk κ-casein has an inhibitory effect on the adhesion of pathogens to the gut and respiratory cell surfaces of the infant. For example, κ-CN inhibited the adhesion of *H. pylori* to human gastric mucosa, and of *Streptococcus pneumoniae* and *Haemophilus influenzae* to human respiratory tract epithelial cells via GlcNAcβ1-3Gal moieties (Aniansson *et al.* 1990). Binding of the oral pathogen *Streptococcus mutans* to saliva-coated hydroxyapatite was also prevented in the presence of κ-CN, an interaction that relied on the presence of sialic acid residues (Vacca-Smith *et al.* 1994).

As κ-CN is present in higher quantities in bovine milk (3.2 g/l) than in human milk (0.4 g/l; Table 1.3), some studies have investigated the relative ability of the bovine and human milk κ-CN for the prevention of pathogen adhesion. The Lewis blood group antigen (Le^b) containing terminal fucose on human κ-CN effectively inhibited the adhesion of *H. pylori* to stomach tissue from a healthy human adult better than κ-CN purified from bovine milk that does not contain this antigen (Strömqvist *et al.* 1995). However, casein from bovine colostrum had a similar ability to inhibit *S. pneumoniae* and *H. influenzae* adhesion to human respiratory tract epithelial cells as casein from human colostrum, although casein from bovine milk was less effective (Aniansson *et al.* 1990).

Glycoproteins from the milk fat globule membrane (MFGM)

Mucins (MUC)

Mucin-mediated adhesion of bacteria to host epithelial cells has been attributed by the highly sialylated *O*-linked glycoprotein MUC1 in the MFGM (Peterson *et al.* 2001). This heavily glycosylated protein of human milk was able to inhibit the binding of S-fimbriated *E. coli* to human buccal mucosal in the mouth (Schroten *et al.* 1992a, 1992b). MUC1 and MUC4 from human MFGM were shown to inhibit *S. enterica* serovar Typhimurium (SL1344) invasion of human intestinal epithelial cells *in vitro* (Liu B *et al.* 2012) with MUC1 inhibiting invasion more strongly than MUC4; however, the specific involvement of the mucin glycans in the inhibition of bacterial invasion was not examined. Experimental support for the potential of human milk mucins to prevent viral gastrointestinal infection has also accumulated,

implicating sialylated and fucosylated structures in the adhesion and infection process of rotavirus and norovirus, respectively (Jiang *et al.* 2004; Yolken *et al.* 1992). Little research has been carried out on mucins from bovine milk; however, in one study, bovine MUC1 inhibited the binding of *E. coli* and *S. typhimurium* to Caco-2 cells grown *in vitro* (Parker *et al.* 2010).

Bile salt-stimulated lipase (BSSL)

The Le^x epitope conjugated to BSSL has been identified as the key glycan on a minor fucosylated glycoprotein component in human milk that can bind to dendritic cell-specific ICAM3-grabbing non-integrin (DC-SIGN) to prevent the transfer of human immunodeficiency virus type 1 (HIV-1) gp120 envelope protein to CD4+ T lymphocytes (Naarding *et al.* 2005, 2006). BSSL from the milk of women with the secretor blood phenotype (producing α1,2-fucose-containing glycans) also inhibited the attachment of recombinant norovirus-like particles to saliva or synthetic H-type 1 oligosaccharides, suggesting that α1-2-linked fucose residues could act as decoy receptors (Ruvoën-Clouet *et al.* 2006). BSSL was also found to inhibit the binding of the oral pathogen *S. mutans* to saliva and salivary agglutinin gp340-coated hydroxyapatite (Danielsson Niemi *et al.* 2009).

Lactadherin

The anti-pathogenic properties of lactadherin for the human infant have been mainly associated with prevention of rotavirus infection. Work carried out by Yolken *et al.* (1992) revealed that a mucin-associated 46 kDa glycoprotein (later designated as lactadherin) inhibited rotavirus infection and sialic acid was the key determinant in the interaction. Later work (Kvistgaard *et al.* 2004), revealed that lactadherin inhibited rotavirus infection of human colon epithelial cells only when incubated with the virus prior to application to the cells, suggesting the effect was due to an inhibitory effect on binding.

1.6 Milk glycolipids

1.6.1 Synthesis and structural features of glycans on human milk glycolipids

After lactose, lipids are the second largest component of human milk (41 g/l; Table 1.3); however, only about 1 % of the human milk lipids are glycosylated. Glycolipids contain glycans that are covalently attached to a ceramide (Cer) lipid core. Ceramide is composed of an 18 carbon sphingosine base and an amide-linked acyl group (Dickson 1998; Varki *et al.* 2009a). Glucosylceramide (GlcCer) is synthesised on the cytoplasmic face of the ER from ceramide and UDP-Glc by UDP-glucose ceramide glucosyltransferase. Further extensions are

made in the Golgi via a series of glycosyltransferases which can add one or more sialic acid and/or galactose or GalNAc residues (Figure 1.6). The most abundant form of glycolipid in human milk are the sialic acid-containing glycosphingolipids, known as gangliosides, which are found exclusively in the MFGM (Keenan and Patton 1995). The hydrophobic ceramide portion of the molecule embeds in the lipid bilayer of the membrane, leaving the glycan chains exposed to the exterior (Pandit and Scott 2007).

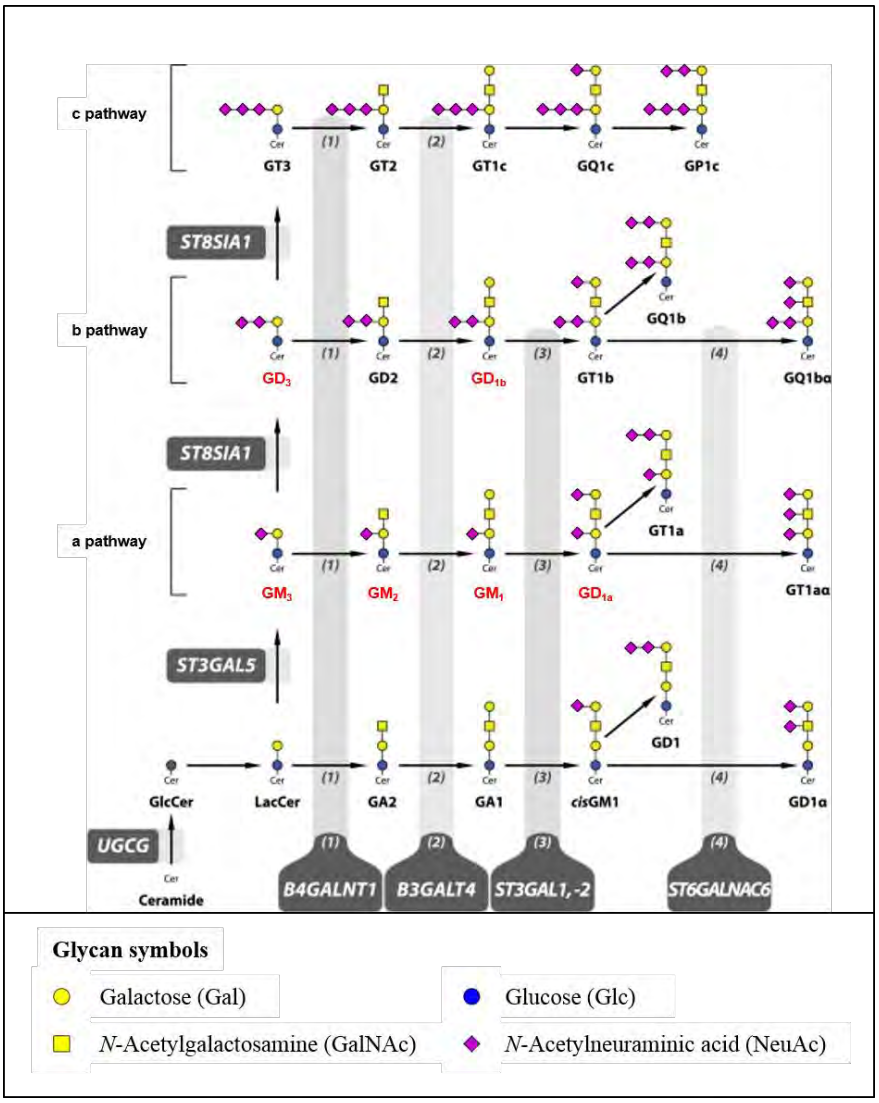


Fig. 1.6. Pathway in glycolipid biosynthesis. Glycolipids reported in human milk are indicated in red font. Gangliosides contain highly sialylated structures that are bound to the Galβ1-3GalNAcβ1-4Galβ1-4GlcβCer core. The first step in the synthesis pathway is the addition of a glucose residue to a ceramide, catalysed by ceramide glucosyltransferase. As the synthesis progresses, a β4-Gal is added to create lactosylceramide (LacCer). This galactose residue may hold a branch of sialic acid residues via a stepwise addition of the monosaccharides by sialyltransferases. Acidic glycolipids: $GD_{1\alpha}$, GD_{1a}, GD_{1b}, GD_2, GD_3, disialylogangliosides; GM_1, GM_2 and GM_3, monosialogangliosides; GP_{1c}, pentasialylogangloside; $GQ_{1b\alpha}$, GQ_{1b}, GQ_{1c}, tetrasialylogangliosides $GT_{1a\alpha}$, GT_{1b}, GT_{1c}, GT_2, GT_3, trisialylogangliosides. Neutral glycolipids: GA_1, GA_2. Figure taken from Potapenko *et al.* 2010.

1.6.2 A comparison of human and bovine milk glycolipids

The major human milk gangliosides include the monogangliosides (GM_1, GM_2 and GM_3) and the disialoganglioside (GD_3; Figure 1.6). GD_3 is the major ganglioside in colostrum (~65 % of total gangliosides; Takamizawa *et al.* 1986), but decreases in quantity during lactation (~25 % of total gangliosides in mature human milk). In contrast, GM_3 increases throughout the lactation period and is the major ganglioside in mature milk (~74 % of total gangliosides; Rueda 2007). Other minor glycolipid components of human milk include the acidic glycolipids GM_2 (~2 %) and GM_l (~0.l %) (Laegreid *et al.* 1987) and the neutral glycolipid Gb_3 (~2 %; Newburg and Chaturvedi 1992).

Human and bovine milk have a relatively similar total lipid concentration (41 g/l and 37 g/l, respectively; Table 1.3) and, similar to human milk, only about 1 % of bovine milk lipids are glycosylated. However, the relative proportions of the major glycolipids vary between the two types of mammalian milk. While GM_3 is the major ganglioside in human milk, GD_3 is the predominant ganglioside in bovine milk (60 - 70 %; Puente *et al.* 1992) and GM_3 and GT_3 are present in much lower amounts (Nakano *et al.*, 2001; Pan and Izumi 2000).

1.6.3 Evidence that glycans on milk glycolipids may influence pathogen adhesion

The fact that glycolipids are present in such low concentrations in human and bovine milk may be a contributing factor to the relatively small amount of research that has investigated the role milk glycolipids may play in preventing pathogen adhesion to host cells. However, several studies on human milk glycolipids have taken place, as summarised in Table 1.7 (and Table 2, Publication I, Appendix B). The few studies that have been conducted on bovine milk glycolipids for the inhibition of pathogen adherence to the gastroinestial tract are described in the text below.

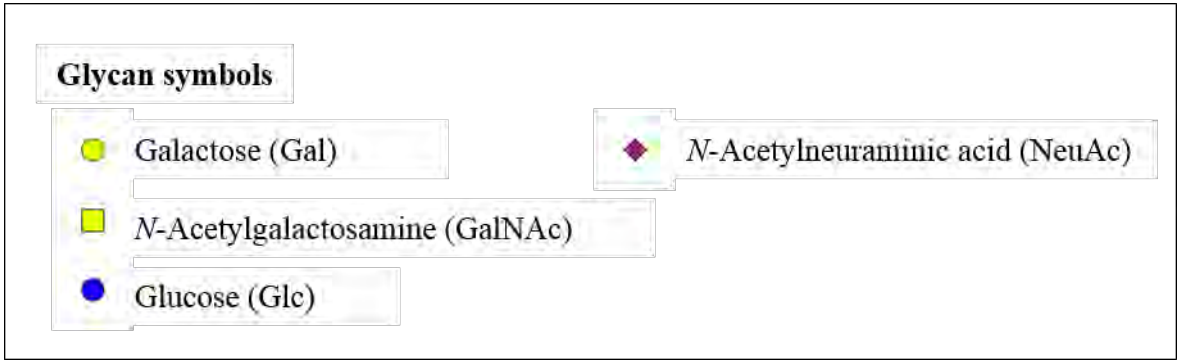
Table 1.7. Human milk glycolipids that may inhibit the adhesion of pathogens and/or their toxins to human epithelial cells

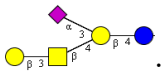
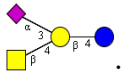
Glycolipid (Binding epitope)[a]	Target	Experimental evidence	Reference
GM_1	Enterotoxigenic *Escherichia coli* (ETEC)	Inhibited binding to Caco-2 (human epithelial colorectal adenocarcinoma) cells - 80% inhibition	Idota and Kawakami 1995
GM_1	*E. coli* heat-labile enterotoxin (LT); *Vibrio cholerae* cholera toxin	Inhibited LT binding to ELISA plates, and reduced the *in vivo* effects of cholera toxin in rabbit intestinal loop experiments	Otnaess *et al.* 1983; Laegreid and Kolstø Otnaess 1987
GM_1, GM_2	*Helicobacter pylori* vacuolating toxin (VacA)	Lyso-GM_1 and lyso-GM_2 neutralized vacuolation activity in AZ-521 (human epithelial gastric carcinoma) cells	Wada *et al.* 2010
GM_2	Human respiratory syncytial virus (RSV)	Inhibited absorption of RSV into HEp-2 (human epithelial laryngeal carcinoma) cells	Portelli *et al.* 1998
GM_3	Enterotoxigenic *E. coli* (ETEC)	Inhibited binding to Caco-2 (human epithelial colorectal adenocarcinoma) cells - 69 % inhibition	Idota and Kawakami 1995
GM_1, GM_3, GD_3, NeuAc	*Campylobacter jejuni*, *Listeria monocytogenes*, *Salmonella*	Inhibited binding to Caco-2 (human epithelial colorectal adenocarcinoma) cells	Salcedo *et al.* 2013

Glycolipid (Binding epitope)[a]	Target	Experimental evidence	Reference
	enterica (Typhi), *Shigella sonnei*, *H. pylori*		
GD_3	Enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC)	Inhibited binding to Caco-2 (human epithelial colorectal adenocarcinoma) cells - 16% inhibition	Idota and Kawakami 1995
Gb_3	*Shigella dysenteriae* Shiga toxin	Bound to Shiga toxin in a solid-phase binding assay	Newburg *et al.* 1992; Gallegos *et al.* 2012
NeuAcα2-3Gal, NeuAcα2-6Gal	Enterovirus 71 (EV71)	Inhibited infection of DLD-1 (human epithelial colorectal adenocarcinoma) cells	Yang *et al.* 2009
Sulfated glycolipids-Sulfatide ceramide, Sulfated lactosyl	HIV gp120	Inhibited the binding of recombinant HIV surface glycoprotein gp 120 in cultured human colonic and vaginal epithelial cells	Newburg and Chaturvedi 1997

Abbreviations: GD_3, disialylloganglioside; EPEC, enteropathogenic *Escherichia* coli; ETEC, enterotoxigenic *E. coli*; EV71, Enterovirus 71; Gal, galactose; GM_1, GM_2, GM_3, monosialogangliosides; Gb_3, globotriaosylceramide; ELISA, enzyme-linked immunosorbent assay; LT, heat-labile enterotoxin; HIV gp120, human immunodeficiency virus glycoprotein 120; RSV, respiratory syncytial virus; NeuAc, *N*-acetylneuraminic acid; VacA,

vacuolating cytotoxin.



[a]Structures: GM1  ; GM2  ; GM3  ; GD3  ; Gb3 

The sialylated human milk gangliosides have been implicated in numerous studies to have protective effects against the adhesion of gastrointestinal pathogens to intestinal epithelial cells (Table 1.7). GM_1 and GM_3 inhibited the binding of enterotoxigenic *Escherichia coli* (ETEC) to human colon cells (Idota and Kawakami 1995), and showed specific inhibitory activity to enterotoxins from both *E. coli* and *Vibrio cholerae* (Laegreid and Kolstø Otnaess *et al.* 1987; Otnaess *et al.* 1983). Furthermore, GM_1 and GM_2 significantly neutralised the activity of *H. pylori* vacuolating cytotoxin (VacA) in human gastric epithelial cells (Wada *et al.* 2010), and GD_3 inhibited the binding of both ETEC and EPEC to human colon cells (Idota and Kawakami 1995). Gb_3 was found to be a receptor for both Shiga toxins (Stx1 and Stx2) and may contribute to the protective effect of human milk against infantile diarrhoea associated with these toxins (Herrera-Insua *et al.* 2001; Newburg *et al.* 1992). Further studies indicated that Stx1 bound primarily to the glycan structure, but Stx2 only bound to the lipid conjugated Gb_3 (Gallegos *et al.* 2012).

Glycolipids from human milk also appear to have inhibitory activity against the binding and invasion of human cells by viruses *in vitro* (Table 1.7). GM_2 exhibited antiviral activity against human respiratory syncytial virus (RSV) in cell culture (Portelli *et al.* 1998). Sialylated human milk glycolipids with NeuAcα2-3Gal and NeuAcα2-6Gal moieties prevented enterovirus 71 (EV71) infections of human epithelial colon cells (Yang *et al.* 2012), and the sulfated glycolipids sulfatide and sulfated lactosyl ceramide inhibited the binding of recombinant HIV surface glycoprotein (gp120) in human colonic and vaginal epithelial cell lines (Newburg and Chaturvedi P 1997).

Few studies have investigated the ability of bovine milk glycolipids to inhibit pathogen adhesion to host cells. In one study, bovine milk gangliosides were compared to human milk gangliosides for their inhibitory activity against the binding of EPEC to Caco-2 cells (Idota and Kawakami 1995). While the human milk gangliosides inhibited binding, the bovine gangliosides did not, indicating that the dominant bovine ganglioside GD_3 is not biologically active against this pathogen. In comparison, ETEC bound to GM_3 and GD_3 from bovine milk and inhibited bacterial hemagglutination (Sánchez-Juanes *et al.* 2009). Furthermore, the galactose-containing GM_1 from bovine milk and skim milk powder, has been identified to inhibit the binding of cholera toxin, and *E. coli* heat-labile enterotoxin of serogroup I (Becker *et al.* 2010).

1.7 Intestinal microflora in infants

The gastrointestinal tract (GIT) of the foetus has been described to be extremely sterile until birth. However, after birth, the newborn's GIT becomes quickly colonised by the mother's intestinal flora. Subsequently, the composition of the intestinal flora of the infant is largely influenced by the dietary intake and the environment. For example, breast-fed infants usually acquire a flora rich in *Bifidobacterium*. Less abundant anaerobes include *Clostridium* and *Bacteroides* species, as well as *Enterobacteria* and *enterococci*. On the other hand, non-breastfed infants are colonised by a more complex microflora with high numbers of *Bacteroides, Bifidobacteria*, *Clostridium, Enterobacteria* and *enterococci*. Within days or weeks, a consistent number of *E. coli* and streptococci become the first colonisers of the infant GIT , where bacterial numbers can reach 10^8 to 10^{10} per gram of faeces (Mountzouris *et al.* 2003). *Lactobacillus* species have also been isolated from infant faeces (Panwar *et al.* 2014), and form part of the healthy gut microbiome into adulthood.

Infection by gastrointestinal pathogens disrupts the healthy microbial balance in the GIT and results in gastrointestinal infection and diarrhoea. This literature review has suggested that *C. jejuni*, *E. coli* and *Salmonella* species are the major bacterial causes of gastrointestinal infections throughout the world, particularly in children (Table 1.2). Consequently, these potential pathogens were chosen for experimental work carried out for this thesis in which their binding to milk proteins, and their conjugated glycans, was investigated. In addition, the binding of *Lactobacillus rhamnosus*, was measured in parallel to provide a comparison of adhesion by commensals.

1.8 Chapter summary and project aims

The information presented in this chapter has revealed that human milk is a complex food that provides nutrition and many bioactives to give protection for the growing infant from disease. Bovine milk, which is used in formulas as an alternative or supplement to human milk, has a significantly different composition to human milk and does not confer the same level of disease prevention. It is evident that one, less explored, aspect that distinguishes human and bovine milk is the diversity of glycan structures that are present in the two forms of milk.

A considerable amount of research suggests that HMOs may bind to and remove pathogens by virtue of their glycan structures that appear to mimic binding receptors on epithelial cells in the gastrointestinal tract (Table 1.5). It is also probable that glycans bound to milk proteins and lipids could act by a similar mechanism. Furthermore, the conformation of the conjugated

glycans may present pathogens with more effective binding epitopes than free oligosaccharides. In agreement with this concept, research is beginning to accumulate that implicates milk glycoproteins and glycolipids as inhibitors of pathogen adhesion to potential host cells (Tables 1.6 and 1.7, respectively). However, little is yet known about the specific glycan epitopes of the milk glycoconjugates that are critical to their ability to inhibit binding or invasion of gastrointestinal cells. Moreover, the comparative availabilities of specific conjugated glycans in human and bovine milk that can serve as decoys for pathogen attachment has not been fully explored.

The present study was undertaken to determine the role of the glycans on milk glycoconjugates in conferring innate immune protection to infants against gastrointestinal infections by measurement of the effectiveness of the binding to intestinal bacteria of different fractions of human and bovine milk. Since glycoproteins are much more abundant in both human and bovine milk (8.9 and 33 g/l, respectively) than glycolipids (0.41 and 0.37 g/l, respectively; Table 1.3), this thesis has a particular focus on milk glycoproteins rather than milk glycolipids in investigating the role of glycans in inhibiting pathogen infection. In addition, human intestinal cells were used as a model system to investigate the involvement of glycans in the adherence and invasion of gastrointestinal pathogens.

The more specific aims were:

1. To develop a quantitative assay to measure milk glycoprotein-bacterial interaction.
2. To demonstrate that the binding of human-specific gastrointestinal bacteria to human and bovine milk was glycan-dependent.
3. To show how glycans on different human and bovine milk fractions affect the attachment of human-specific gastrointestinal bacteria to their milk glycoprotein receptors.
4. To determine the effectiveness of the milk glycans released from human and bovine milk glycoproteins in inhibiting the adhesion and invasion of human-specific gastrointestinal bacteria into human intestinal epithelial cells.

Chapter 2. General materials and methods

Materials and methods that were used regularly throughout the work are described in this chapter (Figure 2.1). Any specific techniques developed or modified as part of this thesis are described in detail in the separate chapters (Table 2.1).

2.1 Bacterial strains and growth conditions

The bacterial strains used in this study included three human gastrointestinal pathogens, namely *Escherichia coli* O111:NM (ATCC 43887), *Salmonella typhimurium* (Group B clinical isolate, provided by Dr J Merlino, Concord Hospital, NSW, Australia) and *Campylobacter jejuni* NCTC11168 (provided by Professor HM Mitchell, University of New South Wales), one commensal gastrointestinal bacterium, *Lactobacillus rhamnosus* (ATCC 53103), and two human oral bacteria, the commensal *Streptococcus gordonii* F552 and pathogen *Streptococcus mutans* LT11.

Escherichia coli, *S. typhimurium* and *L. rhamnosus* were grown overnight at 37 °C in 5 ml of nutrient broth (Sigma-Aldrich, MO, USA) with orbital shaking at 200 rpm. *Streptococcus gordonii* F552 and *S. mutans* LT11 were grown overnight at 37 °C, 200 rpm, in 5 ml of brain-heart infusion broth (BHI; Oxoid, Cambridge, UK). The bacteria were harvested by centrifugation at 4000 × g for 3 min at room temperature (RT), washed twice with phosphate buffered saline (PBS) and resuspended in PBS prior to fluorescent labelling (Section 2.2).

Campylobacter jejuni NCTC11168 was grown on horse blood agar (HBA) derived from blood agar base #2, supplemented with 6 % (v/v) defibrinated horse blood (Oxoid Australia, Thebarton, SA). The inoculated agar plates were placed at 37 °C for 48 h under micro-aerobic conditions (5 % oxygen, 10 % carbon dioxide and 85 % nitrogen) in a sealed 2.5 l anaerobic jar (Oxoid Australia). The micro-aerobic conditions were generated by one sachet of CampyGen (Oxoid Australia), a small bag of silica gel beads and a piece of paper towel soaked with 10 ml of sterile Milli-Q water in the jar. *Campylobacter* cells were harvested from the agar plates, resuspended in 1 ml of PBS, pelleted by centrifugation at 12000 × g for 1 min at RT, and washed twice with 1 ml of PBS before fluorescent labelling.

2.2 Fluorescent labelling of whole bacterial cells

Bacteria were diluted to an OD_{600} of approximately 1.5 (10^6 - 10^7 bacterial cells per ml suspension). A volume of 1 ml of the 1.5 OD cells was pelleted then resuspended in 500 µl of

0.1 % (w/v) SYBR® Green I (S-7567, Invitrogen ™, Life Technologies, VIC, Australia) in PBS for labelling in the dark for 5 min. This nucleic acid stain was chosen because its excitation and emission maxima (Ex 497 nm and Em 520 nm, respectively) does not overlap with the excitation and emission maxima of milk protein autofluorescence (Ex 347 nm, Em 415 nm respectively; Ferrer *et al.* 2005). Bacterial cells were then washed with PBS and resuspended in 1 ml PBS prior to use in the binding assays, as described in Chapters 3 - 5.

2.3 Protein quantification and SDS-PAGE

Total protein concentrations of the samples were determined using the Bradford assay (Sigma-Aldrich, MO, USA) as per manufacturer's instructions. Measurements for three replicates per sample were represented as mean ± standard error of the mean (SEM).

Protein analysis by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using NuPAGE® Novex® 4 - 12 % gradient Bis-Tris gels (1.0 mm thick, 12-well; Invitrogen ™). Electrophoresis was carried out with a fixed voltage of 200 V for 45 min using NuPAGE® 3-(*N*-morpholino) propanesulfonic acid (MOPS) SDS running buffer (Invitrogen ™). To visualise the proteins in the gel after electrophoresis, the proteins were fixed with 10 % (v/v) methanol and 7 % (v/v) acetic acid for 1 h, stained overnight with SYPRO® Ruby Protein Gel Stain (Invitrogen ™) and rinsed in Milli-Q water. The gels were then destained with fixative buffer and the proteins visualised using a Typhoon Trio Variable Mode Imager (GE Healthcare, England, UK) with an emission filter of 610 nm, and bandpass filter of 30 nm. All gel images were scanned using 100 micropixel size with the photomultiplier tube (PMT) value adjusted between 500 V and 600 V.

2.4 Release of *N*- and *O*-linked glycans

BioTrace polyvinylidene difluoride (PVDF) membranes of an AcroWell™ 96-well membrane-bottom plate (Pall Corporation, NY, USA) were pre-wet with 100 % (v/v) methanol to activate the membrane then washed twice with Milli-Q water. Approximately 50 µl of milk proteins (1 mg/ml) were placed into each well and allowed to dry on the PVDF membrane. The membrane was then blocked with 100 µl of 1 % (w/v) polyvinylpyrrolidone (PVP) 40000 in 50 % (v/v) methanol, agitated for 5 min and washed three times with Milli-Q water to remove residual salts and detergents. *N*- and *O*-linked glycans of the milk glycoproteins were reduced and desalted as previously described (Jensen *et al.* 2012). Briefly, *N*-linked glycans were released by incubating for 15 min at 37 °C with 3 U of peptide-*N*-glycosidase F (PNG*ase* F, recombinant cloned from *Flavobacterium meningosepticum* and

expressed in *E. coli*, Roche Diagnostics, Basel, Switzerland). After the 15 min incubation, to avoid evaporation of samples, 10 µl of Milli-Q water was added to the sample wells and a small amount of Milli-Q water was also added to surrounding empty wells before sealing the plate tightly with parafilm and placing in a plastic bag containing a wet paper towel. The plate was incubated overnight at 37 °C.

To recover the *N*-linked glycans, the plate was sonicated in a water bath for 20 min and the *N*-linked glycans were collected in a new 1.5 ml microtube. Wells containing the released *N*-linked glycans were washed twice with 20 µl of Milli-Q water and the combined released *N*-linked glycans were incubated with 10 µl of 100 mM ammonium acetate pH 5.0 for 1 h at RT to remove the amines from the glycans prior to drying in a Savant SC110A SpeedVac® Concentrator (Thermo Scientific, MA, USA). The *N*-linked glycans were then reduced for 3 h with 20 µl of 1 M sodium borohydride in 50 mM potassium hydroxide at 50 °C and subsequently the reaction was quenched with 2 µl of glacial acetic acid. Remaining released *N*-linked glycans were collected from the sample wells with 2 × 20 µl of Milli-Q water. After the *N*-linked glycan release, *O*-linked glycans were released from the same glycoprotein spots by reductive β-elimination. Firstly, 2 µl of 100 % (v/v) methanol was briefly applied to the wells containing the glycoproteins to re-wet the PVDF membrane. Then 20 µl of 0.5 M sodium borohydride in 50 mM potassium hydroxide was added to each well and the plate was incubated at 50 °C overnight. The reaction was quenched with 2 µl of glacial acetic acid.

A clean up of the *N*- and *O*-linked glycan alditols to get rid of salts and detergents was carried out using handmade cation exchange columns consisting of 30 µl of AG50W-X8 cation-exchange resin (BioRad, CA, USA) packed into the top of Perfect Pure C-18 Tips (Eppendorf, Hamburg, Germany) (Schulz *et al.* 2002). Columns were prepared by washing three times with 1 M hydrochloric acid (50 µl) to remove salts and contaminants, followed by three washes of 100 % (v/v) methanol (50 µl) and another three washes of Milli-Q water (50 µl). The reduced *N*- and *O*-linked glycan alditols were then applied to the column and eluted twice with 50 µl of Milli-Q water and dried under vacuum using the Savant SC110A SpeedVac® Concentrator (Thermo Scientific). Residual borate was removed from the glycans by washing with 100 % (v/v) methanol (100 µl) for three to five times prior to drying the glycan samples again in the SpeedVac® Concentrator. Finally, all glycan samples were resuspended in 10 µl of Milli-Q water and stored at -20 °C prior to LC-ESI-MS analysis.

2.5 Mass spectrometry

The analysis of the released *N*- and *O*-linked glycans was carried out as described by Everest-Dass *et al.* (2012). Briefly, the glycan samples were applied to a Hypercarb porous graphitised carbon column (5 µm particle size, 180 µm I.D. × 100 mm, Thermo Hypersil, Thermo Fisher Scientific, MA, USA). The injection volume of each sample was 5 µl. The *N*-linked glycans were separated using a linear gradient with 0 - 45 % (v/v) acetonitrile in 10 mM ammonium bicarbonate for 85 min, whereas *O*-linked glycans were separated using a linear gradient with 0 - 90 % (v/v) acetonitrile in 10 mM ammonium bicarbonate for 45 min at a flow rate of 2 µl/min using an Agilent 6330 series ion-trap mass spectrometry (MS) instrument linked to an Agilent 1100 capillary liquid chromatography (LC; Agilent Technologies, CA, USA). LC-electron spray ionisation (ESI)-MS was performed in negative ion mode at full scan with mass range between *m/z* 200 and *m/z* 2200. The data was analysed using ESI-Compass 1.3 (Bruker Daltonics, MA, USA). Glycan compositions were predicted from the mono-isotopic masses of detected ions using GlycoMod software (http://web.expasy.org/glycomod/) with a mass tolerance of ± 0.5 Da.

Table 2.1. A list of specific techniques performed in this work.

Method	Chapter	Refer to Section
Preparation of glycan microarray to screen for specific bacterial binding epitopes	3	3.2.1
Preparation of polyvinylidene difluoride (PVDF) plate assay to measure bacterial binding to milk proteins	3, 4 and 5	3.2.2, 4.2.8 and 5.2.5.1
Techniques for milk protein precipitation	3	3.2.2.1
Isolation of total milk proteins by methanol/chloroform/water precipitation	3 and 4	3.2.2.1 and 4.2.2
Inhibition of bacterial binding to milk proteins with sialic acid	3 and 4	3.2.2.4 and 4.2.8.1
Neuraminidase treatment of milk proteins	3 and 4	3.2.2.5 and 4.2.8.2

Method	Chapter	Refer to Section
Mass spectrometric analysis of released *N*- and/or *O*-linked glycans from milk glycoproteins	3 and 5	3.2.2.6 and 5.2.6
Monosaccharide analysis of released *N*- and *O*-linked glycans from total human milk proteins	3	3.2.2.7
Fractionation of skim milk using Microflow MF10 system	4	4.2.4
Separation of whey proteins and caseins from bovine and human skim milk by high-speed centrifugation	4 and 5	4.2.5 and 5.2.1
Fast protein liquid chromatography (FPLC) gel filtration of the bovine and human milk whey proteins	4	4.2.6
Preparative release of *N*-linked glycans from bovine and human milk whey proteins	5	5.2.2
Culturing HT-29 cells for cell-based assay	5	5.2.4
PVDF plate assay for measuring bacterial interaction with bovine and human milk glycoproteins and the inhibition of binding by *N*-linked glycans from milk whey proteins	5	5.2.5.1
Cell-based assay for measuring bacterial adherence and invasion of HT-29 cells	5	5.2.5.2
Monosaccharide analysis of released *N*-linked glycans from bovine and human milk whey proteins	5	5.2.7, detailed in 3.2.2.7
Membrane preparation of HT-29 cells prior to glycan analysis	5	5.2.8
PNG*ase* F treatment of HT-29 cell membrane proteins to release *N*-linked glycans for mass spectrometry analysis	5	5.2.9

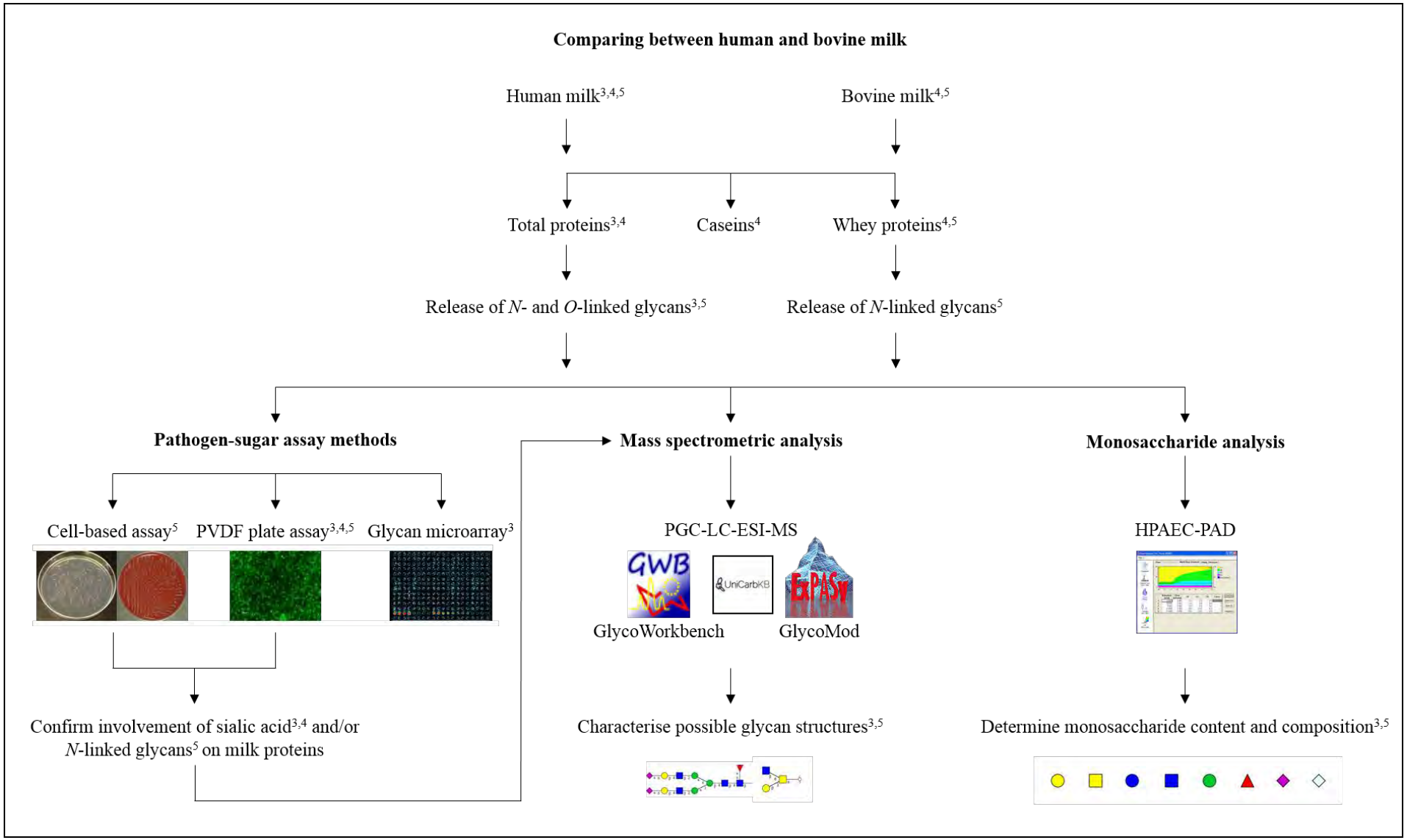

Comparing between human and bovine milk
Human milk[3,4,5]
Bovine milk[4,5]
Total proteins[3,4]
Caseins[4]
Whey proteins[4,5]
Release of N- and O-linked glycans[3,5]
Release of N-linked glycans[5]
Pathogen-sugar assay methods
Mass spectrometric analysis
Monosaccharide analysis
Cell-based assay[5]
PVDF plate assay[3,4,5]
Glycan microarray[3]
PGC-LC-ESI-MS
GWB
UniCarbKB
ExPASy
GlycoWorkbench
GlycoMod
HPAEC-PAD
Confirm involvement of sialic acid[3,4] and/or N-linked glycans[5] on milk proteins
Characterise possible glycan structures[3,5]
Determine monosaccharide content and composition[3,5]

Fig. 2.1. The general workflow used in this thesis to study the sugar-pathogen interaction. [3,4,5]Superscript numbers refer to the chapters of this thesis, in which the specific techniques were developed, used or modified.
Abbreviations: HPAEC-PAD, high-performance anion-exchange chromatography with pulsed amperometric detection; PGC-LC-ESI-MS, porous graphitised carbon-liquid chromatography-electronspray ionisation-mass spectrometry.

Chapter 3. Measuring sugar-pathogen interactions

Rationale

The aim of this chapter was to study the interaction of sugars (glycans) with different strains of bacteria. Two methods were used. The first method developed by the Institute for Glycomics at Griffith University (Gold Coast, Australia) employed a glycan microarray technology that uses a variety of synthetic glycan structures covalently immobilised onto activated glass slides to screen for specific glycan structures recognised by bacterial strains. The second method involved using a 96-well plate, in which total proteins precipitated from human skim milk were immobilised onto polyvinylidene difluoride (PVDF) membranes and the binding of these glycoproteins to bacterial strains can be quantified.

The role of glycosylation of human milk glycoproteins in sugar-pathogen interaction, particularly the involvement of sialic acid, was examined by testing the effect of pre-incubation with free sialic acid, or removal of terminal sialic acids on the immobilised human milk glycoproteins using α2-3,6,8 neuraminidase, on inhibiting the bacterial binding. The activity of the enzyme was verified by mass spectrometry.

To determine the glycan sub-structures that presented the sialic acids as receptors of the bacterial binding, a structural analysis of the released *N*-linked glycans from human milk glycoproteins was also carried out using porous graphitised carbon liquid chromatography-electrospray ionisation mass spectrometry (PGC-LC-ESI-MS) and high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD).

3.1 Introduction

It is known that human milk protects breastfed infants against infections, as outlined in Chapter 1 (Table 1.1). Bacteria can enter the human body through various sites (Jones and Isaacson 1983). The infection process is commonly mediated by adhesins expressed on the bacterial cell surface, which interact with specific host cell surface receptors (Kelly and Younson 2000; Sakarya and Öncu 2003). To protect against infections, the human body employs several defence mechanisms against invading bacteria. As part of the innate immune system, the human body secretes several types of fluids, such as tears (Ben Bacha and Abid 2013; Franklin 1989), mucous (Jackson 2001; Kaliner 1992), saliva (Fábián *et al.* 2012; Tenovuo *et al.* 1986) and sweat (Rieg *et al.* 2005) have anti-adhesive properties, suggesting they may help to prevent the adherence of pathogen binding to epithelial surfaces in the eyes, nose, mouth and skin respectively. There is significant evidence that the drinking of human milk could also prevent the adhesion of pathogens to the gastrointestinal tract of breastfed infants with a similar washing effect (Cederlund *et al.* 2013; Newburg 2005, 2009). Numerous studies have indicated that free oligosaccharides of human milk can promote or inhibit the adhesion of pathogenic bacteria to the gastrointestinal tract of infants, but the protective roles of the large repertoire of complex glycans attached to human milk proteins (glycoproteins) are not well-understood (reviewed in Table 1.6, Chapter 1). It is therefore important to identify the glycan structures on human milk glycoproteins that serve as a first line of defence against the binding of pathogenic bacteria.

Several studies have also indicated that human milk glycoproteins, similar to the innate protection provide by salivary glycoproteins, may also contribute protection against infection in the oral cavity, that is the first component of the gastrointestinal tract. This is achieved by the binding of oral pathogens to the human milk glycoproteins, instead of to the epithelial surfaces in the mouth. For example, several glycoproteins in human milk, including lactoferrin, IgA, IgG, α-lactalbumin and bile salt-stimulated lipase, have been implicated in inhibiting the binding of *Streptococcus mutans* to saliva-coated hydroxyapatite (S-HA) (Danielsson Niemi *et al.* 2009; Wernesson *et al.* 2006). Additionally, bovine milk proteins have been demonstrated to inhibit the adhesion of *S. mutans* to salivary components (Oho *et al.* 2004; Schüpbach *et al.* 1996; Vacca-Smith *et al.* 1994). It has been shown that the Lewis a-antigen (Le^a, Galβ1-3(Fucα1-4)GlcNAc) on salivary agglutinin (SAG) plays a role in binding of *S. mutans* (Ligtenberg *et al.* 2000). Milk glycoproteins including lactoferrin, containing *N*-linked glycans with α2-6 NeuAc linkages, were found to play a role in binding the S-fimbriae of *Escherichia coli* HB101 (Schwertmann *et al.* 1999). Information on the interactions of the

oral colonisers, streptococci, suggests that their interaction with salivary glycoproteins is sialic acid-dependent (Murray *et al.* 1992; Plummer and Douglas 2006). Thus, it is interesting to study the mechanisms by which gastrointestinal and oral pathogens interact with glycans on human milk glycoproteins that may offer novel anti-microbial therapeutics to control infections.

Pathogen-sugar assay methods

To study the interaction between microorganisms and host cell surface receptors, several methods have been previously developed, e.g haemagglutination inhibition assays (Beachy *et al.* 1981) and thin layer chromatography overlay assays (Prakobphol *et al.* 1995), and the more recent bead-based flow cytometry immunoassays (Park *et al.* 2000) and microtitre plate binding assays (Acord *et al.* 2005; Bosch *et al.* 2003). Labelling of the bacteria is frequently used in such assays to measure the adhesion to host receptors, mainly for rapid throughput visualisation of binding and comparison of different binding specificities by individual bacterial strains (Vesterlund *et al.* 2005). These labelling techniques include radioactive labelling (Cannon *et al.* 1995), fluorescent labelling (Acord *et al.* 2005, Hytönen *et al.* 2006) antibody tagging (McArthur *et al.* 2007) and nucleic acid staining (Bosch *et al.* 2003). Staining the nucleic acid of bacteria with a fluorescent probe prior to bacterial adhesion was chosen for this study as it does not interfere with bacterial surface molecules that may be involved in bacterial binding, and it is cost effective and easy to use (Fuller *et al.* 2000; Logan *et al.* 1998). Microscopic observation of the interactions of bacteria with S-HA, for example, has been the adopted method to study the inhibitory effects of human milk on adhesion of *S. mutans* (Danielsson Niemi *et al.* 2009; Hajishengallis *et al.* 1992; Vacca-Smith *et al.* 1994; Wernesson *et al.* 2006), although it is difficult to obtain a quantitative measure by this approach (Cannon *et al.* 1995).

Other approaches described to measure bacterial binding include a BIAcore binding assay that is based on the principle of surface plasmon resonance (SPR). This is achieved by immobilising the ligand onto a sensor surface, where the refractive index near the sensor surface is measured in real time by the accumulation of protein on the sensor surface as the analyte binds to the ligand. Although this system has been applied to screen for the binding properties of probiotic strains to soluble human colonic mucin (sHCM), before and after removal of sialic acid as well as sulphate residues (Huang *et al.* 2013), this method is not recommended for high throughput applications.

Glycan microarrays are emerging as useful screening tools to identify and evaluate the binding specificity of carbohydrate binding proteins (CBPs), including animal and plant lectins, antibodies, toxins and pathogens, such as bacteria and viruses (Heimburg-Molinaro *et al.* 2012; Leppänen and Cummings 2010, Song *et al.* 2009; Tao SC *et al.* 2008) with small quantities of defined glycan structures (pico- to nanogram) on the array. The major advantage of this solid phase approach is that it allows organisms to bind to hundreds of unique glycan structures. Therefore, multiple screening can be performed simultaneously on individual arrays, providing a wide range of glycan structures to which these organisms bind (Lonardi *et al.* 2010). Table 3.1 summarises the advantages and limitations of different applications used to study bacteria-glycan interactions.

Table 3.1. Advantages and disadvantages of technologies available to study bacteria-glycan interactons.

Technology	Advantage	Disadvantage
BIAcore binding assay	Rapid identification and quantification of bacteria-glycan interactions	Not recommended for high throughput applications as expensive equipment Special knowledge needed
Glycan microarrays	Allows organisms to bind to hundreds of unique glycan structures Multiple screening can be performed simultaneously on individual array Sensitive	Not recommended for high throughput applications as expensive equipment Special knowledge needed
Haemagglutination inhibition (HAI) assays	Technically simple and easy to automate Provides visualisation of binding and comparison of different binding specificities by individual bacterial strains	Poor reproducibility Requires considerable experience evaluating antibody response to infection
Flow cytometry immunoassays	Measures distribution of bacterial bindng to glycans	Not recommended for high throughput applications as expensive equipment Special knowledge needed

Technology	Advantage	Disadvantage
Microtitre plate -Binding assay by staining the nucleic acid of bacteria	Direct visualisation of bacteria-glycan interactions Does not interfere with bacterial surface molecules Cost effective and easy to use	Possible cross-well contamination during washing step Microtitre plates cannot be reused
Saliva-coated hydroxyapatite (S-HA)	Simple setup with little preparation required Provides direct visualisation of bacteria-glycan interactions	Not ideal for thick samples that may appear distorted Difficult to obtain a reliable quantitative measurement
Thin layer chromatography (TLC) overlay assays	Simple and rapid method Rapid visualisation of several samples Do not need expensive equipment	Sample must be homogenised and extracted before analysis

In the present study, two high throughput, fluorescence-based methods were used to study sugar-pathogen interactions. The glycan microarray technology was first trialled to identify whether specific glycan structures were recognised by three human gastrointestinal bacterial strains, the opportunistic *Escherichia coli*, pathogenic *Salmonella typhimurium* and the commensal *Lactobacillus rhamnosus*. The second 96-well microplate method was developed to investigate the multivalent interactions of the different human-specific bacterial strains with total human milk proteins immobilised on polyvinylidene difluoride (PVDF) membranes. In addition to the above strains, the binding of *Campylobacter jejuni* was assayed based on its potential to cause infectious diarrhoea in human infants (Kotloff *et al.* 2013), and the adhesion of two oral bacteria involved in the development of dental caries in humans, the commensal *Streptococcus gordonii* and pathogen *S. mutans*, were also measured.

This assay was also used to investigate the role of sialic acids in mediating the interaction of bacteria with human milk glycoproteins. This was tested by pre-incubating the bacteria with sialic acid before bacterial binding and washing the bound bacteria with sialic acid after bacterial binding. Terminal sialic acid residues on human milk glycoproteins were also removed by α2-3,6,8 neuraminidase prior to the binding of bacteria to further investigate the involvement of sialic acid residues of human milk glycoproteins in this binding.

Characterisation of glycan structures

To understand the structural complexity, in addition to sialylation, of the glycans on the milk glycoproteins that may be binding the bacteria, the profile of the *N*-glycosylation of the conjugated oligosachharides was determined. Due to the heterogeneity of the glycan structures, various profiling and characterisation strategies have been applied to determine the structures of glycans released from glycoproteins, such as mass spectrometry (MS; Haslam *et al.* 2006; Nwosu *et al.* 2012) and nuclear magnetic resonance (NMR; Abe *et al.* 2012) analyses in combination with separation techniques, using high-performance liquid chromatography (HPLC; Bao *et al.* 2013). Separation techniques include hydrophilic interaction LC (Domann *et al.* 2007) and capillary electrophoresis (CE; Guttman *et al.* 1996), and reduced unlabelled glycans can be well-separated and analysed by porous graphitised carbon LC electrospray-mass spectrometry (PGC-LC-ESI-MS, Jensen *et al.* 2012). A number of these methods are often combined with exoglycosidase digestion to acquire detailed monosaccharide linkage and branching information of the glycan structure (Aldredge *et al.* 2013; Kobata 2013). Analytical techniques such as gas liquid chromatography (Zanetta *et al.* 1972) and HPLC (Fu and O'Neill 1995), have also been used to quantify the amount of each

component monosaccharide in a mixture of glycans using fluorometric or ultraviolet (UV) detection after derivatisation. High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) is sometimes preferred as samples are not required to be derivatised (Harazono *et al.* 2011).

In this study, PGC-LC-ESI-MS was used to profile the glycomic array of glycans presented on the human milk glycoproteins. The neuraminidase activity was verified using mass spectrometry to monitor the loss of sialic acid from the glycans and the released milk glycans were quantitated using HPAEC-PAD analysis.

3.2 Materials and methods

Two methods were used to investigate the adherence of fluorescently-labelled whole bacterial cells either to synthetic or natural glycan structures in a glycan microarray or using a polyvinylidene difluoride (PVDF) plate assay.

3.2.1 Glycan microarray

The glycan microarray technology described here was developed at the Institute for Glycomics at Griffith University (Gold Coast, Australia), based on Blixt *et al.* (2004) and uses standard robotic microarray printing technology to link amine functionalised glycans to an amino-reactive glass slide. Spacers are attached to the non-reducing end of each glycan for the attachment of glycan to the epoxy surface of the slide. These spacers differ in length and chemistry, however, information about the exact chemical structure of the spacers is not available. The glycans presented on the microarray surface were composed primarily of synthetic and some natural glycan sequences that represent glycan structures of glycoproteins and glycolipids (glycoconjugates). A full list of the glycan structures printed on the array is available in the Supplementary Data (Tables S1 and S2).

3.2.1.1 Preparation of glycan microarray

Glycans were sourced from Dextra Laboratories (Reading, UK) and Glycoseparations Ltd (Moscow, Russia), functionalised and printed on activated SuperExpoxy 2 glass slides (ArrayIt Microarray Technologies, CA, USA) at the Institute for Glycomics (Australia) as previously described (Day *et al.* 2009). Briefly, two identical sub arrays were printed per slide with each sub array consisting of 352 spots (80 spots, eight controls in replicates of four). Slides were blocked in PBS supplemented with 0.1 % (w/v) bovine serum albumin (BSA), 2 mM magnesium chloride and 2 mM calcium chloride, pH 7.4 for 30 min, transferred to an

empty 50 ml polypropylene conical centrifuge tube (BD Falcon™, NJ, USA), followed by centrifugation at 900 rpm for 2 min to remove the buffer. Slides were ready for use in the bacterial binding assay once they were completely dry.

3.2.1.2 Culturing, harvesting and fluorescent labelling of whole bacterial cells

Details of the culturing and harvesting conditions of *E. coli* O111:NM (ATCC 43887), *S. typhimurium* (Group B) clinical isolate and commensal *L. rhamnosus* (ATCC 53103) are provided in Section 2.1. The resulting cell pellets were washed twice with PBS supplemented with 0.1 % (w/v) BSA, 2 mM magnesium chloride and 2 mM calcium chloride, pH 7.4 to remove residual broth. For labelling, the bacterial cells were resuspended in 500 µl of 5 mM carboxyfluorescein diacetate *N*-succinimidyl ester (CFDA-SE; Molecular Probes, CA, USA) prepared in PBS supplemented with 0.1 % (w/v) BSA, 2 mM magnesium chloride and 2 mM calcium chloride, pH 7.4 and incubated in the dark at 37 ^{o}C for 30 min. Excess dye was removed by washing three times in PBS supplemented with 0.1 % (w/v) BSA, 2 mM magnesium chloride and 2 mM calcium chloride, pH 7.4 and resuspended in PBS supplemented with 0.1 % BSA, 2 mM magnesium chloride and 2 mM calcium chloride, pH 7.4 to obtain an OD_{600} between 0.2 and 0.3, containing approximately 1 - 5 × 10^7 of CFDA-SE labelled bacterial cells.

3.2.1.3 Application of CFDA-SE labelled bacterial cells to glycan microarray

Each of the two glycan sub arrays printed per slide was covered with a 1.7 × 2.8 cm Gene Frame (Abgene, St. Leon-Rot, Germany), in which 1 - 5 × 10^7 (65 µl) of CFDA-SE labelled bacterial cells in PBS supplemented with 0.1 % BSA, 2 mM magnesium chloride and 2 mM calcium chloride, pH 7.4 were added. Both sub arrays were covered with GeneFrame coverslips (Abgene) and incubated in a humidified chamber in the dark at 37 ^{o}C for 30 min. The GeneFrames and coverslips were carefully removed by gently submerging the surface of the slide in PBS supplemented with 0.1 % (w/v) BSA, 2 mM magnesium chloride and 2 mM calcium chloride, pH 7.4. Slides were placed in a 50 ml tube containing PBS supplemented with 0.1 % (w/v) BSA, 2 mM magnesium chloride, 2 mM calcium chloride and 0.01 % (v/v) Tween 20, pH 7.4 to remove excess unbound bacterial cells on the slides. The tube was then gently inverted in a continuous motion for about 2 min before washing the slide with fresh PBS supplemented with 0.1 % (w/v) BSA, 2 mM magnesium chloride and 2 mM calcium chloride, pH 7.4. Slides were then fixed with 3.7 % (v/v) paraformaldehyde at RT for 20 min, washed with PBS supplemented with 0.1 % (w/v) BSA, 2 mM magnesium chloride and 2 mM calcium chloride, pH 7.4 to remove the fixative and transferred to the empty 50 ml

polypropylene conical centrifuge tube for centrifugation at 900 rpm for 2 min to remove excess buffer, followed by centrifugation at 900 rpm for 2 min in the empty 50 ml tube to dry the duplicate slides.

3.2.1.4 Fluorescent image acquisition and data processing

The extent of bound, labelled bacteria to the printed glycans was measured by the fluorescence intensities of array spots using the ProScanArray Microarray 4-Laser Scanner (PerkinElmer, MA, USA) with the Blue Argon 488 nm excitation laser set to a fluorescein isothiocyanate (FITC) setting (494 nm excitation and 518 nm emission). Image analysis was performed using ProScanArray imaging software ScanArray Express (PerkinElmer). The extent and level of binding of CFDA-SE labelled bacterial cells to immobilised glycans on the array was confirmed through visual inspection of the scanned images. Statistical analysis of each bacterial binding is defined as a value greater than one-fold increase above the mean background relative fluorescence units (RFU). The mean background was calculated from the average background of empty spots on the array, including three standard deviations. Statistical analysis of the data was performed by a Student's t-test with a confidence level of 99.99 % ($p \leq 0.0001$).

3.2.2 Polyvinylidene difluoride (PVDF) plate assay

The polyvinylidene difluoride (PVDF) plate assay method involved quantitative comparison of the binding of bacteria to human milk glycoproteins. The strains of bacteria comprised the above gastrointestinal-associated *E. coli*, *S. typhimurium*, commensal *L. rhamnosus* and *C. jejuni* NCTC11168, as well as the oral commensal and pathogen, *S. gordonii* F552 and *S. mutans* LT11 respectively. A 96-well plate with PVDF membrane-bottom format was used that immobilised and oriented the human milk glycoproteins via hydrophobic interaction with the PVDF membrane. The presented glycans were then probed with fluorescently-labelled bacteria and the bacterial binding quantified using a fluorescent plate reader.

3.2.2.1 Human milk protein preparation

Pooled human milk was obtained from a breast milk bank (Semmelweiß Frauenklinik (Vienna, Austria). Prior to protein precipitation procedures, whole human milk was centrifuged at 13000 × g for 30 min at 4 ^{o}C as previously described in Wilson NL *et al.* (2008) to separate the cream and skim milk. After removing the cream layer from milk using a spatula, the supernatant containing the protein mixture was divided into four equal sets, which include three sets for protein precipitation and a milk control (without precipitation). Three

protein precipitation methods, including acetone, trichloroacetic acid (TCA) /acetone, and methanol/chloroform/water protein precipitation were compared in this study to investigate the efficiency of these methods for precipitation of total human milk proteins and protein solubilisation in a suitable buffer. The two protein precipitation methods used a nine times volume of ice-cold acetone (Method 1), and ice-cold TCA/acetone ratio of 10:90 (v/v, Method 2) added to the human milk sample and incubated overnight at -20 ^{o}C. Method 3 involved the phase separation of human milk components using a methanol/chloroform/water ratio of 4:2:3 (Wessel and Flügge 1984) with the following modifications. The lower chloroform layer containing milk lipids was discarded, while the upper aqueous phase containing free oligosaccharides with proteins at the interphase of the methanol/water mix were dried together under vacuum by a Savant SC110A SpeedVac concentrator (Thermo Fisher Scientific, MA, USA). The precipitated fractions from all three methods were solubilised in 8 M urea before being concentrated using an Amicon® Ultra centrifugal filter with 100 kDa nominal molecular weight limit (NMWL; Merck Millipore, MA, USA) at 12000 × g for 30 min at 4 ºC to remove low molecular weight proteins, free oligosaccharides and salts. The retentate containing a mixture of concentrated proteins was collected for further analysis. All four samples were analysed by SDS-PAGE. Experimental details for determining total protein concentration and performing SDS-PAGE of human milk protein can be found in Section 2.3.

3.2.2.2 PVDF plate assay of bacterial adhesion to milk proteins

A 96-well microplate was used to quantitatively measure the adherence of 1 - 5 × 10^7 bacterial cells to proteins immobilised from 50 µg of total human milk proteins onto PVDF membrane in each well. The DNA of the live bacterial cells were fluorescently-labelled with SYBR Green that is excited by blue light at 497 nm and emits green light at 520 nm. SYBR Green was chosen as the bacterial strain also because its intracellular nuclear location does not interfere with the bacterial cell membrane. In addition, its excitation/emission properties avoided the autofluorescence of human milk proteins that will excite at 347 nm and emit at 415 nm (Ferrer *et al.* 2005). After correction for the day-to-day and bacterial fluorescence uptake variability, the fluorescent intensity remaining after the wash cycle was used as a quantitative measure of bacterial binding to the milk proteins.

AcroWell™ 96-well membrane-bottom, black plates with 0.45µm BioTrace™ PVDF membranes (Pall Corporation, NY, USA) were used for the assay. Membranes were pre-wet with methanol for 5 - 10 min and washed two times in PBS. A volume of 50 µl human milk proteins (50 µg) was added to each well and incubated on a plate shaker at 400 rpm for 16 h at

4 ^{o}C. Excess solution was removed and the wells were washed three times with PBS. Blocking of the membrane was not necessary as the membrane in the wells was fully coated with milk proteins (Bosch *et al.* 2003). A volume of 50 µl of fluorescently-labelled bacteria at OD_{600} of 1.5 cells (approximately 1 - 5 × 10^7) was added to the wells, incubated for 1 h at RT in the dark on the plate shaker, before being washed six times with PBS to remove unbound bacteria. Fluorescence was measured by the FLUOstar GALAXY fluorescence microplate reader (BMG LABTECH, Ortenberg, Germany). The filters were set at λ_{ex} = 485 nm and λ_{em} = 520 nm for detection of SYBR Green-labelled bacteria. To enable comparison of bacterial binding to unglycosylated proteins 50 µg of BSA (A4919, Sigma-Aldrich, MO, USA) was also bound to some wells of each plate. Each assay was performed in three technical replicates.

3.2.2.3 Fluorescence measurement

The SYBR Green fluorescent dye uptake by bacteria varied between the six bacterial strains and on a day to day basis so that data was corrected for fluorescence uptake when calculating the binding of each bacterial strain. To correct for this, the fluorescence measurement was normalised based on equalising initial fluorescence of the bacteria at the OD_{600} of 1.5. The fluorescence measurements were also corrected for milk autofluorescence by subtracting the background reading of milk proteins and PBS without bacteria. All results were calculated as mean ± standard error of the mean (SEM) of each bacterial strain and/or species using two tailed unpaired Student's t-test with Microsoft Excel, where $p<0.05$ was considered significant.

3.2.2.4 Inhibition of bacterial binding with *N*-acetylneuraminic acid (sialic acid)

Two different approaches were used to study the effect of sialic acid on bacterial binding. In the first approach, bacteria bound to human milk proteins were incubated with free sialic acid for 1 h to determine whether the sialic acid could remove the bound bacteria. A control of washing with an acidic buffer (0.1 M glacial acetic acid, pH 4.5) was also included to ensure that the bound bacteria were not removed simply by the presence of acid at the same pH. In the second approach, labelled bacteria were pre-incubated with free sialic acid for 30 min before binding the bacteria to milk proteins. Pre-incubation of bacteria with PBS instead of sialic acid was used as a control. Three technical replicates were performed for each approach. For each bacterial strain, the percent inhibition of binding of bacteria with (1) competitive inhibition with free sialic acid or (2) pre-incubation of bacteria with free sialic acid was expressed relative to the binding of the same bacteria to human milk proteins, which

was taken as 100 %.

3.2.2.5 Removal of sialic acid residues on total human milk proteins with neuraminidase

Terminal sialic acid residues on human milk glycoproteins were cleaved enzymatically by α2-3,6,8 neuraminidase (N2133, Sigma-Aldrich). Total precipitated human milk proteins immobilised on the PVDF membrane were treated with 50 µl of 0.05 U/ml neuraminidase in 50 mM sodium phosphate, pH 5.5, for 16 h at 37 °C. The binding of each bacterial strain to desialylated human milk glycoproteins was expressed relative to the binding to intact human milk proteins. The workflow of releasing *N*- and *O*-linked glycans from human milk proteins for mass spectrometry (MS) analysis has been described in Sections 2.4 and 2.5, respectively and was used to confirm the cleavage of sialic acid from the glycans. Released glycans from bovine fetuin (F3385, Sigma-Aldrich) were chosen as an internal control as they are well-characterised (Green *et al.* 1988; Hayase *et al.* 1992; Townsend *et al.* 1986).

3.2.2.6 Data analysis of released *N*- and *O*-linked glycans by mass spectrometry

The abundance of each glycan structure was estimated based on peak areas of extracted ion chromatograms (EICs) for each individual glycan mass, where each EIC was smoothed using a Gaussian algorithm before integration. To calculate the relative abundance of each glycan structure, the peak area of the EIC peaks corresponding to one glycan mass (i.e. all mass isomers) was expressed as a percentage of the total peak area of the glycans in the sample.

3.2.2.7 Monosaccharide analysis

Monosaccharide analysis was performed to determine the monosaccharide content and monosaccharide composition of human milk glycoproteins. Approximately 20 µg of total human milk proteins were dot-blotted on to the PVDF membrane and hydrolysed using 2 M trifluoroacetic acid at 100 °C for 4 h (neutral sugar analysis), 4 M hydrochloric acid at 100 °C for 6 h (amino sugar analysis), and 0.1 M trifluoroacetic acid at 80 °C for 40 min (acidic sugar or sialic acid analysis), dried separately under vacuum by the Savant SC110A SpeedVac concentrator (Thermo Fisher Scientific) and reconstituted with an internal standard of 0.1 M 2-deoxy-D-glucose. Three technical replicates were performed for each type of monosaccharide analysis (neutral, amino or acidic). Bovine fetuin (Sigma-Aldrich) was used as a control as it is a well-characterised glycoprotein and prepared the same as the test samples. Monosaccharide content was determined by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) fitted with a CarboPac PA1 column (2 × 250 mm, Dionex) and a CarboPac guard column PA1 (2 × 50 mm) at 25 °C.

The hydrolysates (10 µl) were eluted either by gradient with 60 to 300 mM sodium acetate over 20 min for sialic acids or isocratically for neutral and amino monosaccharides using 18 mM sodium hydroxide for 15 min with a flow rate of 0.5 ml/min. Data was collected and analysed using Chromeleon software (Version 6.70 SP5 Build 1914, Dionex, © 1994-2006 Dionex Corporation, Thermo Fisher Scientific). The amount of each monosaccharide released from human milk glycoproteins was expressed in µmoles and the molar ratio of each monosaccharide was determined.

3.3 Results

3.3.1 Glycan microarray analysis of CFDA-SE labelled bacteria

The glycan microarray was performed to elucidate the specific glycan structures recognised by *Escherichia coli* O111:NM (ATCC 43887), *Salmonella typhimurium* (Group B) clinical isolate and *Lactobacillus rhamnosus* (ATCC 53103), which are commonly associated with the human gastrointestinal tract. More than 350 different synthetic glycans were printed onto the array (Table S1 and Table S2, Supplementary Data). Glycans that were bound by the three human gastrointestinal bacteria on the array are listed in Table 3.2

3.3.1.1 *Escherichia coli* O111:NM (ATCC 43887)

The opportunistic gastrointestinal pathogen, *E. coli* O111:NM (ATCC 43887) bound to only 11 diverse structures of different composition and linkage as determined by using ProScanArray imaging software ScanArray Express with statistical analysis of four replicates calculated with $p \leq 0.0001$ (refer Section 3.2.1.4). A value greater than one-fold increase above the mean background relative fluorescence units (RFU) indicates a positive bacterial binding on the array. The bound structures of *E. coli* included those terminating with a Galβ1-3 and Galβ1-4 (with and without sulphate) as well as GlcNAcβ1-3 and GlcNAcβ1-4 (as part of the chitobiose core), GalNAcβ1-3 (including the glycolipid globotetraosylceramide, Gb_4) receptor which is a common human glycan receptor of the gastrointestinal tract and glycosaminoglycan related structures with glucuronic acid (GlcA). The *E. coli* also bound to single monosaccharide residues (Galα or Fucα), but surprisingly not to the numerous complex neutral glycans containing fucosylated and galactosylated structures on the array.

3.3.1.2 *Salmonella typhimurium* (Group B) clinical isolate

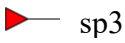
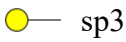
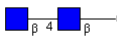
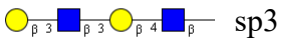
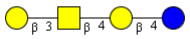
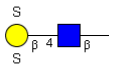
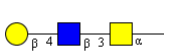
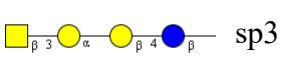
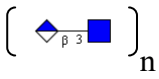
Similarly, pathogenic *S. typhimurium* (Group B) clinical isolate bound convincingly to only 5 structures with little in common; a ganglioside asialo-GM_1 receptor containing a terminal neutral (Galβ1-3) residue, a glycosaminoglycan containing GlcA and a heparin sulphate-like

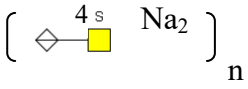
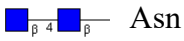
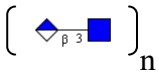
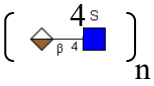
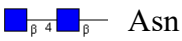
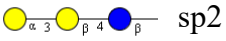
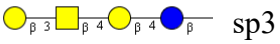
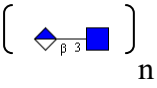
structure, as well as two glycans containing GlcNAcβ1-4GlcNAc residues, as part of the chitobiose core of all *N*-linked glycans.

3.3.1.3 *Lactobacillus rhamnosus* (ATCC 53103)

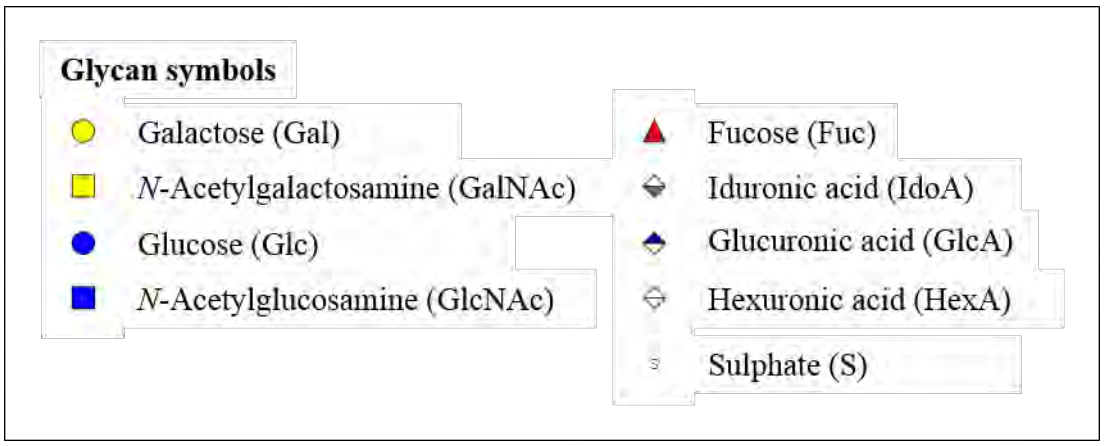
The binding pattern of the commensal bacteria *L. rhamnosus* (ATCC 53103) to glycans was also to the chitobiose core, similarly to the two gastrointestinal pathogens, with binding affinity to a terminal Galα1-3- and Galβ1-3-containing glycan as well as to a glycosaminoglycan structure containing glucuronic acid. The only glycan recognised by *L. rhamnosus* which was not recognised by the two pathogenic bacterial strains was a complex neutral glycan structure containing Galα1-3Galβ1-4Glcβ-sp2.

Table 3.2. Glycans recognised by the selected strains of human gastrointestinal bacteria.

Common names	Text nomenclature	Glycan structures
***Escherichia coli* O111:NM (ATCC 43887)**		
L-α-Fucose	Fucα-sp3	
α-Galactose	Galα-sp3	
Chitobiose-Asn	GlcNAcβ1-4GlcNAcβ-Asn	
LeCb3'LN	Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ-sp3	
Not known	Galβ1-3GalNAcβ1-4Galβ1-4Glc	
3',6'-di-O-Su-LacNAc (LN3'6'Su_2)	3,6-O-Su_2-Galβ1-4GlcNAcβ-sp2	
LN3Tn	Galβ1-4GlcNAcβ1-3GalNAcα-sp3	
Globotetraosylceramide (Gb_4, P antigen)	GalNacβ1-3GalαGalβ1-4Glcβ-sp3	
HA 107000 Da	GlcAβ1-3GlcNAcβ1-4	
HA 190000 Da		

Common names	Text nomenclature	Glycan structures
ΔUA→GalNAc-4SNa$_2$ (Δ Di-4S)	$C_{14}H_{19}NO_{14}SNa_2$ (produced from various chondroitin sulphates By the action of chondroitinases ABC, B and AC-1)	
***Salmonella typhimurium* (Group B) clinical isolate**		
Chitobiose-Asn	GlcNAcβ1-4GlcNAcβ-Asn	
Chitobiose	GlcNAcβ1-4GlcNAcβ-sp4	
Asialo-GM$_1$ (GM$_1$)	Galβ1-3GalNAcβ1-4Galβ1-4Glcβ-sp3	
HA 1600000 Da	GlcAβ1-3GlcNAcβ1-4	
Heparin sulfate		
***Lactobacillus rhamnosus* (ATCC 53103)**		
Chitobiose-Asn	GlcNAcβ1-4GlcNAcβ-Asn	
Aa3'Lac-C2	Galα1-3Galβ1-4Glcβ-sp2	
Asialo-GM$_1$ (GM$_1$)	Galβ1-3GalNAcβ1-4Galβ1-4Glcβ-sp3	
HA 107000 Da	GlcAβ1-3GlcNAcβ1-4	

Abbreviations: Asn, asparagine; Da, dalton; GM$_1$, ganglioside 1; Gb$_4$, globotetraosylceramide; HS; heparan sulphate; HA, hyaluronic acid; Na$_2$, sodium; Su, sulfate; sp, spacer; ΔUA, unsaturated hexuronic acid.
The spacers (sp2 - 4) used in this glycan microarray study differ in length. However, information regarding the exact chemical structure of these spacers is not publicly available.



3.3.1.4 Variations in glycan microarray results

Overall, less than 5 % of the printed glycans were bound by the three bacteria, making it difficult to establish bacterial binding structures for each strain. Based on the visual images obtained of the glycan arrays after incubating with the bacteria, there was also poor reproducibility of bacterial binding to specific glycans. Figure 3.1 shows an example of an image taken in which the grid containing the position of the printed glycans is shown, particularly, two quadruplicate glycan spots produced high fluorescence in one array (sub array 1), while fluorescence was undetected for the same glycan spots on the replicate array (sub array 2) on the same slide. The proximity of the glycan structures on the array can affect their conformation and presentation to the bacteria as discussed in Section 3.4.

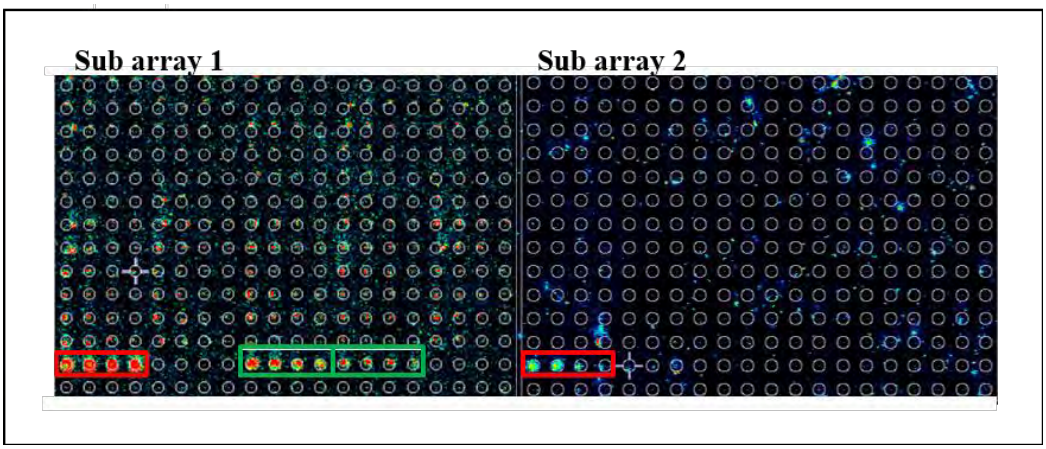


Fig. 3.1. Comparison of images taken from two identical sub arrays of the slide, showing inconsistent fluorescence for replicates of glycan binding spots. The control fluorescein isothiocyanate (FITC) spots (four replicates) on the array are boxed in red, whereas spots indicating a positive bacterial binding to a specific printed glycan are boxed in green.

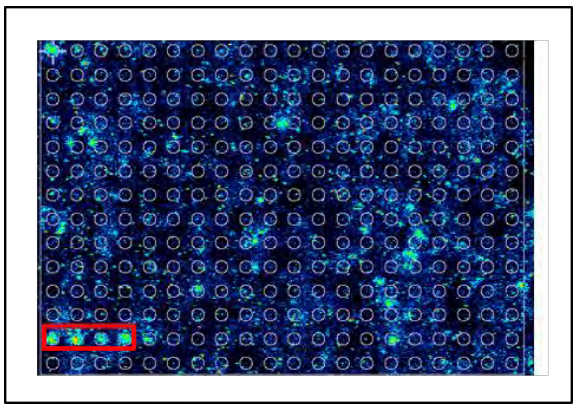


Fig. 3.2. High background of non-specific fluorescence obtained by incubating with bacteria. The bacteria appear to clump together on the array. The control FITC spots (four replicates) are well-defined and boxed in red.

Variations in the fluorescent intensity values of the replicate glycan spots were consistently observed. Apart from the well-defined FITC spots, non-specific bacterial binding produced random fluorescence on the array. Figure 3.2 shows the binding of bacteria to the slide with the printed glycan spots overlaid, where intense background can be seen outside the array spot overlay. The high background fluorescence shown is likely due to bacterial clumping together. It was apparent that this glycan array method, although it is routinely used to measure protein and viral interactions with immobilised glycans, did not appear to be suitable for measuring intact bacterial binding to the glycans.

3.3.2 PVDF plate assay

A second high throughput assay was thus developed to provide a better quantitative measurement of the sugar-pathogen interaction, where the immobilised glycoproteins contained glycan receptors present in a more natural environment. The PVDF plate assay enabled 1) investigation of the binding of different bacterial strains to human milk glycoproteins, 2) the capacity to modify the glycan structures presented on the human milk glycoproteins to help characterise the bacterial binding partners and 3) the application of glycans as competitive inhibitors of the bacterial binding to identify mimics of this interaction.

3.3.2.1 Comparing protein precipitation methods

In the assay, glycoproteins adhere to the PVDF membrane at the bottom of a 96-well plate. Three different methods were trialed to precipitate and concentrate the total proteins in human milk. The amount of proteins precipitated was quantified by the Bradford protein assay and verified by SDS-PAGE. Table 3.3 shows the amount of protein precipitated by each technique as a proportion of the total protein content in the same volume of human skim (delipidated) milk, where the highest protein amount in the precipitate was obtained using methanol/chloroform/water (74 %), followed by acetone (43.3 %) and TCA/acetone (35.6 %)

precipitations.

Table 3.3. Comparison of protein precipitation methods for human milk.

Methods	**Protein amount (mg)**	**Yield (%)**
Skim milk control	10.4±0.1	100
Acetone precipitation	4.5±0.3	43.3
TCA/acetone precipitation	3.7±0.1	35.6
Chloroform/methanol/water precipitation	7.7±0.2	74

Each protein amount was calculated as mean ± SEM of three 1 ml replicates.

The precipitated human milk proteins were also analysed using 1D SDS-PAGE which substantiated the difference in yield from the methods. As shown in Figure 3.3, the protein bands precipitated from the three methods showed a similar banding pattern as the skim milk in lane 4 although with different SYPRO Ruby stain intensities. Amongst the three precipitation methods, acetone precipitation (lane 2) yielded a slightly different mixture of proteins as compared to chloroform/methanol/water precipitation (lane 3) and TCA/acetone precipitation (lane 5). The former method precipitated abundant proteins in the 80 kDa range, but precipitated fewer lower (< 80 kDa) and higher (> 260 kD) molecular weight proteins. Chloroform/methanol/water precipitation (lanes 3) and TCA/acetone precipitation (lane 5) yielded similar protein banding patterns with different amounts. There was a poor protein recovery by TCA/acetone precipitation (lane 5), of proteins under 50 kDa. Thus, it appeared that the chloroform/methanol/water precipitation method was the most efficient protein precipitation method as it also has the advantage of removing the lipid and concentrating the protein at the same time. From these results, the chloroform/methanol protein precipitation method was chosen for the preparation of total milk glycoproteins for the subsequent binding assays.

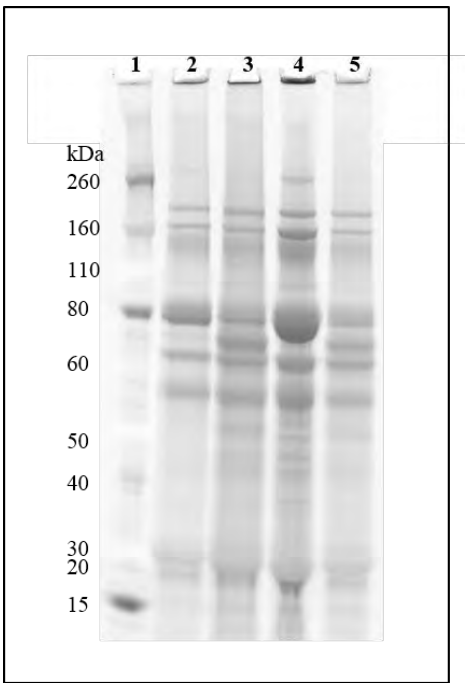


Fig. 3.3. Comparing milk protein precipitation methods with SYPRO Ruby staining. Lane 1, Novex® sharp pre-stained protein standard; lane 2, acetone precipitation; lane 3, methanol/chloroform/water precipitation; lane 4, human skim milk and lane 5, TCA/ acetone precipitation.

3.3.2.2 Comparison of binding efficiencies of fluorescently-labelled gastrointestinal tract- and oral-associated bacteria to total human milk proteins

The PVDF plate assay was used to quantitate the gastrointestinal tract (including oral and gastrointestinal) bacterial adhesion to total human milk proteins. Four human gastrointestinal bacteria, *C. jejuni*, *E. coli*, *S. typhimurium* and *L. rhamnosus* were labelled with SYBR Green prior to binding to the immobilised milk proteins, which consisted of a mixture of glycosylated and unglycosylated proteins. Figure 3.4 shows significant ($p<0.05$) binding of all bacterial strains to human milk proteins, as compared to unglycosylated BSA. Although unglycosylated proteins such as β-casein, lysozyme and serum albumin are present in milk (Table 1.3), this data suggests that glycosylation plays a role in the binding of bacteria to milk proteins. Among the gastrointestinal-associated bacteria, it was also observed that the non-pathogenic, gastrointestinal (probiotic) *L. rhamnosus* bound most efficiently to milk proteins and the binding was slightly better than *C. jejuni* and *S. typhimurium*. The opportunistic non-motile gastrointestinal *E. coli*, bound least to the total human milk proteins.

By comparison with the initial exposure of the milk in the mouth, the two oral streptococci species, *S. gordonii* and *S. mutans* bound less efficiently to the total human milk proteins than the gastrointestinal bacteria, with *S. gordonii* binding 33 % more efficiently than *S. mutans* (Figure 3.4). This indicates that the interaction between bacteria and human milk proteins varies between species. *Streptococcus gordonii* had a similar binding to total human milk proteins as *E. coli*, while the binding of *S. mutans* to total human milk proteins was the lowest

amongst all six bacteria. This suggests that the bacterial binding epitopes recognised by the oral streptococci on human milk proteins may be different from the ones bound to by gastrointestinal pathogens, thus influencing bacterial binding specificities in different parts of the digestive tract.

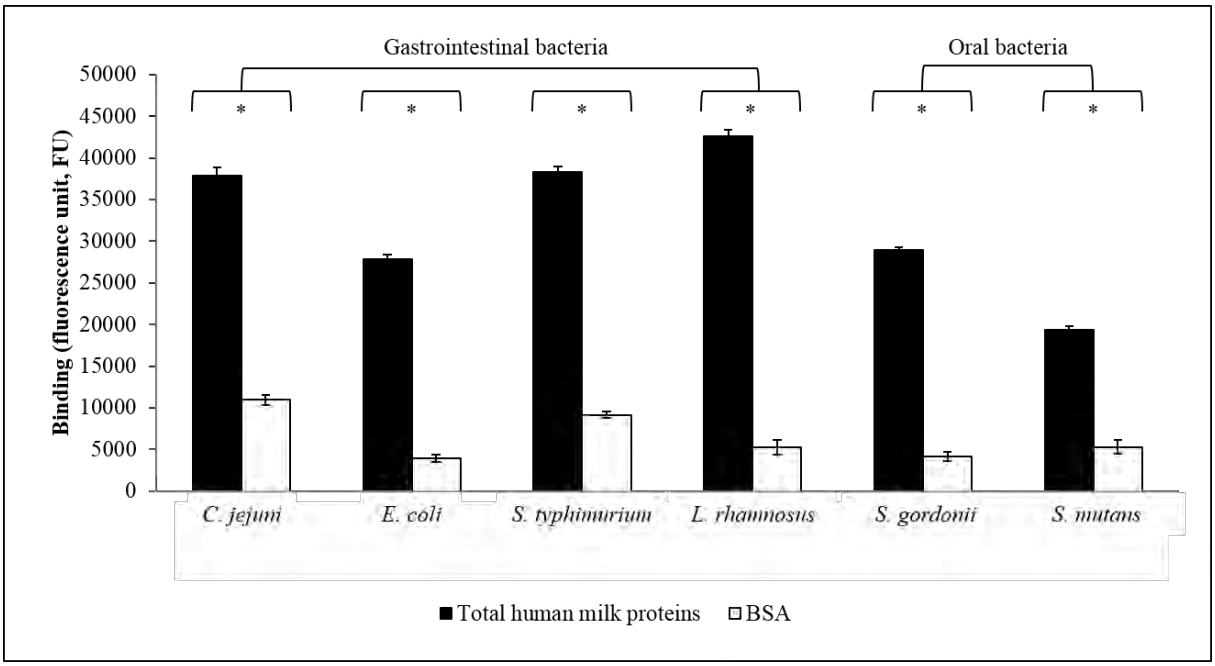


Fig. 3.4. Comparison of the binding (represented by fluorescence unit, FU) of the gastrointestinal and oral bacteria to total human milk proteins immobilised on PVDF membranes. All six bacteria bound to human milk proteins significantly more than to the same amount of unglycosylated bovine serum albumin (BSA). Three technical replicates were used and data were adjusted for fluorescence uptake before calculating the mean ± SEM for each bacterial strain. Error bars represent SEM, where significance (* = $p<0.05$) was compared to individual BSA control.

3.3.2.3 Sialic acid involvement in the binding of bacteria to human milk proteins

The involvement of sialic acid, i.e. the abundant terminal monosaccharide on the milk glycoproteins, in mediating bacterial binding was investigated using the PVDF plate assay. Five concentrations (0, 0.05, 0.1, 0.2, 0.4 µg/µl) of 50 µl of *N*-acetylneuraminic acid (or sialic acid; A0812, Sigma-Aldrich) were used to determine the concentration of sialic acid required to inhibit bacterial binding to human milk proteins on the PVDF membrane.

A volume of 50 µl of free sialic acid (0 to 0.4 µg/µl) was added to the bound bacteria to determine whether this monosaccharide could compete with the binding of oral streptococci to total human milk proteins and remove the bacteria. For both species, the bacterial binding to human milk proteins (Figure 3.5) showed that washing the protein bound bacteria with 0.1 µg/µl of free sialic acid was sufficient to reduce the binding of *S. gordonii* to human milk

proteins by 24.4 % and *S. mutans* by 15.3 %. Higher amounts of free sialic acid (0.2 µg/µl) did not show a further effect and thus, 0.1 µg/µl of free sialic acid was determined as the minimal inhibitory concentration. A volume of 50 µl of glacial acetic acid (0.1 M, pH 4.5, equivalent to 0.1 µg/µl of free sialic acid) was included as a negative control to exclude the possibility that the bound bacteria can be removed by the acidic washing process or whether bacterial cell lysis is caused by acid entering the cell. Almost no effect (< 1% decrease in binding) with acetic acid was found.

In an alternate approach, pre-incubation of the bacteria with free sialic acid appeared to have a greater effect on the binding of *S. gordonii* to total human milk proteins than washing the bound bacteria from milk proteins with free sialic acid. Pre-incubating the bacteria with free sialic acid (0.1 µg/µl) before adding the *S. gordonii* to the milk proteins effectively reduced the binding by 37.3 % and did not show increased inhibition of binding with higher amounts of free sialic acid were used (Figure 3.5). In contrast, *S. mutans* binding was not inhibited at all by pre-incubation of the bacteria with free sialic acid, as indicated by an actual slight increase of about 6 % of bacteria bound. This suggests that sialic acid may not be involved in the binding of *S. mutans* to human milk proteins. Both approaches indicated that the binding of *S. gordonii* to human milk proteins was sialic acid-dependent with *S. mutans*, showing a lesser involvement of sialic acid in binding to milk proteins.

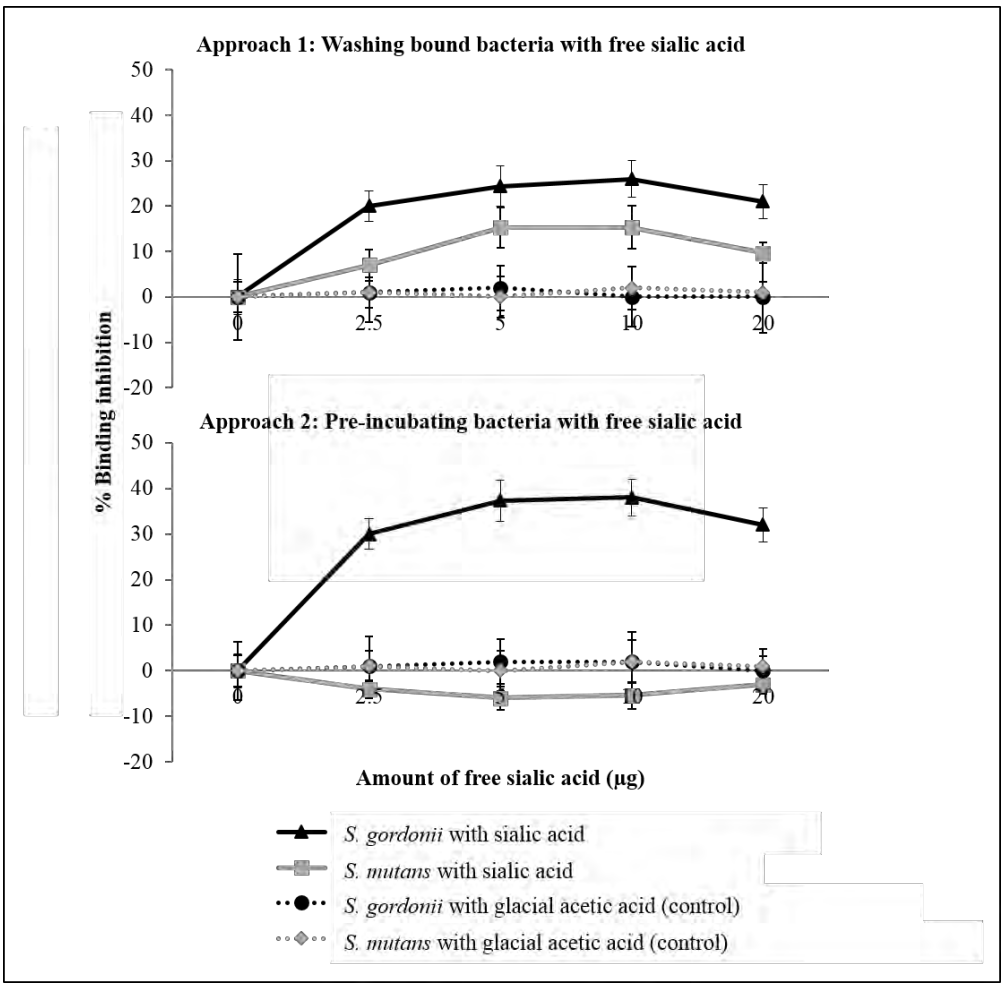


Fig. 3.5. Inhibition of oral streptococci binding to human milk proteins with increasing amounts of sialic acid by 1) washing bound bacteria with sialic acid and 2) pre-incubating bacteria with sialic acid before binding. Varying amounts of sialic acid (0 - 0.4 µg/µl) were tested to determine the inhibition of the binding of *S. gordonii* and *S. mutans* to human milk glycoproteins by the monosaccharide. A weak glacial acetic acid solution (0.1 M, pH 4.5, equivalent to 0.1 µg/µl of free acid) served as a control in both approaches to provide support that the binding of bacteria to human milk proteins is sialic acid-dependent and not just acid affected. Each data point on the curve was corrected for fluorescence uptake by the different bacteria and represents the mean ± SEM of triplicate measurements from the PVDF plate assay.

3.3.2.4 Validation that sialic acid is involved in binding of bacteria to human milk proteins

To further investigate the role of sialic acid in the bacterial binding to milk glycoproteins, α2-3,6,8 neuraminidase was used to remove the terminal sialic acids on total human milk proteins prior to measuring the binding of the six bacterial strains. Figure 3.6 shows that the neuraminidase treatment of the human milk glycoproteins for 16 h prior to bacterial binding, significantly ($p<0.05$) reduced the attachment of all four intestinal bacterial strains, particularly *C. jejuni* (reduced by 44.7 % compared to before neuraminidase treatment), followed by *S. typhimurium* (34.3 %), *L. rhamnosus* (28.3 %) and *E. coli* (14.1 %).

Also, in agreement with the competitive inhibition effect of free sialic acid on the bacterial binding (as shown in Section 3.3.2.3), the removal of sialic acid had a differential effect on the binding of the oral streptococci. The binding of oral *S. gordonii* to desiaylated human milk proteins was reduced by 28.9 %, but desialylation did not affect the binding of *S. mutans* (actually causing a slight increase in binding of 5.6 %; Figure 3.6). Significant ($p<0.05$) differences were also observed between the binding of *S. gordonii* and *S. mutans* after either washing the bound bacteria or pre-incubating the bacteria with free sialic acid (Figure 3.5), in which the *S. gordonii* binding to human milk glycoproteins appeared to involve sialic acid, while the binding of *S. mutans* was not affected by the presence of sialic acid. These combined data support the observation that *S. mutans* binds to different bacterial epitopes on milk proteins to those recognised by *S. gordonii*. These data also support the hypothesis that the interaction of many bacteria with human milk is at least partially (and differentially) reliant on binding to the terminal sialic acid residues on the milk glycoproteins.

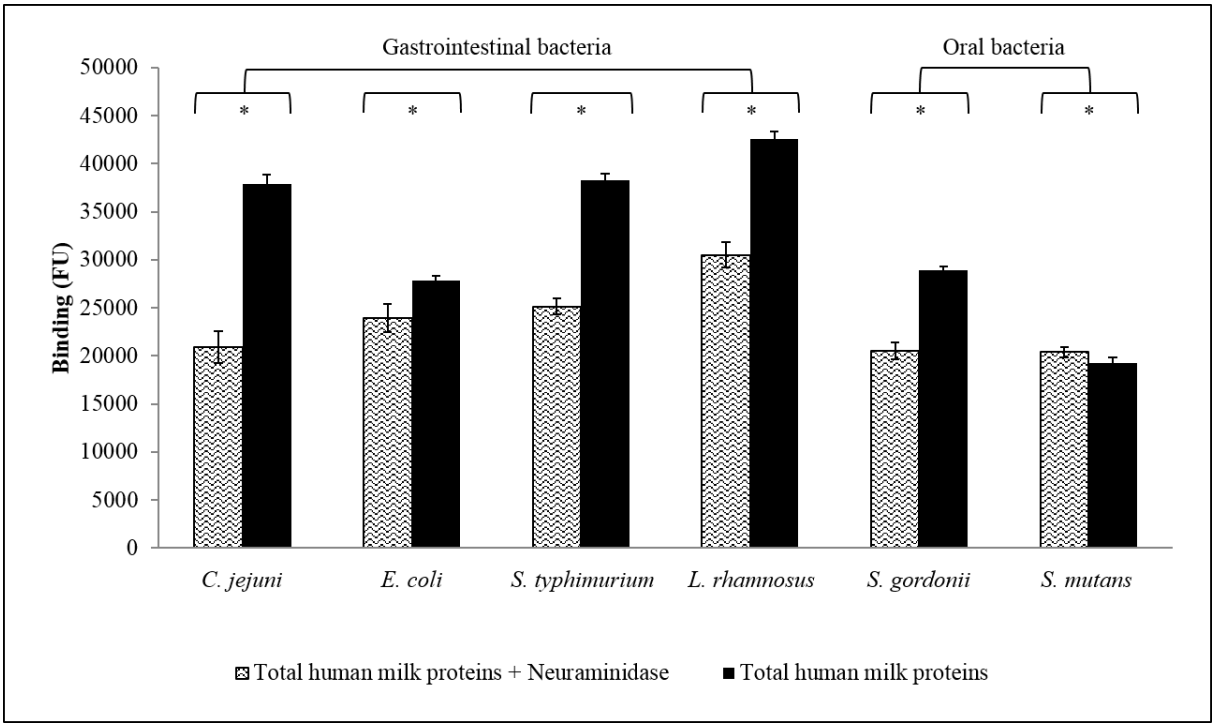


Fig. 3.6. Desialylation of the milk glycoproteins on the PVDF membrane by neuraminidase digestion for 16 h decreased all bacterial binding efficiencies, except *S. mutans*, the binding of which was not affected by desialylation of the milk glycoproteins. Three technical replicates were used and data were corrected for fluorescence uptake before calculating the mean ± SEM of each bacterial strain. Error bars represent SEM, where significance (* = $p<0.05$) was compared to total human milk proteins only. Abbreviation: FU, fluorescence unit.

3.3.2.5 Confirmation of desialylation of human milk glycoproteins by neuraminidase

To substantiate that the changes in bacterial binding observed after treatment of the milk

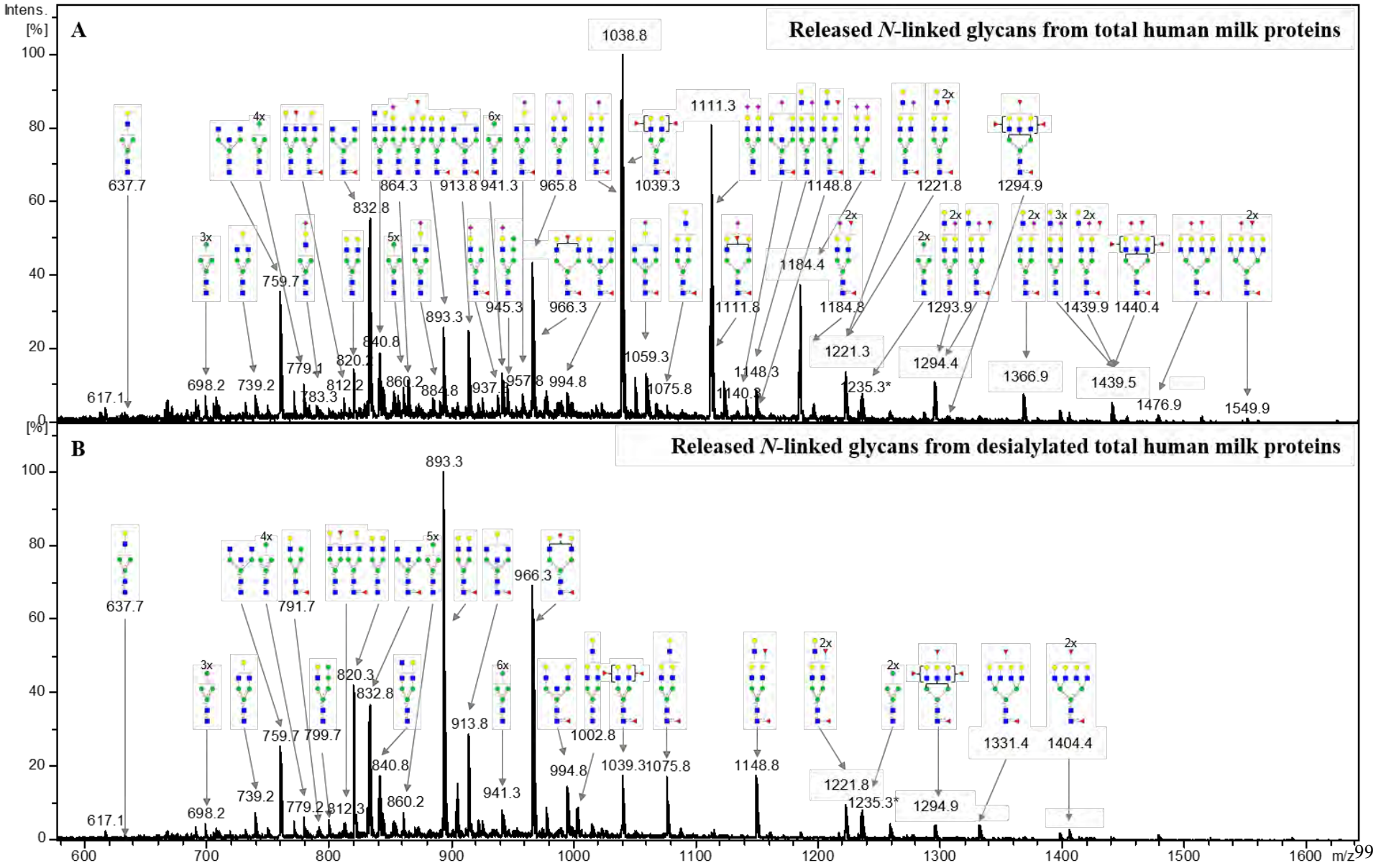
proteins with α2-3,6,8 neuraminidase were due to the absence of terminal sialic acid, the *N*- and *O*-linked glycans were released from the milk proteins and compared before and after desialylation using LC-ESI-MS (Table 3.4 and Figure 3.7 A and B). The relative abundances (ion intensities) of the sialylated glycan structures were compared before and after neuraminidase digestion based on the extracted ion chromatograms for each sialylated *N*-linked glycan. The presence of sialic acid was confirmed by MS/MS analysis by the loss of a sialic acid residue with *m/z* of 291.

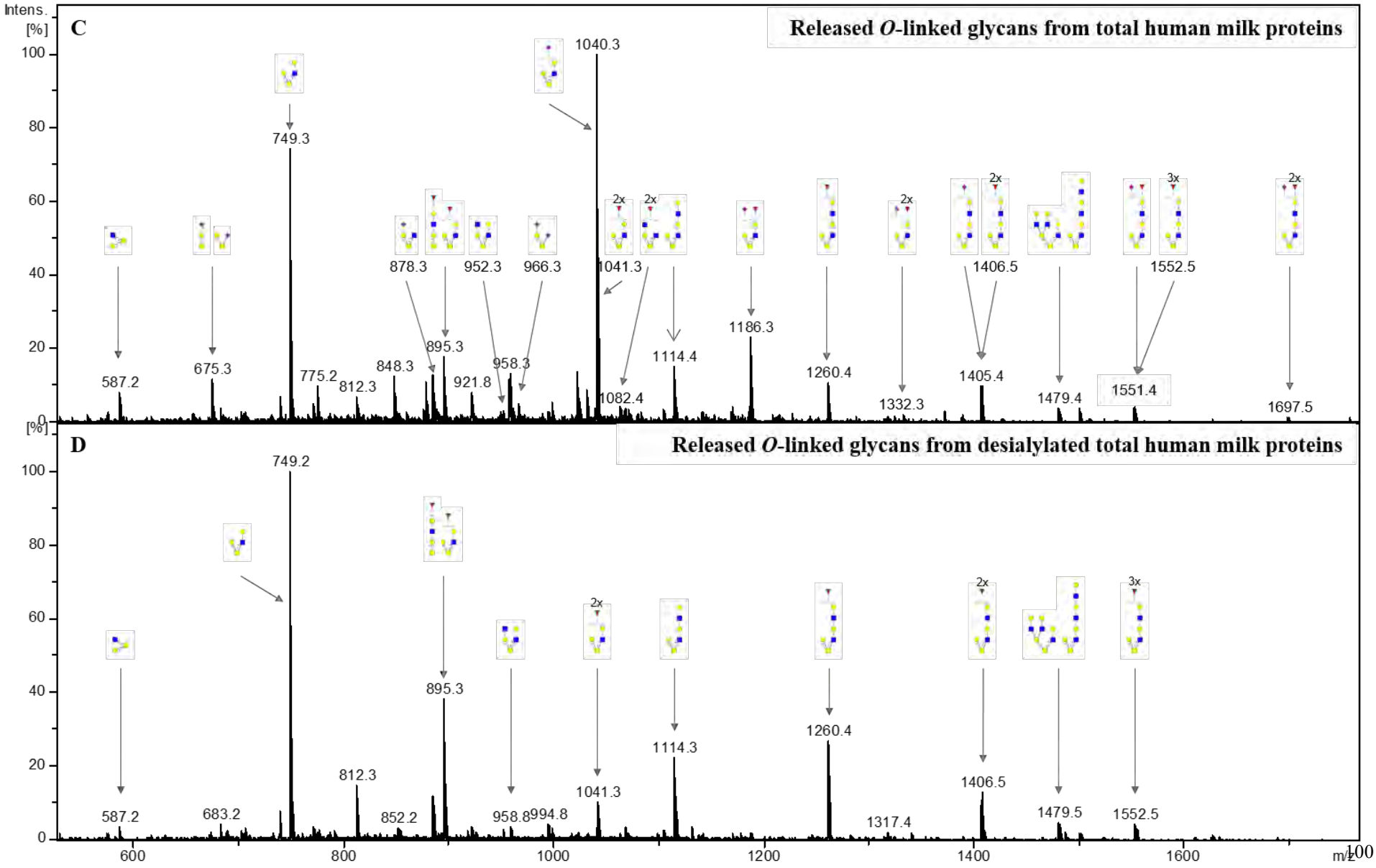
There were significant differences between the *N*- (Figures 3.7 A and B) and *O*- (Figures 3.7 C and D) glycome after desialylation of the human milk glycans. A total of 65 glycan structures (45 *N*- and 20 *O*-linked glycans) were identified before desialylation. Details of these structures were determined in the next section (Section 3.3.3) and are provided in the Supplementary Data (Table S3). Figures 3.7 A and C show the summed average mass of *N*- and *O*-linked glycans released from human milk proteins before neuraminidase treatment. While Figures 3.7 B and D show the summed average mass spectra after neuraminidase treatment. The desialylation of human milk glycans by α2-3,6,8 neuraminidase after 16 h showed a decrease in abundance of structures with sialic acid residues and resulted in an increase in the non-sialylated glycan products (see also Figure S1, Supplementary Data).

For example, after desialylation, two abundant doubly sialylated bi-antennary *N*-linked glycans, *m/z* $[1111.3]^{2-}$ and *m/z* $[1184.4]^{2-}$ (Figure 3.7 A) in the released *N*-linked glycans from the total human milk glycoproteins decreased significantly after neuraminidase treatment with a concomitant decrease in their mono-sialylated products, *m/z* $[965.8]^{2-}$ and *m/z* $[1038.8]^{2-}$ respectively, followed by an increase in the abundance of their fully desialylated products, *m/z* $[820.2]^{2-}$ and *m/z* $[893.3]^{2-}$ (Figure 3.7 B). Three singly sialylated *N*-linked glycans, *m/z* $[945.3]^{2-}$, *m/z* $[1059.3]^{2-}$, *m/z* $[1140.3]^{2}$ (Figure 3.7 A) were completely missing after desialylation, with an increase in the corresponding desialylated products, *m/z* $[799.7]^{2-}$, *m/z* $[913.8]^{2-}$ and *m/z* $[994.8]^{2-}$ (Figure 3.7 B). It was noticed that there was an increase in the possible Lewis type fucosylated *N*-linked structure of *m/z* $[966.3]^{2-}$ after desialylation. This indicates that there may be limited resolution by the ion-trap mass spectrometer of the mono-sialylated bi-antennary structure *m/z* $[965.8]^{2-}$ from the di-fucosylated structure *m/z* $[966.3]^{2-}$ that have a mass difference of 1 Da in the released *N*-linked glycan average mass spectrum. This data thus may result in an underestimation of the Lewis fucosylated *N*-linked glycans, but the global removal of sialic acid substantiates the activity of the α2-3,6,8 neuraminidase.

Similarly, three abundant *O*-linked mono-sialylated glycans, *m/z* $[1040.3]^{1-}$, *m/z* $[1186.3]^{1-}$ and *m/z* $[1551.4]^{1-}$ (Figure 3.7 C) released from human milk proteins also decreased with a concomitant increase in the percentage abundance of the respective desialylated species, *m/z* $[749.2]^{1-}$, *m/z* $[895.3]^{1-}$ and *m/z* $[1260.4]^{1}$ (Figure 3.7 D) when treated with α2-3,6,8 neuraminidase. Note that the two proposed sialylated glycan isomers of *m/z* $[675.3]^{1-}$ are completely removed after neuraminidase treatment, indicating the loss of the attached sialic acid residue; the desialylated disaccharide products would not be seen in the mass window chosen.

This study confirmed that the α2,3,6,8, neuraminidase has removed the sialylation from both the *N*- and *O*-linked glycoproteins in human milk. These results substantiate the conclusion that sialylation of the milk glycoproteins is an important factor in the binding of both commensal bacteria and those that are potentially pathogenic to the human gastrointestinal tract, where the extent of this adhesion varies between different strains of bacteria.


Intens.
[%]
A
Released N-linked glycans from total human milk proteins
1038.8
1111.3
637.7
864.3
913.8
941.3
965.8
1039.3
1148.8
1221.8
1294.9
832.8
759.7
893.3
945.3
966.3
1184.4
1111.8
1184.8
1293.9
1439.9
1440.4
1059.3
1221.3
1294.4
1366.9
1439.5
698.2
739.2
779.1
820.2
840.8
860.2
884.8
937.8
957.8
994.8
1075.8
1148.3
1140.8
1235.3*
1476.9
1549.9
617.1
783.3
812.2
B
Released N-linked glycans from desialylated total human milk proteins
893.3
637.7
791.7
966.3
759.7
799.7
820.3
832.8
913.8
1002.8
994.8
1039.3
1075.8
1148.8
1331.4
1404.4
698.2
739.2
779.2
812.3
840.8
860.2
941.3
1221.8
1235.3*
1294.9
617.1
600
700
800
900
1000
1100
1200
1300
1400
1500
1600
m/z


Intens.
[%]
C
Released O-linked glycans from total human milk proteins
1040.3
749.3
878.3
952.3
966.3
1041.3
1406.5
1552.5
1186.3
895.3
958.3
1114.4
587.2
675.3
775.2
812.3
848.3
921.8
1082.4
1260.4
1332.3
1405.4
1479.4
1551.4
1697.5
[%]
D
Released O-linked glycans from desialylated total human milk proteins
749.2
895.3
812.3
1114.3
1260.4
587.2
683.2
852.2
958.8
994.8
1041.3
1317.4
1406.5
1479.5
1552.5
100
80
60
40
20
0
600
800
1000
1200
1400
1600
m/z

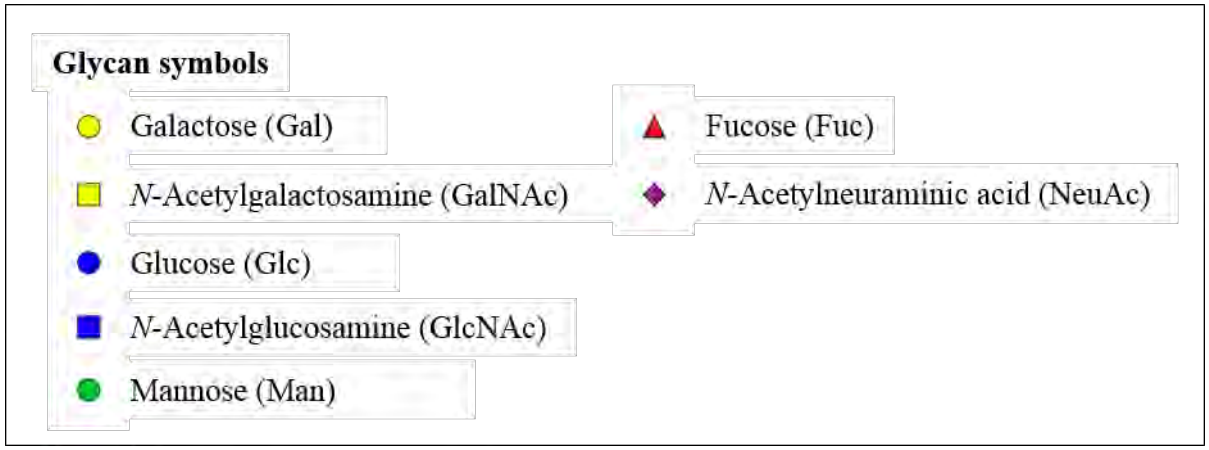
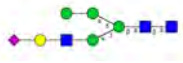
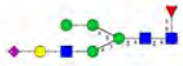
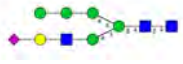
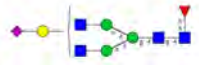
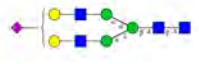
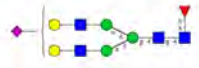
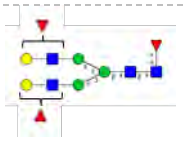
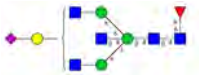
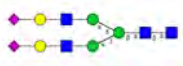
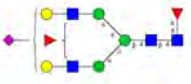
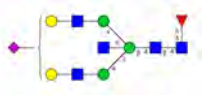
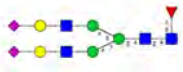
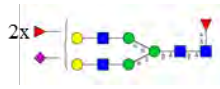
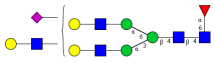
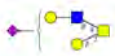
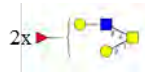
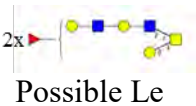


Fig. 3.7. Comparison of the summed mass spectra of *N*- and *O*-linked glycans from human milk glycoproteins before (A and C) and after (B and D) digestion with α2-3,6,8 neuraminidase. Before desialylation a total of 65 *N*- and *O*-linked glycan structures were identified by retention times over all samples using bovine fetuin of known retention time as an internal standard, where 45 *N*-linked glycans were eluted between 32.7 -58.4 min retention time and 20 *O*-linked glycans eluted between 23.4 -35.3 min retention time. *N*- and *O*-linked glycans were separated using different gradients, in which the *N*-linked glycans were separated using a linear gradient with 0 - 45 % (v/v) acetonitrile in 10 mM ammonium bicarbonate for 85 min, whereas *O*-linked glycans were separated using a linear gradient with 0 - 90 % (v/v) acetonitrile in 10 mM ammonium bicarbonate for 45 min at a flow rate of 2 µl/min using an Agilent 6330 series ion-trap MS instrument linked to an Agilent 1100 capillary LC (Agilent Technologies, CA, USA). Details of these structures are presented in Table S3, Supplementary Data. The * denotes this particular *N*-linked glycan, *m/z* $[1235.3]^{1-}$ is singly charged. Representative $[M-2H]^{2-}$ and $[M-H]^{1-}$ ions correspond to identified structures of released *N*- and *O*-linked glycans respectively from total human milk glycoproteins. Presence of sialic acid (291 Da) was confirmed by the presence of *m/z* $[291]^{1-}$ in the MS/MS of sialylated structures.

Table 3.4. Comparison of total relative abundances of sialylated structures in total human milk proteins before and after desialylation. Structure numbers refer to structures characterised in the milk (see Table S3, Supplementary Data).

S/ No.	Calculated experimental mass $[M-H]^{1-}$	Proposed glycans on total human milk proteins in this study	Relative abundance before desialylation (%)	Relative abundance after desialylation (%)	Structural confirmation
N 7	1567.6		0.28	ND	Mono-sialylated
N 13	1729.6		0.79	ND	Mono-sialylated
N 17	1875.6		0.63	ND	Mono-sialylated
N 19	1891.6		0.87	ND	Mono-sialylated
N 20	1916.6		0.78	ND	Mono-sialylated
N 21	1932.6		11.21	ND	Mono-sialylated
N 24	2078.6		10.64	ND	Mono-sialylated
N 25	2079.6	 Possible Le	0.16	2.25	Di-fucosylated
N 26	2119.6		1.06	ND	Mono-sialylated
N 28	2223.6		25.82	ND	Di-sialylated
N 29	2224.6	 Le/possible SLe	15.52	ND	Mono-sialylated

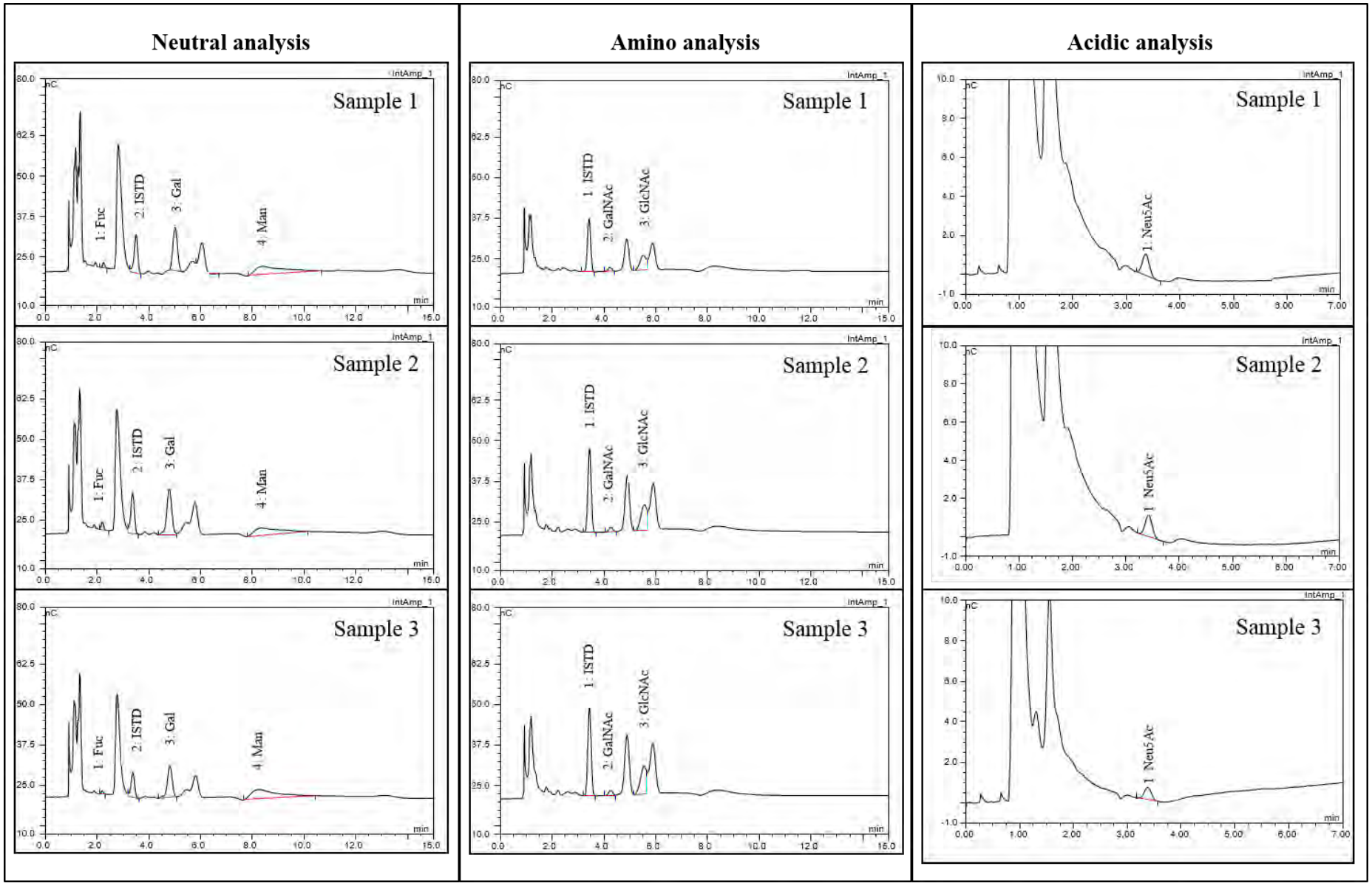
S/ No.	Calculated experimental mass $[M-H]^{1-}$	Proposed glycans on total human milk proteins in this study	Relative abundance before desialylation (%)	Relative abundance after desialylation (%)	Structural confirmation
N 30	2281.6		0.36	ND	Mono-sialylated
N 33	2369.8		4.47	ND	Di-sialylated
N 34	2370.6	 Possible Le/SLe	0.78	ND	Mono-sialylated and di-fucosylated
N 35	2443.6		0.41	ND	Mono-sialylated
O 2	675.3		2.42	ND	Mono-sialylated
O 4	878.3		2.46	ND	Mono-sialylated
O 7	966.3		1.06	ND	Di-sialylated
O 8	1040.3		27.25	ND	Mono-sialylated
O 9	1041.3	 Possible Le	7.72	9.97	Di-fucosylated
O 15	1405.4		3.72	ND	Mono-sialylated
O 16	1406.5	 Possible Le	0.24	5.64	Di-fucosylated

S/ No.	Calculated experimental mass $[M-H]^{1-}$	Proposed glycans on total human milk proteins in this study	Relative abundance before desialylation (%)	Relative abundance after desialylation (%)	Structural confirmation
O 18	1551.4	 Possible Le/SLe	1.45	ND	Mono-sialylated

Abbreviations: ND, not detected; S/No., structure number.

3.3.3 Monosaccharide analysis of released *N*- and *O*-lnked glycans from human milk glycoproteins

To further verify that the possibility of over-estimating the sialylation of this human milk sample by the presence of di-fucosylated structures, the monosaccharide composition of released *N*- and *O*-linked glycans on total human milk glycoproteins was analysed by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Each type of monosaccharide analysis (neutral - Gal, Man, Fuc; amino - GlcNAc, GalNAc; acidic - NeuAc) was carried out to quantify the six mammalian glycoprotein monosaccharides (Figure 3.8). Based on molar ratio, the total *N*- and *O*-linked glycans contained a high relative amount of Gal (56.9 % of total monosaccharides), Man (28.2 % of total monosaccharides) and GlcNAc (11.5 % of total monosaccharides), but a low relative amount of GalNAc (0.6 % of total monosaccharides). Although in relatively low abundance, the HPAEC-PAD analysis detected a Fuc:NeuAc ratio of approximately 4:1, which is even higher than the fucosylation of milk proteins found by Dallas *et al.* 2011 (65 % fucosylation and 38 % sialylation) and Nwosu *et al.* 2012 (75 % fucosylation and 57 % sialylation). The higher total monosaccharide fucosylation shown in our study may be due to the number of fucosylated *O*-linked glycans identified since the two literature reports only analysed the relative abundance of fucosylation in the *N*-glycome of human milk whey proteins.


Neutral analysis
Amino analysis
Acidic analysis
Sample 1
Sample 2
Sample 3
1: Fuc
2: ISTD
3: Gal
4: Man
1: ISTD
2: GalNAc
3: GlcNAc
1: Neu5Ac
nC
min

Monosaccharide amount (µM)	**Gal**	**GalNAc**	**GlcNAc**	**Man**	**Fuc**	**NeuAc**	**Total**
Sample 1	260.6	3.8	57.4	132.0	9.4	2.6	
Sample 2	297.1	2.9	57.7	194.0	8.1	2.8	
Sample3	286.6	3	55.5	92.4	15.6	1.5	
Mean	**281.4±18.8**	**3.2±0.5**	**56.9±1.2**	**139.5±15.2**	**11.0±4.0**	**2.3±0.7**	**494.3**
Relative amount (%)	**56.9**	**0.6**	**11.5**	**28.2**	**2.2**	**0.5**	**100**

Fig. 3.8. Monosaccharide (neutral, amino and acidic) analysis of total *N*- and *O*-linked glycans that were hydrolysed from total human milk glycoproteins. The mean values are shown as the mean ± SEM of triplicate measurements from each monosaccharide analysis. Abbreviations for: Fuc, fucose; Gal, galactose, HPAEC-PAD, high-performance anion-exchange chromatography with pulsed amperometric detection; ISTD, internal standard (i.e. 2-deoxy-D-glucose); Man, mannose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; NeuAc, *N*-acetylneuraminic acid; %, percentage.

3.4 Discussion

This study provides evidence for the hypothesis that human milk may prevent gastrointestinal and oral bacterial colonisation in breastfed infants. Two fluorescent-based adhesion methods were used to study the binding mechanism, by which proteins or glycoproteins in human milk can bind to bacteria in order to facilitate or inhibit bacterial colonisation. The first method used a glycan array technology to identify the binding by three gastrointestinal bacteria: *E. coli*, *S. typhimurium* and *L. rhamnosus* to specific immobilised glycan structures. The printed glycan microarrays have been used by others mainly for measuring glycan recognition by purified proteins, antibodies, lectins and viruses (Fazio *et al.* 2002; Fukui *et al.* 2002; Houseman and Mriksich 2002; Nimrichter *et al.* 2004; Schwarz *et al.* 2003; Wang *et al.* 2002) as it is a sensitive and robust technique and requires low quantities of synthetic glycans (Leppänen and Cummings 2010). Although whole bacterial cells have been used in array binding assays (Day *et al.* 2009, 2012), in this study it was found that the assay was not suitable for measuring whole bacterial binding to sugars possibly because the assay conditions were not fully optimised, and the assay results lacked reproducibility and reliability.

In addition, the results from the glycan microarray for all three bacterial strains in this study, did not agree with the glycan-binding specificity as reported in the literature. *Escherichia coli* did not bind to mannosylated and/or sialylated glycans, although acidic oligosaccharide structures have been identified as binding sites for this bacteria on human intestinal mucins (Robbe *et al.* 2004). Several studies have also reported that *E. coli* binding to terminal galactose, mannosylated, fucosylated and sialylated glycan structures, in which some are natural glycan structures of the human gastrointestinal tract, such as GD_3, GM_2, LNT, Le^y, sialyl-Le^x (Ashkenazi *et al.* 1991; Cravioto *et al.* 1991; Idota and Kawakami 1995; Newburg 1997; Newburg *et al.* 1990). However, these types of structures were not recognised by *E. coli* in this work, in which the bacterium only bound to one fucose residue on the array. Likewise, the pathogenic *S. typhimurium* did not bind to any of the fucosylated structures on the array, although in previous research *Salmonella enterica* serotype typhimurium has been shown to bind to terminal α1-2 Fuc residues present in the cecal mucosa (Chessa *et al.* 2009), mannose-rich carbohydrate structures (Grzymajlo *et al.* 2010) and glycoproteins terminating with sialic acid residues (Vimal *et al.* 2000). Although not much information is known about the interaction of *L. rhamnosus* with carbohydrates, the binding specificities of this bacterium to the array in this study are not in agreement with a recent study using other *Lactobacillus* strains ME-522 and *L. gasseri* ME-527, which recognised sialylated residues on sHCM using BIAcore analysis (Huang *et al.* 2013). Further studies also showed that the interaction of *L.*

plantarum with human colonic HT-29 cell line (Adlerberth *et al.* 1996) and porcine jejunal epithelium (Gross *et al.* 2008) could be mannose-specific, which was not found in this assay. Bacterial binding to glycolipid-like structures was also observed on the array (Table 3.2, Chapter 3). The pathogenic *S. typhimurium* and the commensal *L. rhamnosus* bound to asialo-GM_1 with a terminal neutral (Galβ1-3) residue, and *E. coli* bound to Galα1-4 antigen, which is present in glycosphingolipids with previous studies, suggesting that the Galα1-4 antigen may play an important role for *E. coli* adherence to the core of glycosphingolipids (Sung *et al.* 2001a, 2001b). It is therefore very clear that the lack of repeatability of data was the reason that this method was not pursued further.

Many technical factors could have potentially influenced the bacterial binding to the glycan structures on the array: 1) glycan density on the array, which may have affected the number of binding sites (multivalent interactions) presented to the bacteria and the distance between binding sites (Oyelaran and Gildersleeve 2009; Oyelaran *et al.* 2009), 2) excretion of enzymes by bacteria, which may have digested existing sialic acid residues attached to the array (Dallas *et al.* 2012). 3) changes in incubation time, (Lewallen *et al.* 2009), temperature and growth conditions (Day *et al.* 2009), 4) manner in which the structural conformation of the glycan structures on solid phase were presented to the bacteria (Manimala *et al.* 2007) and 5) ratio of glycans printed on each spot to number of bacterial cells. In terms of data analysis, manual interpretation of the microarray array data can be rather difficult and time-consuming. Thus, it was clear that this method required further optimisation to overcome these apparent problems including the non-reproducibility, inconsistency of recognised structures and amount of printed glycans on the array. In addition, the native *N*- and *O*- linked glycans that are actually found on human gastrointestinal and human milk glycoproteins are not well-represented by the synthetic structures displayed on the arrays. Due to these considerations, it was decided that a more effective and reliable method could be developed that used a complex mixture of glycans as a more authentic multivalent biological model, and which included the natural binding partners.

A second assay, which involved the use of milk glycoproteins arrayed in a PVDF-based 96-well plate, was thus developed. This method is based on the binding of the hydrophobic proteins to the PVDF membrane (Jensen *et al.* 2012) to provide a more natural presentation of the glycans on the milk proteins to study the interactions between microorganisms and specific glycans on human milk proteins. This plate assay also enabled quantification of the fluorescently-labelled bacterial binding in a high throughput manner. The immobilisation of

the human milk glycoproteins presents a more complex glycan structural substrate as compared to single, smaller and limited amount of synthetic glycan structures on the glycan microarrays. This method also allowed for the modification of the attached glycans on the human milk glycoproteins to allow investigation of the involvement of specific glycans in the binding of gastrointestinal and oral bacteria.

All four human gastrointestinal bacterial strains bound to human milk proteins, but not to the unglycosylated BSA, thus indicating the role of glycans in bacterial binding. The human commensal bacteria, *L. rhamnosus* bound slightly more efficiently to milk proteins when compared to the human pathogens, *E. coli*, *C. jejuni* and *S. typhimurium*. For the oral bacteria, *S. gordonii* showed a strong binding affinity to the milk proteins than *S. mutans*, but both bacteria bound less effectively when compared with the human gastrointestinal pathogens. One possible explanation for the differential binding of the oral bacteria is that *S. gordonii* is known as an initial enamel coloniser, which provides an anchor for other oral microorganisms in the cascade of colonisation (Chalmers *et al.* 2008), whereas *S. mutans* is a plaque-forming bacterium which colonises later in oral biofilms (Kreth *et al.* 2008; Kuramitsu and Wang 2006). Upon desialyation of human milk proteins with α2-3,6,8 neuraminidase, the attachment of all four gastrointestinal bacterial strains was significantly ($p<0.05$) reduced, indicating that the interaction of these bacterial strains with human milk glycoproteins is partially reliant on binding to terminal sialic acid residues on the glycans. *Salmonella typhimurium*, *L. rhamnosus* and *E. coli* showed less inhibition of binding by desialylation than *C. jejuni*. Interestingly for the oral environment, *S. gordonii* recognised sialic acid as part of the binding epitope structure, but *S. mutans* binding did not appear to be dependent on the presence of sialic acid. The lack of dependency on sialic acid for binding of *S. mutans* was confirmed by the fact that free sialic acid could not completely remove the interaction or prevent the binding of *S. mutans*.

While *S. mutans* is known to bind to salivary glycoproteins, the oral bacterium binds to other epitopes and not to terminal sialic acid residues (Levine *et al.* 1978). For instance, Gibbons and Qureshi (1979) have previously shown that galactose and melibiose inhibited eight strains of *S. mutans* binding to saliva-coated hydroxyapaptite (S-HA) surfaces. Another study showed that *S. mutans* bound only to Le^a, Galβ1-3(Fucα1-4)GlcNAc containing the fucose residue and not to the other blood group antigens such as Le^b, Le^x, Le^y, H1, H2, A, B and sialylated Le^a (Ligtenberg *et al.* 2000). Ligtenberg *et al.* (2000) showed that the Le^a blood group antigens were important for binding and found that *S. mutans* bound to Le^a without

galactose (Fucα1-4GlcNAc), but not to Le^a without fucose (Galβ1-3GlcNAc), confirming that fucose and not sialic acid plays a major role in the binding of *S. mutans*. Therefore, further experiments involving enzyme digestion with other exoglycosidases need to be carried out to identify the specific sugar residue(s) responsible for *S. mutans* binding to human milk glycoproteins.

From the comparative binding studies described in Figures 3.4 and 3.6, it is interesting to note that the commensal bacteria, *L. rhamnosus* and *S. gordonii*, had a slightly higher binding affinity to the human milk proteins than the pathogenic bacteria. This was unexpected since it seems to suggest that human milk proteins may bind and remove commensals from the gut just as well, or even better, than it does for the pathogens. Nevertheless, as shown by the literature review (Chapter 1), there is strong evidence that human milk consumption correlates with a healthy infant gut microbiota. It is possible that the binding of commensals to human milk may aid in their delivery to the infant mouth or gut from external sources, such as the lactating mother. Meanwhile, the adhesion to milk destined for excretion may facilitate a regular turnover of the commensal gut microbiome that promotes genetic diversity and resilience. Clearly the process is complex and multifactorial. Exactly how this translates *in vivo* is difficult to determine but could be explored further in future studies.

In past research, identification of most of the proposed human milk glycan structures have been based on MS-based analysis of released glycans from either a proportion of glycoproteins in human milk fractions or specific proteins (Table S3, Supplementary Data). A few studies have characterised *N*-linked glycans of human skim milk proteins (Dallas *et al.* 2011) and human milk whey proteins (Nwosu *et al.* 2012). Although the *N*- and *O*-linked glycans of human milk fat globule membrane proteins have been reported (Wilson *et al.* 2008), these proteins account for less than 5 % of total human milk protein content (Fortunato *et al.* 2003). Hence in the present study, LC-ESI-MS was used to identify the binding partners on total human milk proteins that interacted with the six different bacterial strains, in particular investigating the involvement of sialic acid in bacterial binding. This LC-ESI-MS/MS method, which requires low quantities of starting material (down to fetomole range) has been widely used for the analysis of glycan structures (Jensen *et al.* 2012; Karlsson *et al.* 2004a, 2004b; Kolarich *et al.* 2012; Pabst *et al.* 2007). In addition, the porous graphitised carbon (PGC) column also has the advantage of differentiating glycan isomers and separating neutral and acidic glycans (Packer *et al.* 1998). It should be noted that in this study, the instrument was operated in a negative-ion mode rather than in positive-ion mode for better

ionisation of the acidic glycans (Sagi *et al.* 2005).

From the glycans analysed in this study, the glycans characterised in total human milk glycoproteins consisted mainly of sialylated bi-antennary *N*-linked glycans, while a majority of the *O*-linked glycans were core 2 structures, with mono-fucosylated and mono-sialylated structures accounting for most of the *O*-linked glycans (see Table S3, Supplementary Data). The bi-antennary and di-sialylated glycan (N28) was detected as the most abundant *N*-linked structure, while the mono-sialylated core 2 type structure (O8) was the most abundant *O*-linked glycan. It was noted that 28 *N*- and 16 *O*-linked glycans have been previously reported in literature as glycans found on human milk glycoproteins (see Table S3, Supplementary Data). These glycan structures included mostly core fucosylated and sialylated *N*-linked glycans, while the *O*-linked glycan structures were primarily sialylated core 2 type. Similarities were observed between the proposed structures in this study and previously reported human milk glycan structures from the literature. For instance, the core-fucosylated bi-antennary structure (N15) was also found in three other studies on whey proteins (Nwosu *et al.* 2012), bile salt-stimulated lipase (Mechref *et al.* 1999) and human lactoferrin (Matsumoto *et al.* 1982; Samyn-Petit *et al.* 2001). Interestingly, this structure was absent in a previous study on human skim milk proteins (Dallas *et al.* 2011) that reported the fucose attached to a GalNAc residue on the arm (as Lewis-type epitope), rather than to the core. Twenty-one low level *N*- and *O*-linked glycan masses (Supplementary Data, Table S3: N1, N7, N14, N20, N23, N27, N30, N31, N32, N35, N36, N37, N38, N39, N41, N44, N45, O9, O16, O19 and O20) found in this study have not been previously reported in the three studies used for comparison of human milk proteins.

Some differences are also seen in the structural analysis of the total human milk glycoproteins, for example, the proposed structure O13 was assigned as a fucosylated Galβ1-3GlcNAc attached to the Gal in a β1-3 linkage on the branched β1-6 arm of GalNac of core 2. However, this composition has been shown to be a different terminal sequence on sIgA found in human milk in a previous study (Pierce-Crétel *et al.* 1989), in which the fucosylated Galβ1-3GlcNAc attached to the Gal in a β1-3 linkage on the branched β1-3 arm of the GalNac. In this study, three sialylated core 2 *O*-linked glycans (O2, O7 and O8) on the total human milk glycoproteins shared a similar composition with known structures on the bovine MFGM proteins, but not reported on MFGM proteins of human milk (Wilson *et al.* 2008). It is possible that the pooled human milk samples collected from some breastfeeding mothers who had consumed cow's milk prior to breastfeeding, since early studies have shown that β-

lactoglobulin, which is a major whey protein in bovine milk, was found in human milk samples of mothers who have ingested cow's milk (Jakobsson *et al.* 1985; Sorva *et al.* 1994). However, the acidic residue, *N*-glycolylneuraminic acid (NeuGc), only found in non-human mammals (Nakao *et al.* 1991; Nowak *et al.* 1986; Nwosu *et al.* 2012; Suzuki *et al.* 2000) such as cows was not identified in the human milk analysed here.

This study shows predominately highly sialylated, core fucosylated structures in the released *N*- and *O*-linked glycan structures of the total human milk proteins. The sialylation was confirmed by MS/MS analysis (loss of mass of sialic acid - 291 Da) and by desialylation with α2-3,6,8 neuraminidase. Total monosaccharide analysis using HPAEC-PAD showed greater fucosylation than sialylation in the total released *N*- and *O*-linked glycans, which is in agreement with the high fucosylation on *N*-linked glycans of human milk whey proteins (Dallas *et al.* 2011; Nwosu *et al.* 2012) with over 50 % of the fucosylated *N*-linked glycans containing terminal fucose residues bearing a possible Lewis structure. It is not clear whether the high fucosylation (75 %) distribution observed by this group referred to core or Lewis fucose residues as the relative abundance of these structures was not presented. In this present study, the glycan analysis was carried out using negative mode ionisation, which increases the detection of the acidic (sialylated) glycans. Hence, a greater proportion of sialylated glycan structures may have been seen compared with the positive mode ionisation of the permethylated *N*-linked glycans used by Nwosu *et al.* (2012). Additionally, the glycan composition of human milk proteins could also vary in individual mothers depending on the lactation stage, like that found in free oligosaccharides (Section 1.4, Chapter 1). To date, there is little information on both the *N*- and *O*-glycosylation of human milk proteins, with only one paper on the glycan profile of human MFGM proteins (Wilson *et al.* 2008).

The results in Figure 3.7 (see Table S3, Supplementary Data) from this study suggest that high sialylation is a general feature of glycosylation on total human milk proteins. The discrepancy between this work and that of Nwosu *et al.* (2012), which showed high fucosylation and low sialylation as the general characteristic of human milk, might be explained by the data in Figure 3.7, which shows several masses after desialylation that correspond to a di-fucose substituent (292 Da) remaining after desialylation (loss of 291 Da). It is suggested that before desialylation (Figures 3.7 A and C), the apparent relative abundances of some of the sialylated *N*- and *O*-linked glycan types (see Table S3 and Figure S1 in Supplementary Data) are comprised of a mixture of sialylated and di-fucosylated species and thus, do not represent the actual relative quantification of sialylation and fucosylation in human milk. To account for the

inaccurate mass measurement of the ion trap MS analysis that cannot easily distinguish this mass difference, the total relative distribution of masses that could be representing di-fucosylated and sialylated structures on human milk proteins before and after desialylation was compared (Table 3.4). It is shown that the proposed sialylated *N*-linked glycan masses were all significantly reduced after desialylation, confirming these masses were glycans containing sialic acid and not di-fucose residues. Di-fucosylated assigned structures, such as N25 did not decrease, but increased (see Figure S1, Supplementary Data). Likewise, the *O*-linked glycans O9 and O16 did not decrease (and actually increased in abundance in the latter case) after desialylation, suggesting that these were correctly assigned as di-fucosylated structures. The increase in O9 and O16 after desialylation could be due to the desialylated products of a parent sialylated structure, such as *m/z* $[1697.5]^{1-}$ and *m/z* $[1332.3]^{1-}$, respectively after neuraminidase treatment (Figure 3.7).

Protein bound glycans in human milk were thus shown to be recognised as binding partners by bacteria that typically inhabit the digestive tract from the mouth to the intestine. The involvement of the terminal sialic acids on the bacterial binding is variable, suggesting that different bacterial species/strains discriminate in the attachment to glycan substructures on the milk glycoproteins. These data add to accumulating evidence about the importance of sugar determinants on glycoproteins in human milk, which can confer a natural defence mechanism by binding to and expelling human gastrointestinal pathogens from the intestine, as well as oral pathogens from the mouth, of breastfed infants, before they can bind and infect the epithelial linings.

Chapter 4. Binding of human gastrointestinal bacteria to bovine and human milk proteins

Rationale

The work described in this chapter aimed to investigate and compare the binding of human gastrointestinal bacteria to bovine and human milk proteins. Methods were explored to effectively fractionate milk to help elucidate which proteins play the greatest role in bacterial adhesion. One fractionation method involved a preparative electrophoresis instrument, MicroFlow MF10, designed to separate complex protein mixtures under native conditions across hydrophilic membranes into different fractions by charge and size. The second method used high-speed centrifugation with pH adjustment for separation of whey and casein fractions. The third method utilised fast protein liquid chromatography (FPLC) gel filtration (size exclusion) to further separate the proteins in the soluble whey fraction.

The PVDF plate assay developed in Chapter 3 was used to compare the binding of gastrointestinal bacteria to bovine and human milk proteins (total proteins, casein, whey and whey sub fractions). The involvement of conjugated milk glycans in bacterial adhesion, and specifically the contribution of sialic acid, was investigated by 1) washing the bacteria bound to the protein fractions with sialic acid, 2) pre-incubating the bacteria in sialic acid prior to measuring their binding to the milk fractions and 3) removal of terminal sialic acid residues on milk glycoproteins by α2-3,6,8 neuraminidase.

4.1 Introduction

Human milk is known to provide human infants with greater protection against gastrointestinal disease than bovine milk or cow's milk based infant formulas (Section 1.1, Chapter 1). Part of this phenomenon has been attributed to the human immunoglobulins, particularly sIgA, which are present at very high concentrations in human colostrum and then decrease as milk, and the immune system of the infant, matures (Goldman *et al.* 1982; Ogra and Ogra 1978). Free oligosaccharides are also more abundant in human milk (5 - 15 g/l) than bovine milk (0.05 g/l; Table 1.3, Chapter 1) and are thought to play a significant role in inhibiting pathogen adhesion to the gastrointestinal tract via a decoy mechanism (Figure 1.2, Chapter 1). The ability of other components of human and bovine milk to bind to gastrointestinal bacteria and inhibit their adhesion to the gastrointestinal tract is not well-understood and thus warrants further investigation. In particular, it is hypothesised that the glycans attached to the milk proteins may also contribute to the pathogen adhesion function of milk, and further, that there may be differences in the extent of pathogen adhesion between bovine and human milk milk glycoproteins.

Bovine milk differs from human milk in a number of ways, as summarised in Table 1.3, Chapter 1. The protein content of bovine milk is typically over three times that of human milk; hence, the increased health benefits of human milk cannot be positively correlated with the abundance of total protein *per se*. There is also a much higher amount of caseins in bovine milk than human milk, including the glycosylated κ-casein. Bovine and human milk have been reported to have similar total whey protein content. However, the specific components of the whey fraction are slightly different in the two types of milk. The glycoproteins lactoferrin and sIgA form substantial components of human milk whey, but not of bovine milk whey. In contrast, β-lactoglobulin, a protein that has not been reported as glycosylated, represents almost half of bovine milk whey proteins (Table 1.3). Differences in the glycan structures of milk glycoproteins from bovine and human milk have also been established (Nwosu *et al.* 2012; Wilson *et al.* 2008).

The relative contribution of the different components of bovine and human milk to bind to and remove pathogens from the digestive tract has not been well studied, particularly beyond the possible decoy functions of human free milk oligosaccharides. Some studies have emerged demonstrating the abilities of milk glycoproteins to bind to pathogens (Table 1.6, Chapter 1). However, the work was typically carried out using purified glycoproteins at arbitrary concentrations appropriate for maximum effect in a particular assay, and rarely was

carried out using concentrations similar to those in the original milk source. Furthermore, there appears to be a lack of research that assesses the contribution to pathogen binding of crude fractions of milk, such as total whey or casein fractions, in which several glycoproteins/proteins feature together in combinations and concentrations typical of real milk. The specific contribution of the array of conjugated glycans presented by each of these fractions has also received little attention.

In the work described in this chapter, the adhesion of gastrointestinal bacteria to various protein fractions of human and bovine milk was compared to determine which components of each type of milk could have potential to remove pathogenic bacteria from the human gastrointestinal tract. The first stage of the work involved the establishment of effective methods to isolate different protein fractions from bovine and human milk. Several techniques were explored: 1) a selective electrophoresis, designed to separate milk proteins by size and charge across variable pore size polyacrylamide membranes when a voltage is applied; 2) a high-speed centrifugation technique with pH adjusted to separate the soluble whey proteins from the insoluble caseins, and 3) size exclusion (fast protein liquid chromatography, FPLC gel filtration) fractionation of the whey proteins. The fractions of bovine and human milk proteins were immobilised onto the PVDF plate in the quantities representative of their concentrations in the original milk source and the binding of gastrointestinal bacteria to the milk protein fractions was compared. The involvement of conjugated milk glycans in bacterial binding, and specifically the importance of sialic acid as a binding epitope, was also examined by washing or pre-incubating with free sialic acid, and removing the terminal sialic acid residues on milk glycoproteins using neuramindase prior to the binding of gastrointestinal bacteria. This study provides important information on the protein components of bovine and human milk which may have potential to bind to gastrointestinal pathogens and thus prevent their adhesion to the gastrointestinal tract, and the role that the glycosylation of the milk proteins may play in this interaction.

4.2 Materials and methods

4.2.1 Milk samples

Pooled human milk was obtained from the breast milk bank of Semmelweiß Frauenklinik, Vienna, Austria. Bovine milk was obtained from a Jersey cow at the Calmsley Hill City Farm, Sydney, Australia, two months after calving.

4.2.2 Methanol/chloroform/water precipitation for isolation of total milk proteins

Total proteins of bovine and human milk were precipitated from raw bovine and human milk by methanol/chloroform/water precipitation according to Section 3.2.2.1.

4.2.3 Preparation of skim milk

To obtain skim milk, bovine and human milk were centrifuged at 13000 × g, 4 °C for 30 min (Section 3.2.2.1). The cream layer was removed and the skim milk was supplemented with protease inhibitor (Roche Diagnostics, NSW, Australia). Skim milk samples were freshly prepared prior to application in each of the following fractionation techniques.

4.2.4 Fractionation of skim milk using the Microflow MF10 system

Bovine and human skim milk (Section 4.2.3) were fractionated using the selective electrophoresis MicroFlow MF10 system (NuSep Ltd, Sydney, Australia), designed to separate proteins by molecular weight (Horvath *et al.* 1994). Four chambers were assembled using five polyacrylamide membranes (2.7 × 2.2 cm) with increasing pore size. All five membranes (5 kDa × 2, 75 kDa × 1, 150 kDa × 1 and 500 kDa × 1) were soaked twice with Milli-Q water and twice with 1X Tris-ε-aminocaproic acid (EACA)/urea buffer, consisting of 90 mM Tris (hydroxymethyl) aminomethane, 10 mM EACA and 4 M urea. The chamber of the instrument, tubing and cartridges designed to hold the polyacrylamide membranes were also washed in Milli-Q water for 10 min. Membranes were placed into the cartridges in order of increasing pore size from the anode to the cathode, forming four separate sub-chambers, as illustrated in Figure 4.1. The two 5 kDa membranes were placed at both ends of the assembly. The whole system was equilibrated in a buffer solution of 1X Tris/EACA/urea before loading the freshly prepared skim milk sample (Section 4.2.3).

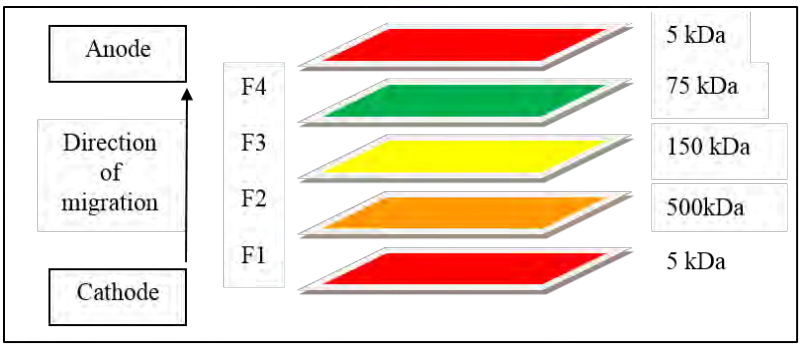


Fig. 4.1. A schematic diagram of the polyacrylamide membranes assembled in the Microflow MF10 chamber for protein fractionation. Based on the principle of polyacrylamide gel electrophoresis, proteins migrate across the membranes from cathode to anode according to their size and charge when an electric field is applied (Wasinger *et al.* 2005).

A volume of 140 µl of skim milk was loaded into the sub-chamber at the cathode end of the apparatus between the 5 kDa and 500 kDa membranes using gel loading pipette tips (Proxeon Biosystems A/S, Odense, Denmark). An equal volume (140 µl) of 1X Tris/EACA/urea buffer was added to the remaining three sub-chambers. The run was set at 50 V for 30 min and increased to 200 V for another 2 h. Fractionated milk samples from the sub-chambers between the polyacrylamide membranes were then collected into separate tubes and designated as fractions F1 to F4 (Figure 4.1). The proteins in each collected milk fraction were precipitated with nine volumes of ice-cold acetone at -20 °C for 2 h. After centrifugation at 16000 × g, 4 °C for 15 min, the supernatant was removed from the protein pellet, which was then stored at -20 °C prior to analysis by SDS-PAGE (Section 2.3). In addition, all of the polyacrylamide membranes were stained with Ponceau S solution (Sigma Aldrich) to detect any residual proteins that adhered to the membranes.

4.2.5 Separation of whey and casein from skim milk by high-speed centrifugation

The whey and casein fractions of bovine and human skim milk (Section 4.2.3) were separated by high-speed centrifugation using a method described by Kunz and Lönnderal 1989, with slight modifications. Briefly, the pH of the skim milk was adjusted to 4.6 with 1 M hydrochloric acid, followed by the addition of 60 mM of calcium chloride/l prior to ultracentrifugation at 100000 × g, 4 °C, for 60 min. The supernatant containing the soluble fraction (including whey proteins and free oligosaccharides) was removed from the casein pellet and placed in a separate tube. The soluble fraction was mixed with five volumes of ice-cold acetone and stored at -20 °C for 2 h, then centrifuged at 16000 × g, 4 °C for 15 min. The supernatant was removed and the pellet containing precipitated whey proteins was carefully washed with 50 % (v/v) acetone. The washed protein pellet was air dried at RT to remove residual acetone, dissolved in a small volume of 100 mM ammonium bicarbonate, pH 8.0 and thoroughly mixed by vortexing. PBS was then added up to a volume equivalent of the original milk sample prior to use in the PVDF plate assay (Section 4.2.8).

The insoluble fraction (casein pellet) obtained from the ultracentrifugation was washed three times with PBS containing 0.01 M sodium hydroxide, followed by centrifugation of the solution at 8500 × g for 30 min. The casein sample was then dried and reconstituted with PBS to a volume equivalent of the original milk sample prior to use in the PVDF plate assay (Section 4.2.8). Protein concentration of the whey and casein fractions in bovine and human milk was measured by Bradford assay (Section 2.3). Results were recorded as mean ± SEM from three replicates per sample. SDS-PAGE was carried out to compare the protein content

of the casein and whey fractions from bovine and human milk (Section 2.3).

4.2.6 Fast protein liquid chromatography (FPLC) gel filtration of milk whey

The proteins in the whey fractions of human and bovine milk (Section 4.2.5) were further fractionated by fast protein liquid chromatography (FPLC) using an ÄKTA purifier 10 system (GE Healthcare, Buckinghamshire, United Kingdom) fitted with a pre-packed Superose 12 size exclusion column (10/300 GL, GE Healthcare) for gel filtration of molecules with molecular weights between 10 and 300 kDa as per the protocol of Kunz and Lönnerdal (1989), which was developed specifically for fractionation of human milk on a FPLC system. The running buffer contained 20 mM Tris-hydrochloric acid, 0.1 M sodium chloride, pH 8.0, and was degassed for 10 min and filtered through a Millipore membrane filter with a pore size of 0.22 µm before use. The system was flushed with 5 ml of the running buffer prior to injection of 1 ml of milk whey proteins at a concentration of 1 mg/ml. The elution flow rate was set at 0.4 ml/min (1 ml per fraction) and the pressure was maintained at less than 1.5 MPa. Proteins were detected at 280 nm and fractions were pooled based on the main peaks formed in the elution profile, resulting in three pooled samples designated A1, A2, and A3 for the bovine milk whey fractionation, and B1, B2, and B3 for the human milk whey fractionation (Figure 4.5). Each of these samples was filtered and buffer exchanged with PBS using NMWL cutoff Amicon ultrafiltration spin filters (Merck Millipore, Australia). A 100000 MW cutoff was used for A1 and B1, whereas a 10000 MW cutoff was used for A2, A3, B2 and B3 (Figure 4.5). The final volume of each sample was adjusted to 1 ml with PBS (volume equivalent to the 1 ml sample of whey that was injected onto the column). This process was repeated several times for bovine and human milk whey to obtain enough of the fractionated samples for the plate assay (Section 4.2.8).

4.2.7 Bacterial strains and growth conditions

The two gastrointestinal pathogens, *Escherichia coli* O111:NM (ATCC 43887) and *Salmonella typhimurium* (Group B) clinical isolate, and the commensal *Lactobacillus rhamnosus* (ATCC 53103) were used for the work described in this chapter. The growth conditions for the bacterial strains are described in Section 2.1.

4.2.8 PVDF plate assay

The PVDF plate assay established in Chapter 3 (Section 3.2.2) was used to compare bacterial binding to bovine and human milk fractions. Briefly, after the PVDF membranes were pre-wet with methanol and washed with PBS, a volume of 50 µl of milk fraction (proteins obtained as

described in Sections 4.2.2, 4.2.5 and 4.2.6) was added to each well and incubated on a plate shaker at 400 rpm for 16 h at 4 °C. The liquid was removed and the wells were washed three times with PBS. A volume of 50 µl of fluorescently-labelled bacteria at OD_{600} of 1.5 was added to the wells, incubated for 1 h at RT in the dark on the plate shaker, before all the liquid was removed and the well washed with PBS. Fluorescence of bacterial adhesion to the milk protein fractions was then measured. Data was adjusted for the difference in fluorescence uptake of the bacterial species (Section 3.2.2.3). The above basic procedure was also carried out with additional steps to potentially inhibit bacterial binding, as described below (Section 4.2.8.1 and 4.2.8.2).

4.2.8.1 Inhibition of bacterial binding with *N*-acetylneuraminic acid (sialic acid)

To determine the involvement of sialic acid in bacterial binding to proteins of bovine and human milk, the two approaches developed in Chapter 3 were used (detailed in Section 3.2.2.4). In brief, in the first approach, bacteria were bound to the milk whey proteins and caseins immobilised on the PVDF plate, then washed with free sialic acid to assess the ability of the free sialic acid to bind to and remove the bacteria. In the second approach, the bacteria were incubated with free sialic acid prior to measuring the binding of bacteria to the milk protein fractions. Controls were included, in which the respective incubation step was carried out in PBS alone.

4.2.8.2 Neuraminidase treatment of bovine and human milk proteins

The immobilised casein and whey proteins of bovine and human milk on the PVDF plate (prepared as described in Section 4.2.8) were treated with 50 µl of 0.05 U/ml α2-3,6,8 neuraminidase (Sigma-Aldrich) in 50 mM sodium phosphate, pH 5.5, for 16 h at 37 °C, as described in Section 3.2.2.5, to remove terminal sialic acid residues on the milk glycoproteins. The binding of fluorescently-labelled bacteria to the desialylated bovine and human milk protein fractions was then compared to the binding to non-desialylated control protein fractions, which had been incubated for 16 h at 37 °C in PBS alone.

4.3 Results

4.3.1 Skim milk fractionation by molecular size electrophoresis

The Microflow MF10 system was used for the fractionation of bovine and human skim milk based on molecular size. It was expected that proteins would be enriched in the following fractions: F1 > 500 kDa, F2 150 - 500 kDa; F3, 75 - 150 kDa and F4 5 - 75 kDa. However, as shown in Figure 4.2, all of the fractions from both (A) bovine and (B) human milk contained

many proteins of molecular weights other than those intended. There were indications of slight selectivity towards proteins of the desired molecular weight for some fractions. For example, very high molecular weight proteins were evident in the F1 fraction (> 500 kDa) of bovine milk (Figure 4.2 A, lane 2) but not in fractions F2 - 4, whereas proteins of 20 - 30 kDa was most abundant in the F4 fraction (5 - 75 kDa) of bovine milk (Figure 4.2 A, lane 5). However, the only proteins visible on the gel from the F4 fraction of human milk were between 80 and 115 kDa (Figure 4.2 B, lane 2), and proteins across all molecular weight ranges were evident in the human milk fractions F1, F2 and F3, resulting in a similar banding profile to that of total human skim milk (Figure 4.2 B, lane 4).

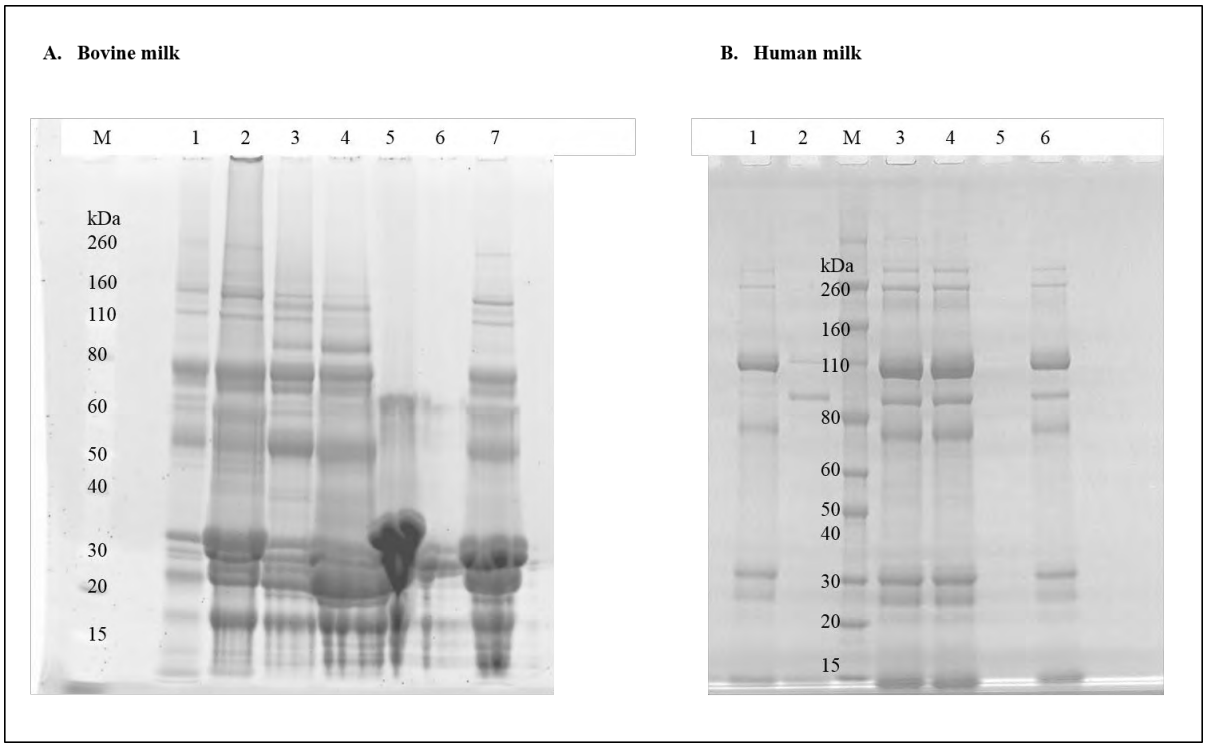


Fig. 4.2. SDS-PAGE analysis of protein fractions from (A) bovine and (B) human skim milk by the Microflow MF10 system. (A) Lane 1, pooled samples of that precipitated onto the membranes during separation; lanes 2 - 5, Fractions F1 - F4, respectively; lane 6, empty well; lane 7, crude bovine skim milk. (B) Lane 1, Fraction F2; lane 2, Fraction F4; lane 3, Fraction F1; lane 4, crude human skim milk; lane 5, empty well; lane 6, Fraction F3. Proteins of bovine and human milk were compared to a Novex® sharp pre-stained protein standard (M) and the gel was stained with SYPRO® Ruby Protein Gel Stain (Invitrogen ™). The decreasing pore size of the polyacrylamide membranes in the Microflow MF10 chamber were F1 > 500 kDa; F2 150 - 500 kDa; F3 75 - 150 kDa; and F4 5 - 75 kDa. Each fraction was loaded with standardised volume (2.5 µl) per well.

It was evident from the above gel analysis that fractionation of the skim milk samples according to molecular size had not been successfully achieved using the Microflow MF10 system. This may have been due to leakage of the milk sample from one fraction to another as

a result of a “bubbling” of the sample around the membrane cartridges that was observed during the electrophoresis. Staining of the polyacrylamide membranes with Ponceau S also revealed that a significant amount of protein had also precipitated onto the membranes, in particular the 5 kDa, 75 kDa and 150 kDa membranes (Figure 4.3; Figure 4.2 A, lane 1). Not only could this have resulted in protein loss, but also a partial clogging of the membrane pores, forcing some sample to “escape” around the membrane cartridges bypassing the gel filtration system.

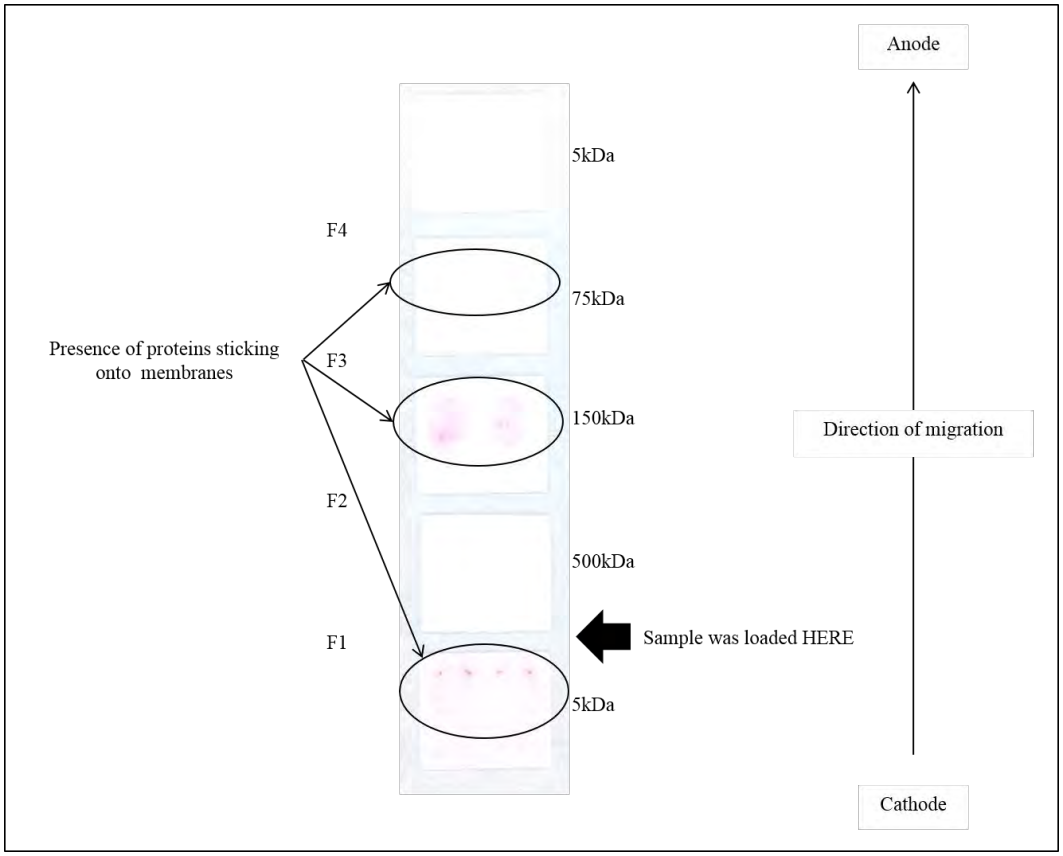


Fig. 4.3. Ponceau S staining of the polyacrylamide membranes after MicroFlow MF10 fractionation. Some milk proteins precipitated onto the membranes, especially the 150 kDa membrane and thus were not collected in the soluble fractions (F1 - F4).

It was concluded from the above work that the Microflow MF10 system, although promising as a method to separate protein mixtures in their native state in solution, was not appropriate for the fractionation of milk in our hands. Therefore, other techniques were trialled for more efficient separation of the protein components of bovine and human milk for further analysis.

4.3.2 Milk protein separation by centrifugation

The whey and casein fractions of bovine and human skim milk were separated by high-speed centrifugation (Section 4.2.5) and resuspended in PBS to volume equivalents of the original

skim milk samples. The protein concentrations of the bovine and human skim milk and their whey and casein fractions were determined by the Bradford protein assay (Table 4.1). The protein content of the bovine milk sample (15.1 mg/ml) was almost twice that of human milk sample (7.9 mg/ml). Caseins accounted for the majority of bovine milk proteins (11.4 mg/ml), whereas the casein content of human milk was much lower (2.8 mg/ml). In contrast, the protein content of human milk was predominantly in the whey fraction (4.9 mg/ml), whereas there was a slightly lower abundance of whey proteins in the bovine milk (3.2 mg/ml).

Table 4.1. Comparison of protein fractions of bovine and human milk.

	Protein concentration (mg/ml)	
Milk fractions	**Bovine milk**	**Human milk**
Skim milk	15.1 ± 0.2	7.9 ± 0.1
Whey	3.2 ± 0.6	4.9 ± 0.1
Casein	11.4 ± 0.6	2.8 ± 0.2

Data is represented as mean ± SEM for three replicates per sample.

SDS-PAGE analysis of the bovine and human skim milk proteins and their casein and whey protein fractions, separated by ultracentrifugation, is shown in Figure 4.4. The total proteins of bovine skim milk (Figure 4.4 A, lane 1) was dominated by proteins in the 20 - 35 kDa range, most probably consisting predominantly of casein subunits (Kunz and Lonnderdal, 1989). Another prominent band was evident at approximately 18 kDa and likely to represent β-lactoglobulin (MW 18 kDa, http://www.uniprot.org/). Total skim milk proteins of human milk (Figure 4.4 B, lane 4) also contained abundant proteins in the 20 - 35 kDa range suggestive of casein. In addition, bands of almost equal prominence were evident at a MW of approximately 80 kDa and 14 kDa, which were thought to represent the other most abundant proteins of human milk (Table 1.3), namely lactoferrin (unglycosylated MW 78 kDa, http://www.uniprot.org/) and α-lactalbumin (unglycosylated MW 14 kDa, http://www.uniprot.org/).

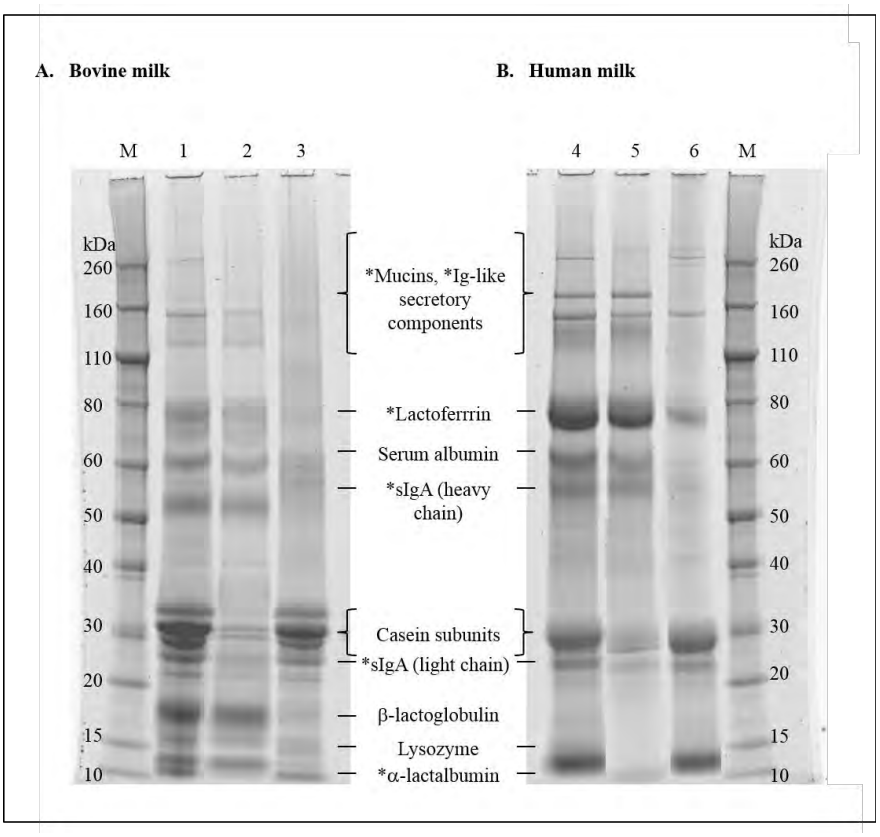


Fig. 4.4. SDS-PAGE analysis of (A) bovine and (B) human milk proteins. Lanes 1 and 4: total skim milk proteins; lanes 2 and 5: protein from the supernatant recovered after high-speed centrifugation of the skim milk with pH adjusted to 4.6 and the addition of calcium chloride ("whey fraction"). Lanes 3 and 6: proteins from the pellet formed by the ultracentrifugation procedure ("casein fraction"). Gels were stained with SYPRO® Ruby (Invitrogen ™). M: Novex® sharp pre-stained protein standard. The main proteins in bovine and human milk are labelled according to their expected molecular weight, and based on a similar gel resulting from milk fractionation by ultracentrifugation presented in the publication of Kunz and Lönnderdal (1989), in which the major proteins were subsequently identified by immunoelectrophoresis. The * denotes the milk protein is glycosylated.

Ultracentrifugation of the bovine and human skim milk for the enrichment of the casein and whey fractions resulted in a substantial separation of proteins. Proteins in the 20 - 35 kDa MW range, corresponding to the casein subunits, were strongly evident in the protein originating from the acid-precipitated pellet from both bovine and human milk samples (Figure 4.4, lanes 3 and 6, respectively). Individual bands within this molecular weight range have masses corresponding to glycosylated κ-casein (approximately 28 - 30 kDa, Yamauchi *et al.* 1981), and the unglycosylated components β-casein (24 kDa), α_{s1}-casein (22 - 24 kDa), and α_{s2}-casein (25 kDa), of which only β-casein is present in human milk (Chatchatee *et al.* 2001; Wal 1998). A significant reduction of proteins of other molecular weights was evident in the casein fractions of bovine and human milk, except for the prominent band at about 14 kDa in the human milk casein fraction. It is likely that the human α-lactalbumin (MW ~14 kDa) co-precipitated with casein as discussed in Section 4.4.

A corresponding massive reduction of proteins in the molecular weight range of the casein subunits could be seen in the soluble "whey fraction" retrieved from the supernatant after ultracentrifugation (Figure 4.4, lanes 2 and 5). Conversely, the abundance of proteins of the other molecular weight proteins was maintained, demonstrating substantial separation of these whey proteins from the casein. In particular, the band of ~ 80 kDa, with the molecular mass of lactoferrin, was one of the most abundant whey proteins in human milk as expected (lane 5), whilst the 18 kDa protein (β-lactoglobulin mass), was the dominant protein in the bovine milk whey fraction (lane 2). The other major protein in the whey of both types of milk is α-lactalbumin (unglycosylated MW 14 kDa). Protein of this approximate molecular weight was not clearly evident in the human milk whey fraction (lane 5) due to apparent co-precipitation with the casein (lane 6, as noted above), but could be strongly observed in the bovine milk whey fraction (lane 2).

The ultracentrifugation technique thus resulted in a better separation of the milk proteins than the MicroFlow technique (Section 4.3.1). Crude whey and casein fractions of bovine and human milk with only a low level of contaminating proteins from the opposing fractions were obtained, thereby enabling subsequent further fractionation of the whey (Section 4.3.3) and assessment of the relative binding of bacteria to the different milk protein fractions using the PVDF assay (Section 4.3.4).

4.3.3 Fractionation of milk whey by size exclusion on the ÄKTA FPLC system

After separating the soluble whey proteins from the acid-insoluble caseins (Section 4.3.2), fractionation of the bovine and human milk whey was carried out by size exclusion chromatography on the ÄKTA FPLC system such that larger proteins were eluted first from the column, followed by smaller proteins. To assist in identification of the eluted proteins, the elution profiles (Figure 4.5) were compared to the published elution profile obtained from human milk whey using a similar size exclusion and a FPLC technique (Kunz and Lönnderdal 1989).

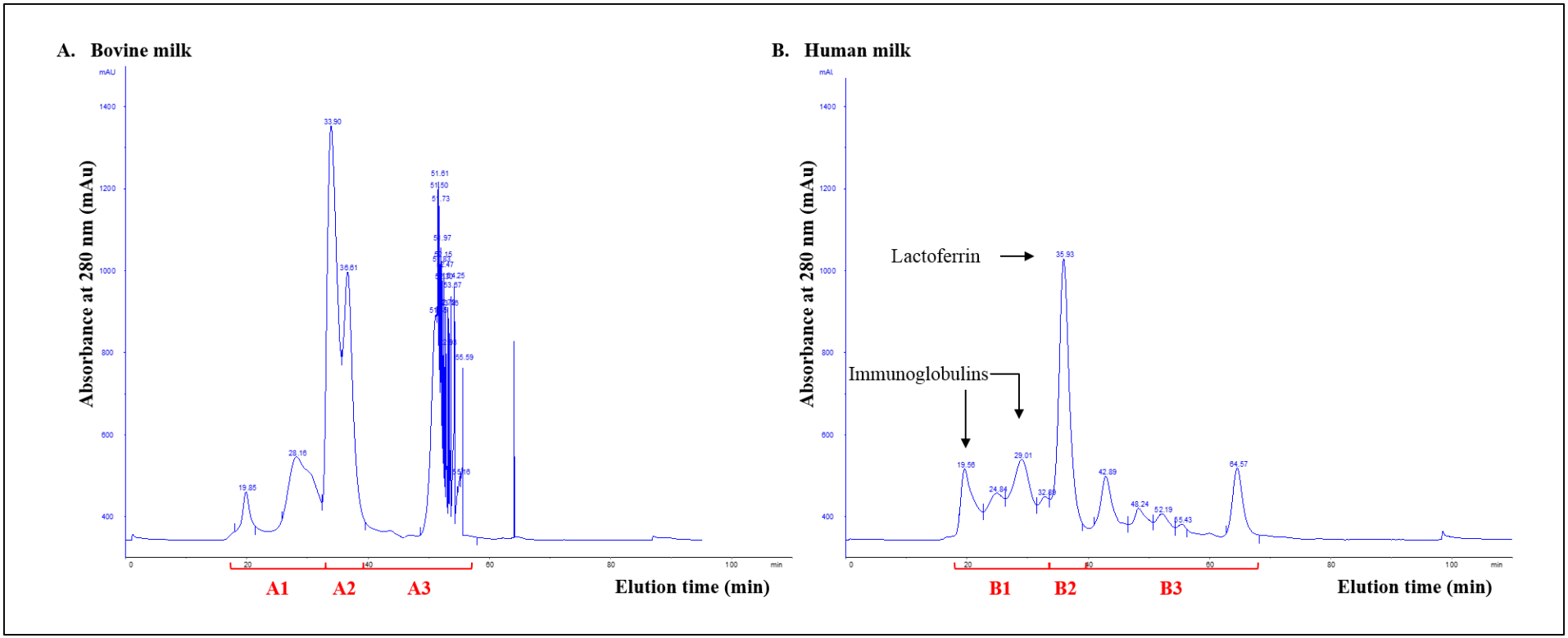


Fig. 4.5. Elution profiles of 1 mg of (A) bovine and (B) human milk whey proteins subjected to FPLC using a size exclusion Superose 12 (1000 - 300000) column. Proteins were detected by absorbance at 280 nm. The flow rate was 0.4 ml/min and 1 ml fractions were collected and combined according to the major elution peaks, resulting in three samples A1, A2 and A3 (from bovine milk whey) or B1, B2 and B3 (from human milk whey). Peaks in the elution profile of human milk have been tentatively labelled based on a published elution profile of human milk whey, in which proteins were subsequently identified and quantified by immunoelectrophoresis and immunodiffusion (Kunz and Lönnderdal 1989). The pooled fractions were buffer exchanged with PBS (final volume 1 ml per pooled fraction A1, A2 etc) using NMWL cutoff Amicon ultrafiltration spin filters prior to application of the protein in the PVDF plate bacterial adhesion assay.

The elution profile of human milk whey (Figure 4.5 B) was very similar to that obtained by Kunz and Lönnderdal (1989). Hence, the major proteins in the pooled fractions B1, B2 and B3 could be tentatively assigned as consisting of predominantly immunoglobulins (B1), lactoferrin/serum albumin (B2) and lower molecular weight proteins (B3). The probable identity of proteins represented by the peaks in the elution profile of bovine whey (Figure 4.5 A) were assigned based on comparison with the human milk whey profile (Figure 4.5 B), the known molecular weights and the profile of bovine milk α-lactalbumin and β-lactoglobulin by cation exchange separation (El-Sayed and Chase 2010). Fractions A1, A2 and A3 would thus comprise predominantly immunoglobulins (A1), serum albumin (A2) and lower molecular weight proteins, possibly including β-lactoglobulin and α-lactalbumin (A3).

The separation of the whey into molecular mass fractions was used to compare the relative contribution of the different protein fractions to the bacterial binding potential of the two milk origins.

4.3.4 Bacterial binding assays

4.3.4.1 Bacterial binding to total proteins of bovine and human milk

The total precipitated proteins of bovine and human milk (Section 4.2.3) were resuspended in PBS to the volume equivalent of the original milk samples, and 50 µl was applied per well of a PVDF 96-well plate. After the total milk proteins were immobilised on the plate and washing steps were carried out, the binding of the two gastrointestinal pathogens *E. coli* and *S. typhimurium* and the commensal *L. rhamnosus* was measured (Figure 4.6).

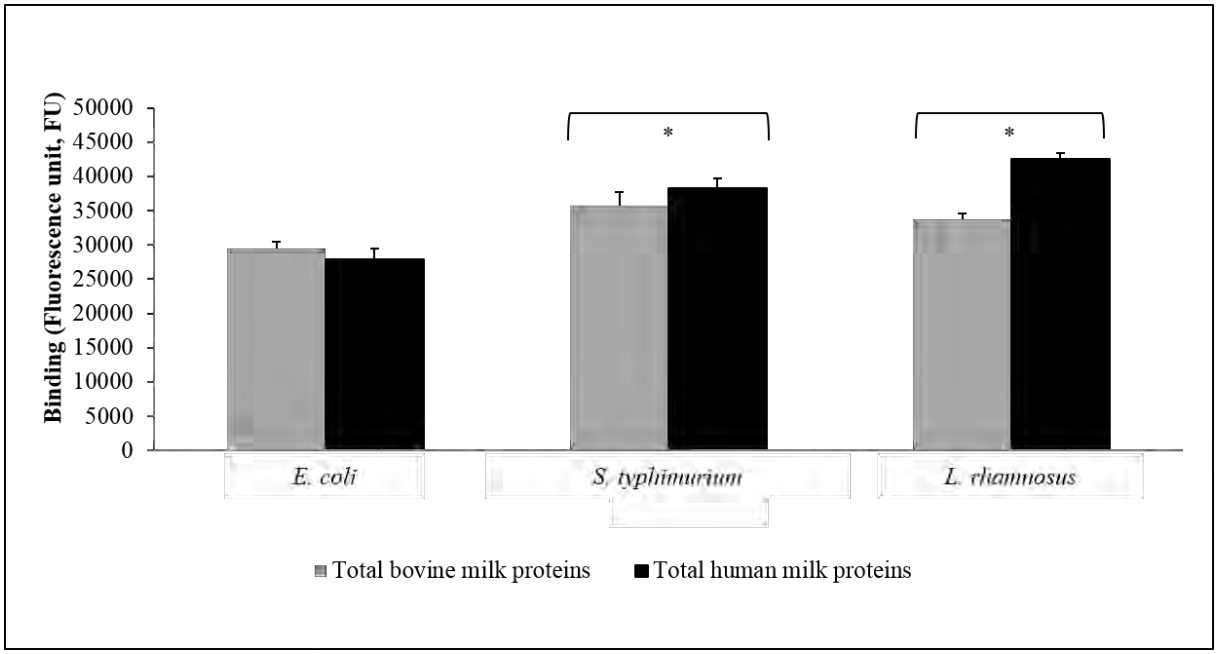


Fig. 4.6. Binding of gastrointestinal bacteria to bovine and human milk total proteins precipitated from 50 µl of milk (represented by fluorescence unit, FU). Data was corrected for the varied fluorescence uptake by the different bacteria and represents the mean ± SEM of three technical replicates. * = the binding of bacteria to total human milk proteins was significantly ($p<0.05$) higher compared to total bovine milk proteins precipitated from the equivalent volume of milk for *S. typhimurium* and *L. rhamnosus*.

As shown in Figure 4.6, *S. typhimurium* and *L. rhamnosus* bound significantly ($p<0.05$) better to the total human milk proteins than the total bovine milk proteins, despite the higher protein content of bovine milk (15.1 mg/ml) than in the equivalent volume of human milk (7.9 mg/ml; Table 4.1). No significant difference was found in the binding of *E. coli* to the bovine or human milk proteins. The binding of the commensal *L. rhamnosus* to human milk proteins was significantly ($p<0.05$) higher than the binding of the pathogenic bacteria to both human and bovine milk proteins. *Escherichia coli* bound slightly less efficiently to either total bovine or total human milk proteins compared to the other two bacteria (Figure 4.6).

4.3.4.2 Bacterial binding to major protein fractions of bovine and human milk

To determine the relative contribution of the major protein fractions to the capacity of the total milk proteins to bind the gastrointestinal bacteria, the casein and whey fractions of bovine and human milk were separated by centrifugation (Section 4.3.2) and resuspended in PBS to volume equivalents of the original milk samples. A volume of 50 µl of each of the milk samples was applied to the wells of a PVDF plate to allow immobilisation of the proteins. After washing steps, the binding of bacteria to the casein and whey fractions of bovine and human milk was measured (Figure 4.7).

All three gastrointestinal bacteria bound significantly ($p<0.05$) better to the human milk whey proteins than the bovine milk whey proteins. The binding of *S. typhimurium* to human milk casein was also higher than to bovine milk casein, whereas *E. coli* bound similarly to the two casein fractions. The commensal bacterium, *L. rhamnosus* bound slightly more to bovine milk casein than to human milk casein. However, it should also be noted there was a much higher amount of proteins in the bovine milk casein fraction (11.4 mg/ml) than in the volume equivalent of the human casein fraction (2.8 mg/ml; Table 4.1).

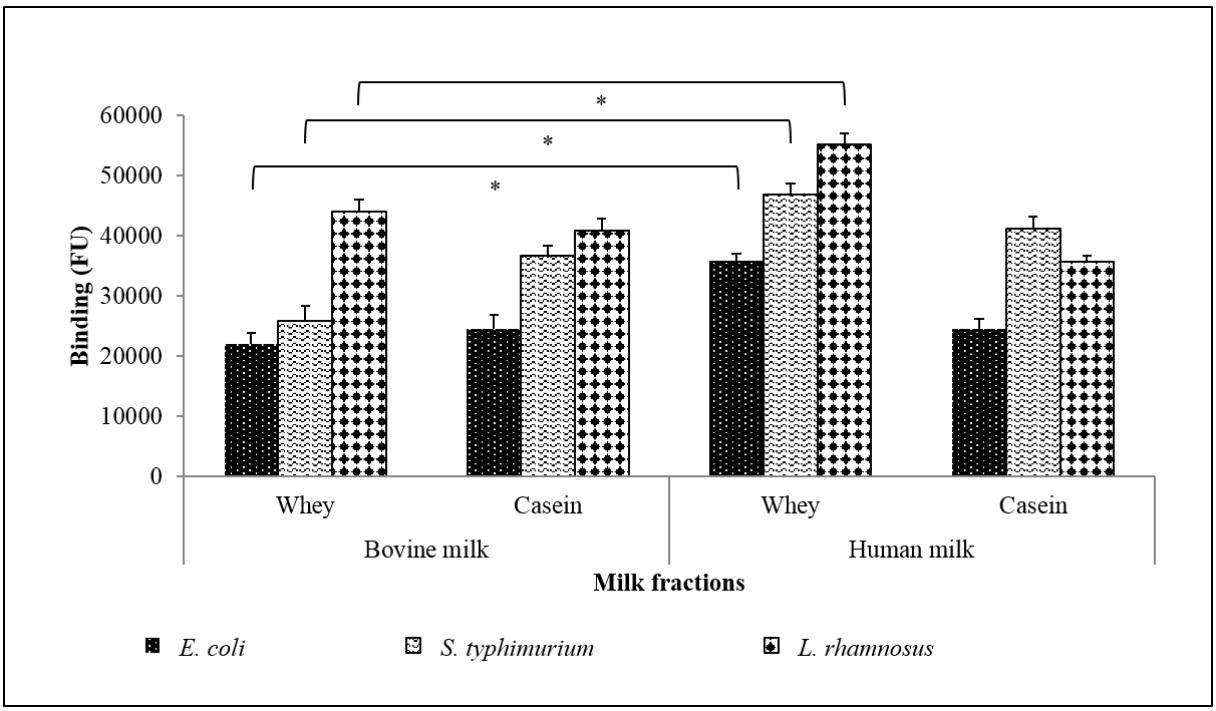


Fig. 4.7. Comparison of binding efficiencies of gastrointestinal bacteria to bovine and human milk whey and casein protein fractions extracted from 50 µl of bovine and human milk. Data was corrected for the varied fluorescence uptake by the different bacteria and represents the mean ± SEM of three technical replicates. * = the binding of all three bacterial strains to human milk whey proteins was significantly ($p<0.05$) higher compared to bovine milk whey proteins. Abbreviation: FU, fluorescence unit.

4.3.4.3 Bacterial binding to fractions of bovine and human milk whey

The whey contains the majority of the glycoproteins in milk (Table 1.3, Chapter 1) so to determine the contribution of the glycoproteins to bacterial adhesion, the whey proteins of bovine and human milk were further fractionated on a molecular mass basis by FPLC using a size exclusion column. Proteins from each of the pooled whey fractions (Section 4.2.6) were resuspended in equal volumes of PBS and 50 µl were loaded per well of the PVDF plate. After the proteins had been immobilised to the PVDF membrane, bacterial binding to each of the whey fraction was then assessed (Figure 4.8).

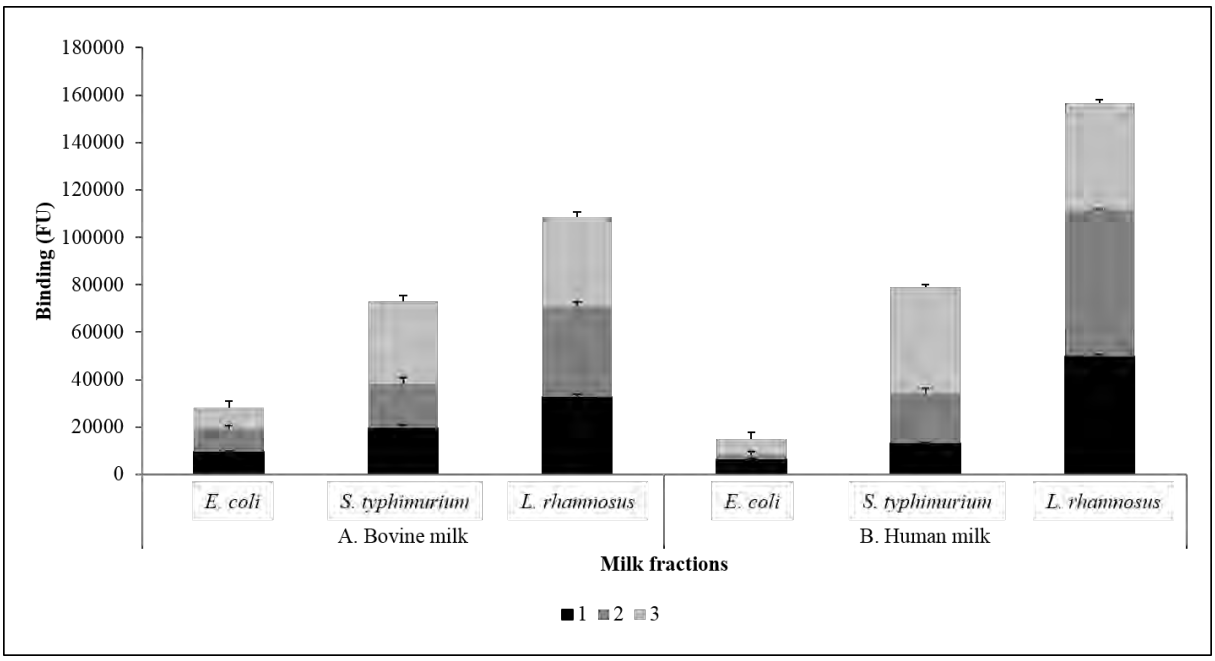


Fig. 4.8. Binding of bacteria to fractions of bovine and human milk whey obtained by FPLC using a size exclusion column (Figure 4.5). The molecular weight of proteins contained in each fraction decreases from fraction 1 to fraction 3. Data was corrected for the varied fluorescence uptake by the different bacteria and represents the mean ± SEM of three technical replicates. Abbreviations: FPLC, fast protein liquid chromatography; FU, fluorescence unit.

The binding of the three gastrointestinal bacteria to the fractions of bovine and human milk whey is presented in Figure 4.8, such that the total binding to the whey (ie. the three fractions, combined) can also be observed. Bacterial binding to both the total bovine milk whey and the total human milk whey increased from *E. coli*, to *S. typhimurium,* to *L. rhamnosus*, as observed previously (Figure 4.7). Also, the previously observed trend for increased binding to total human milk whey proteins compared to total bovine milk whey proteins was also evident for *L. rhamnosus,* but not for the pathogenic bacteria. Part of the reason why the sum of the parts is not equal to the total may be that equal protein amounts of bovine milk whey and human milk whey (1 mg) were used as the starting point for the whey fractionation by FPLC (Section 4.2.6). In comparison, Figure 4.7 relates to equal volume equivalents of the milks, where human milk contains slightly more whey (4.9 mg/ml) than bovine milk (3.2 mg/ml; Table 4.1).

A comparison of the bacterial binding to the whey fractions comprising different molecular weight ranges (Figure 4.8), revealed differences between the bacterial species and their binding affinities to the whey components from the two types of milk. The binding of *L. rhamnosus* was lower to each of the bovine milk whey fractions than to the corresponding fractions from human milk whey. The commensal bacterium displayed a fairly even binding

between each whey fraction from bovine milk, whereas there was a preference of binding to fraction 2 of human milk whey, containing the molecular mass of lactoferrin (Figure 4.5 B).

The binding of *S. typhimurium* was higher to the higher molecular weight proteins of human milk whey than to the other human milk whey fractions, and also higher than to the corresponding high molecular weight proteins of bovine milk whey. Binding of *S. typhimurium* to the mid and lower molecular weight fractions of human milk whey was equal or less than the binding of the pathogen to the corresponding fractions of bovine milk whey. Therefore, the higher overall binding of *S. typhimurium* to the total human milk whey than the bovine milk whey (Figures 4.7 and 4.8) appeared to be mainly attributable to binding to the higher molecular weight proteins of human milk whey that would comprise predominantly human milk immunoglobulins; Figure 4.5 B).

The binding of *E. coli* to all of the bovine milk whey fractions was higher than to the corresponding fractions of human milk whey. The total binding of *E. coli* to the bovine milk whey (fractions combined) was higher than that to human milk whey. An approximately equal binding of *E. coli* to the different whey fractions of bovine milk was observed, however the pathogen bound more to the high and low molecular weight proteins (Figure 4.8, fractions 1 and 3) of the whey than to the mid-range molecular weight proteins (Figure 4.8, fraction 2).

The capacity of both the bovine and human milk proteins to bind bacteria was seen to be due to both the whey and casein components of the milks, with the human milk protein binding generally better to the human gastrointestinal bacteria, particularly to the commensal bacterial species. The different molecular mass fractions of the whey from the milks of both human and bovine origin appeared to all contribute to the total binding capacity.

4.3.4.4 Inhibition of bacterial binding to milk fractions by free sialic acid

To investigate whether there was involvement of the glycans on the proteins in the binding of the bacteria to bovine and human milk whey and casein, free sialic acid was used as a potential competitive decoy. Whey and casein proteins were adhered to the wells of a PVDF plate, as described previously (Section 4.3.4.2). The involvement of sialic acid in bacterial binding to the milk fractions was determined by two approaches: 1) washing bound bacteria with free sialic acid after bacterial binding and 2) pre-incubating the bacteria with free sialic acid before bacterial binding (Section 3.2.2.4). The results presented here are relative to the binding of control samples, in which the bacteria were washed or pre-incubated, respectively,

in PBS alone.

As shown in Figure 4.9 A, all of the bacteria bound to the bovine and human milk whey and casein fractions decreased significantly ($p<0.05$) when washed with free sialic acid. Notably, the sialic acid wash removed more bacteria from the human milk whey and casein than from the bovine milk whey and casein fractions.

Pre-incubating the bacteria with free sialic acid produced an even greater reduction in bacterial binding (Figure 4.9 B) than washing with sialic acid. The same trend was observed in which pre-incubation with sialic acid inhibited the binding of the bacteria to the human milk whey and casein proteins more than to the bovine milk whey and casein proteins.

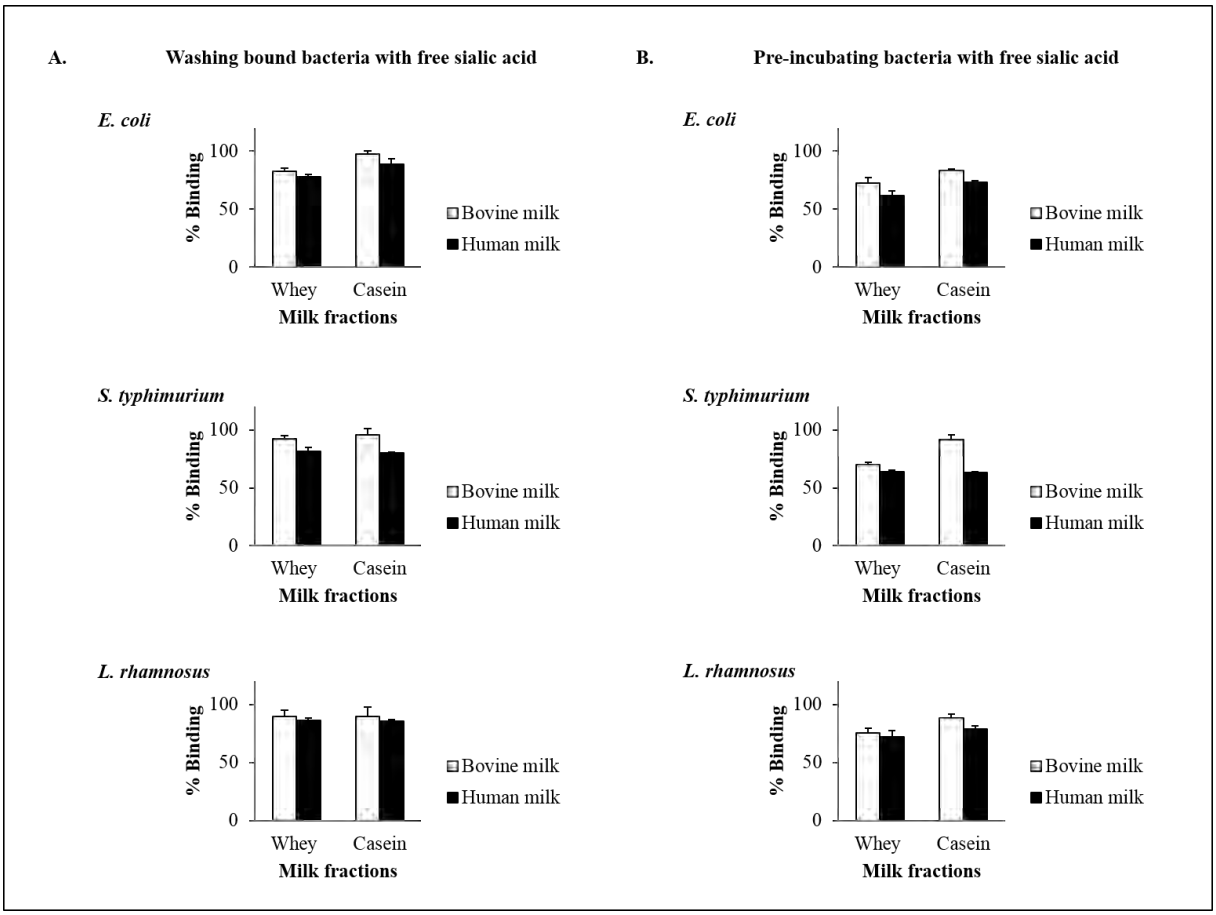


Fig. 4.9. (A) The binding of gastrointestinal bacteria to bovine and human milk whey and casein fractions after washing of pre-bound bacteria with free sialic acid. Binding is represented as a percentage (%) relative to the binding of controls, in which the pre-bound bacteria were washed with PBS alone (B) Bacterial binding to the milk fractions after pre-incubation of the bacteria with free sialic acid. Binding is represented as % relative to the binding of controls, in which the bacteria were pre-incubated in PBS alone. The mean ± SEM is shown for three technical replicates. The binding of all bacteria under both conditions (approaches A and B) to bovine and human milk proteins was significantly ($p<0.05$) reduced compared to the binding of PBS control samples.

The above results (Figure 4.9) gave a clear indication that there was glycan, in particular, sialic acid involvement in bacterial binding to the milk fractions. Specifically, it appeared that sialic acid provided a binding epitope for all the gastrointestinal bacteria. By inference, it appeared that the milk fractions contained sialic acid residues that were involved in bacterial binding, and that the binding to the human whey and casein fractions was more sialic acid-dependent than the binding to the bovine whey and casein fractions.

4.3.4.5 Binding of bacteria to bovine and human milk whey proteins and casein following neuraminidase treatment

To directly substantiate the involvement of sialic acid displayed on both bovine and human milk glycoproteins in the bacterial adhesion, the enzyme α2-3,6,8 neuraminidase was used to cleave the terminal sialic acid residues from the conjugated glycans of the bovine and human milk whey and casein proteins immobilised on the PVDF plate. Because the neuraminidase treatment involved a 16 h incubation period at 37 °C, controls (identical milk fractions immobilised to other wells of the plate that did not undergo neuraminidase treatment) were incubated in PBS for the same length of time at 37 °C.

There was a significant reduction in the binding of all of the bacteria following neuraminidase treatment to the whey and casein fractions of bovine and human milk (Figure 4.10). The effect of neuraminidase treatment on the inhibition of bacterial binding was significantly greater for the human milk whey and casein fractions than the bovine milk whey and casein fractions. These results were consistent with those of Section 4.3.4.4 and provided further evidence for sialic acid involvement in the adhesion of gastrointestinal bacteria to milk glycoproteins, particularly for the glycoproteins of human milk.

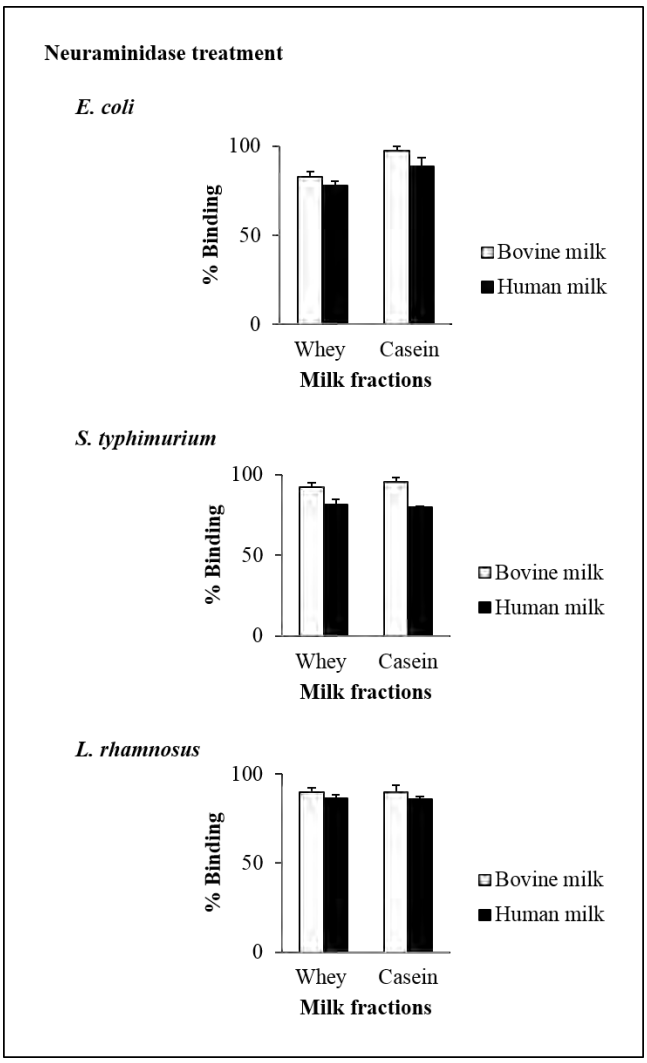


Fig. 4.10. Bacterial binding to bovine and human milk whey and casein after removal of terminal sialic acid residues from the immobilised milk glycoproteins by neuraminidase treatment at 37 °C for 16 h. Binding is represented as a % relative to the binding to control samples (immobilised milk proteins incubated for 16 h at 37 °C in PBS only). The mean ± SEM is shown for three technical replicates. The binding of all bacteria to desialylated bovine and human milk proteins with neuraminidase was significantly ($p<0.05$) reduced compared to non-desialylated bovine and human milk proteins (i.e. PBS control).

4.4 Discussion

The superior ability of human milk to protect infants against gastrointestinal disease compared to cow's milk and infant formulas has been well-documented (Section 1.1, Chapter 1). Although this may be partly attributed to the anti-microbial and immunoregulatory functions of the immunoglobulins and other unique human milk components, inhibition of bacterial adhesion to the gastrointestinal tract by components of human milk has also been demonstrated. In particular, human milk free oligosaccharides have been shown to adhere to gastrointestinal pathogens (Table 1.5, Chapter 1), and thus are hypothesised to act as decoys to remove the pathogens from the gastrointestinal tract before infection can occur. There has been little research to date in which comparisons have been made of the contribution of the

glycan component of bovine and human milk glycoproteins to bind to different gastrointestinal bacteria. Therefore, the work described in this chapter aimed to establish whether different protein components of bovine and human milk (whey and casein fractions) bind differentially to gastrointestinal pathogens, and then to gain an indication of the involvement of the glycan components in this interaction by specifically investigating the involvement of sialic acid as a possible bacterial binding epitope.

In order to study bacterial binding to different components of milk, it was first necessary to establish effective methods of milk fractionation. The first method trialled was a selective electrophoresis MicroFlow MF10 system, designed to separate native proteins by molecular size as they were passed through polyacrylamide membranes of decreasing pore size when a voltage was applied. Previous work using this approach has demonstrated successful separation of proteins from human plasma (Wasinger *et al.* 2005) and tears (Zhao *et al.* 2010) and assisted in the isolation of specific proteins from cell lysates and other complex samples (Catzel *et al.* 2003; Cheung *et al.* 2003; Gee *et al.* 2003; Ly and Wasinger 2010). However, the separation of milk protein using the MicroFlow MF10 system in this work was not so successful and discrete isolation of proteins according to molecular size was not fully achieved. This may have been due to sample leakage around the membranes, as evidenced by a bubbling around the membrane cartridges. The leakage may have been due to the precipitation of protein onto the membranes, which was later demonstrated by Ponceau S staining. The precipitated proteins may have blocked the membrane pores, forcing some the sample around the membrane cartridge, thereby avoiding selection by molecular size. The MicroFlow MF10 system was therefore not considered appropriate for the milk fractionation and other techniques were investigated.

An alternative method was used to precipitate the acid-insoluble caseins from soluble whey proteins in bovine and human milk. The process was based on previous work by Kunz and Lönnderdal (1989) and involved high-speed centrifugation with pH adjustment to precipitate the bovine and human caseins based on the protein isoelectric point (pI). SDS-PAGE analysis of the resulting protein fractions revealed a much better separation than had been achieved by the MicroFlow MF10 system. In particular, proteins in the expected molecular weight of the casein subunits predominated in the protein recovered from the pellet following ultracentrifugation, and were substantially reduced in the soluble whey fraction. Furthermore, proteins of the expected molecular weight of the most abundant proteins of bovine and human milk whey (Table 1.3) were evident on the gel; in particular, there was a high abundance of

protein of the approximate molecular weight of lactoferrin in the whey fraction of human milk, and a prominent band at the expected molecular weight of β-lactoglobulin in bovine milk whey. However, there was little indication of protein of the expected molecular weight of α-lactalbumin (~14 kDa) in the human milk whey sample, whilst a strong band of about 14 kDa was evident in the casein fraction. This may have indicated that the α-lactalbumin co-precipitated with the casein in this study. Precipitation of α-lactalbumin at a pH around its pI 4.2 at moderately warm temperatures (35 °C) has been reported previously (Bramaud *et al.* 1997).

The protein concentrations of the whey and casein fractions of bovine and human milk (Crittenden RG and Bennett LE 2005; Räihä 1985; Wal 1998, also see Table 1.3) were determined after resuspension of the separated protein in PBS to volume equivalents of the original milk samples. This gave an indication of the protein content contributed by the whey and casein fractions of each type of milk. The literature review reported in Chapter 1 had revealed considerable variation in the results of past research comparing the protein content of whey and casein fractions of milk. However, the majority of studies indicated that bovine milk contained approximately 26 g/l of casein compared to approximately 2.5 g/l casein in human milk (Table 1.3). The casein content of the bovine milk in this study (11.4 g/l) was substantially higher than in the human milk, but less than half that typically reported of bovine milk in the literature. Likewise the whey content of bovine milk in this study (3.2 g/l) was lower than typically reported (7 g/l; Table 1.3). As the bovine milk used in this study was sourced from only one cow, the differences in protein content compared to the average reported in the literature may simply be a factor of individual variation. The casein and whey content of the human milk in this study (2.8 g/l and 4.9 g/l, respectively) were very similar to that reported in past research. The fact that the human milk came from a pooled milk source from numerous mothers (Section 4.2.1) may have increased the likelihood of the protein content being similar to that of pooled milk samples in past research.

After obtaining the whey fractions of bovine and human milk, size exclusion was utilised to further separate the whey on the basis of the molecular weight of the milk proteins. An elution profile similar to that obtained from human milk using a similar method by Kunz and Lönnerdal (1989) was used to confirm the separation of the milk whey proteins. Confirmation was also made by SDS-PAGE gel analysis of the whey fractions obtained in this work (Figure 4.4) and that of Kunz and Lönnerdal (1989), in which protein identification and quantification was achieved by immunoelectrophoresis and immunodiffusion. As a result, the high mass

immunoglobulins; mid-range molecular weight proteins, lactoferrin and serum albumin; and low molecular weight protein, α-lactalbumin; were separated. The assignment of possible proteins to the eluted fractions of bovine milk was high molecular weight proteins containing immunoglobulins; mid-range molecular weight proteins including serum albumin; and low molecular weight proteins of β-lactoglobulin and α-lactalbumin; based on the known composition and molecular weights of the proteins in bovine milk.

The PVDF plate assay developed in Chapter 3 was used to compare the binding of the gastrointestinal pathogens *E. coli* and *S. typhimurium*, and the commensal *L. rhamnosus* to bovine and human milk proteins and their casein and whey components. The protein fractions were prepared such that their concentration was representative of their concentration in the original milk source. In this way, the relative contribution of the various components as they would appear in whole bovine and human milk could be compared. The order of binding affinities of the three bacteria to the total milk proteins followed the same trend for both bovine and human milk, with the highest binding recorded for *L. rhamnosus,* followed by *S. typhimurium* and then *E. coli*. *Lactobacillus rhamnosus* and *S. typhimurium* bound significantly ($p<0.05$) better to the total human milk protein than the total bovine milk protein, even though the protein content in human milk (8 mg/ml) was only just above half that bovine milk (15 mg/ml). This suggested that it was not the total quantity of protein in the respective milk samples as such that could have conferred the binding advantage of human milk proteins to some of these bacteria. There was no significant difference in the binding of *E. coli* to the total protein of the two types of milk, which implied that *E. coli* may use different mechanisms and/or different binding epitopes than the other two bacterial species.

Assessment of the binding of the gastrointestinal bacteria to the whey and casein fractions of bovine and human milk helped to elucidate which components of each type of milk are most important in the binding interaction. All the bacteria bound better to the whey fraction of human milk than to the casein fraction. Furthermore, human milk whey was superior to bovine milk whey in adherence to all three bacteria. This effect may be partly attributable to slightly higher whey content in the human milk used in this study (4.9 mg/ml) compared to the whey content of the bovine milk (3.2 mg/ml). However, the differences in binding of the bacteria to human and bovine milk whey was quite substantial, implying that there may also be other factors involved beyond the total protein content of the whey. Notably, human and bovine milk whey contain different proteins and in different proportions. For example, the glycoprotein lactoferrin is highly abundant in human milk whey but only present at a very low

abundance in bovine milk whey, whereas the unglycosylated β-lactoglobulin is the most abundant protein in bovine milk whey but is absent in human milk whey (Table 1.3, Chapter 1). Differences have also been established in the glycosylation profiles of human and bovine milk whey proteins (Nswosu *et al.* 2012), which may also be directly related to bacterial adhesion. Further investigation of the glycosylation profiles of the bovine and human whey proteins in this work is described in Chapter 5.

The binding of the bacteria to the fractions of bovine and human whey gave an indication of the broad molecular weight ranges of the constituent whey proteins that played a role in bacterial adhesion. All of the bacteria adhered to some extent to each of the whey fractions, indicating that proteins across a broad molecular weight range contributed to the overall bacterial binding affinity of the total whey. However, the relative binding affinity of the different species of bacteria used in this study to the various whey fractions did not follow the same pattern. The commensal *L. rhamnosus* bound most strongly to the human milk whey proteins in the mid-range molecular weight fraction, which most likely contained lactoferrin and serum albumin. The binding of gastrointestinal bacteria to human milk lactoferrin has been reported from numerous studies (Barboza *et al.* 2012; Bessler *et al.* 2006; de Araújo and Giugliano 2001; Giugliano *et al.* 1995; Gomez *et al.* 2003). In contrast, the binding of *L. rhamnosus* to the mid-range molecular weight proteins of bovine milk whey, which contains only very low levels of lactoferrin, was significantly less than that of human milk whey, again suggesting that it may be the lactoferrin that plays an important role in the increased adhesion of the commensal to human milk whey. However, the pathogenic *S. typhimurium* bound more efficiently to the lower molecular weight proteins of bovine and human milk whey, whilst *E. coli* adhered approximately equally to the high and low molecular weight proteins of the whey of both types of milk. Hence, although there may be some contribution to bacterial binding from whey proteins across the entire molecular weight range, the extent of binding to the different whey fractions appeared to be species specific.

To investigate the involvement of sialic acid in bacterial binding to the bovine and human milk whey and casein, the binding of *E. coli, S. typhimurium* and *L. rhamnosus* to the milk proteins was challenged by competitive inhibition with free sialic acid. This was achieved by three approaches: 1) washing the bound bacteria with free sialic acids, 2) pre-incubating bacteria with free sialic acid before binding, and 3) removal of sialic acid from the whey and casein proteins. All approaches significantly ($p<0.05$) reduced binding of the three bacterial strains to both human and bovine milk whey and casein. This implies that the adhesion of the

bacterial strains used in this study to milk glycoproteins from both species is at least partially sialic acid-dependent. Furthermore, as the inhibitory effect of free sialic acid and neuraminidase treatment of the glycoproteins on bacterial binding was greater for the whey and casein fractions of human milk than the respective fractions of bovine milk it can be inferred that sialic residues may play a greater role in the adhesion of the bacteria to the human milk glycoproteins than the bovine milk glycoproteins. However, small reductions in inhibition of bacterial binding to milk fractions with free sialic acid were observed (Fig 4.9), suggesting other mechanisms of adhesion. This may involve bacterial binding to different types of adhesion molecules such as other glycan structures or other parts of cells and proteins (Table 1.6).

The work in this chapter showed that gastrointestinal bacteria adhere to proteins of both bovine and human milk. Although the bacteria bound more strongly to human milk proteins than bovine milk proteins, there was evidence that both types of milk have the potential to bind to and remove pathogens from the gastrointestinal tract, and thus prevent gastrointestinal infection and diarrhoeal diseases. The strong adherence of the commensal bacterial species to milk may conversely offer a transport mechanism from the infant's mouth to gut colonisation. Compared to the pathogenic *E. coli* and *S. typhimurium*, the commensal bacterium, *L. rhamnosus*, consistently showed a higher binding affinity for bovine and human milk whey proteins and caseins. As discussed in Chapter 3, this highlights that the relationship between the microbes and milk proteins within the infant gut is a lot more complex than the assumption that consumption of milk will protect infants against infectious diarrhoea by selectively clearing just the pathogens from their body. The data clearly indicates that other complex factors could be involved that promote the colonisation of the commensals rather than the pathogens in the healthy gastrointestinal tract.

Fractionation of the milk helped to elucidate that all the milk component proteins may play a role in bacterial adhesion. It was evident that there was a preference of bacterial binding to human milk whey proteins than to the human milk caseins or either component of bovine milk. Glycan involvement, specifically sialic acid, in the bacterial adherence was also strongly indicated in this work. Further investigation of the glycan components of bovine and human milk whey and their involvement in adhesion and invasion of gastrointestinal bacteria were carried out in the work described in Chapter 5.

Chapter 5. Involvement of *N*-linked glycans from milk whey proteins in inhibition of pathogen adhesion and invasion of the gut

Rationale

It was evident from the work described in Chapter 4 that gastrointestinal bacteria bind to bovine and human milk whey proteins and that this binding is at least partially dependent on sialic acid. Furthermore, the bacterial binding to human milk whey was observed to be greater than to bovine milk whey. The whey proteins represent the main *N*-linked glycosylated proteins in milk and so the work described in this chapter aimed to determine more specifically the contribution that the *N*-linked glycans of bovine and human milk whey could play in pathogen adhesion and invasion of the gastrointestinal tract.

It has been proposed that milk glycans may prevent pathogen adhesion to the human gut by mimicking glycan receptors for pathogens on gastrointestinal epithelial cells, hence the work described here investigated the ability of the milk protein glycans to inhibit pathogen adhesion to, and invasion of, the human gastrointestinal epithelial cells (HT-29 cell line). In addition, monosaccharide analysis and mass spectrometry were used to gauge similarities and differences between the *N*-linked glycans of bovine and human milk whey proteins and the human gastrointestinal cell membrane proteins. This study aimed to achieve more insight into the role that *N*-linked glycans attached to bovine and human milk whey glycoproteins may play in protecting the gastrointestinal tract of infants against bacterial infection.

5.1 Introduction

In human milk, the predominant glycoproteins are found in the soluble whey fraction (Fortunato *et al.* 2003; Froehlich *et al.* 2010; Liao *et al.* 2011; Lönnerdal 2008; Peterson *et al.* 1998; Picariello *et al.* 2008; Smolenski *et al.* 2007). There is typically a similar abundance of total whey proteins in bovine and human milk, but the components of the whey vary. The major glycosylated whey proteins in human milk include immunoglobulins, lactoferrin and α-lactalbumin. Although bovine milk whey also contains immunoglobulins, α-lactalbumin and lactoferrin, the quantities of these glycoproteins are much lower than in human milk whey (Table 1.3, Chapter 1). The most abundant protein in bovine milk whey is β-lactoglobulin, which has not been reported to be glycosylated (Kalidas *et al.* 2001).

The work carried out in Chapter 4 revealed that specific human gastrointestinal bacteria bind significantly more to human milk whey proteins than to bovine milk whey proteins. Sialic acid involvement in this interaction was strongly established, and had a more pronounced effect on the binding of bacteria to human milk whey proteins than bovine milk whey proteins despite the fact that bovine milk has been reported to be more heavily sialylated than human milk glycoproteins (Nwosu *et al.* 2012). Hence, in the work carried out in this chapter, further investigation of the complete *N*-linked glycan structures of the whey proteins from bovine and human milk was carried out to help determine similarities and differences between the two *N*-linked glycan sets that could help to explain the differences observed in their pathogen interactions.

A number of studies have suggested that the protective effects of human milk against gastrointestinal infection may be due to the presence of the attached glycan structures on human milk glycoproteins that mimic glycan epitopes on gastrointestinal cell surfaces that normally form recognition and attachment sites for pathogenic bacteria (Georgi *et al.* 2013; Gopal and Gill 2000; Newburg 1999). Cultured human intestinal cells are commonly used as a model to simulate the human gastrointestinal tract (Gagnon *et al.* 2013; Nickerson *et al.* 2001). Several *in vitro* bacterial adherence and invasion assays have been conducted using bovine and human milk oligosaccharides as competitive inhibitors of adhesion (Coppa *et al.* 2006; Crane *et al.* 1994; Lane *et al.* 2012a; Yang *et al.* 2012), while the inhibitory activities of milk glycoproteins have been less examined. Hence, in the work described in this chapter, the ability of the *N*-linked glycans of the milk whey proteins to inhibit pathogen adhesion to human gastrointestinal cells (cell line HT-29) was investigated. Furthermore, the ability of the glycans to inhibit pathogens invading the cells after adhesion was also studied, a potential that

has received less attention in past research.

To further examine the similarities and differences in the *N*-linked glycan profiles of bovine and human milk whey proteins and human gastrointestinal epithelial cells, the work described in this chapter also involves analysis of the structures of the released *N*-linked glycans from each of these sources by mass spectrometry. Additionally, the total *N*-linked glycan yield from both mammalian milks was measured by monosaccharide analysis of the released *N*-linked glycans. Results from the work in this chapter will give further insight as to the involvement of *N*-linked glycans on milk glycoproteins in affecting the competitive interactions between bacteria and the epithelial cell surface of the gastrointestinal tract and the ingested milk that bathes the intestinal cells.

5.2 Materials and methods

5.2.1 Milk sample preparation for whey protein extraction

Approximately 10 ml of raw bovine milk (obtained from a Jersey cow after calving for two months at the Calmsley Hill City Farm, Sydney, Australia) and pooled human milk (donated by healthy mothers to a breast milk bank (Semmelweiß Frauenklinik, Vienna, Austria) were thawed and divided into 1 ml aliquots per tube. Whey proteins were then extracted from bovine and human milk using the protocol in Section 4.2.5 and resuspended in volume equivalents of the original milk samples.

5.2.2 Preparative release of *N*-linked glycans from bovine and human milk whey proteins

Protein concentration of bovine and human milk whey proteins (Section 5.2.1) was determined by Bradford assay. Approximately 3.2 mg/ml and 4.9 mg/ml of bovine and human milk proteins were calculated respectively. To equalise the amount of protein in each 1 ml sample of bovine and human milk whey to be used for the glycan release, the human milk whey sample was diluted to 3.2 mg/ml. The 1 ml (3.2.mg) samples of bovine or human milk whey proteins were then reduced in solution by the addition of 750 µl of 10 mM dithiothreitol (DTT; Bio-Rad Laboratories, CA, USA) in 100 mM ammonium bicarbonate and incubation for 45 min at 56 °C. Proteins were then alkylated by adding 750 µl of 55 mM iodoacetamide (IAA; Bio-Rad Laboratories, CA, USA) in 100 mM ammonium bicarbonate and incubating in the dark for 30 min at RT. The milk whey proteins were then incubated overnight at 37 °C with sequencing grade modified trypsin (V5111; Promega, WI, USA) using a trypsin-to-protein ratio of 1:50 (w/w). The samples were stored at -20 °C, and brought to RT before

glycan release.

N-linked glycans were released by overnight digestion at 37 °C with 10 U/mg of PNG*ase* F cloned from *Flavobacterium meningosepticum* and expressed in *E. coli* (Roche Diagnostics, with gentle agitation. Upon digestion, the released *N*-linked glycans were separated from the deglycosylated milk whey proteins by passing through an Amicon® centrifugal filter unit with 10 kDa NMWL (Millipore). The *N*-linked glycans were reduced according to Section 2.5 and desalted on homemade cation exchange column with a bed volume of 200 µl packed on top of a 1 ml Sep-Pak® C18 cartridge (Waters, Hertfordshire, UK), as previously described (Everest-Dass *et al.* 2012). The flow through the filtrate containing *N*-linked glycans was loaded onto a graphitised carbon column (GCC) solid phase extraction (SPE) cartridge for further purification as earlier described (Packer *et al.* 1998). The *N*-linked glycans were eluted from the cartridge with 0.05 % (v/v) trifluoroacetic acid in 40 % (v/v) acetonitrile and evaporated to dryness. After drying, the released *N*-linked glycans from each original 1 ml sample of whey proteins (3.2 mg/ml) were reconstituted in 1 ml of Milli-Q water. A volume (200 µl) of these purified *N*-linked glycans was set aside for mass spectrometry (Section 5.2.6) and monosaccharide analysis (Section 5.2.7). The remaining released *N*-linked glycans were then tested in two pathogen binding assay methods (Section 5.2.5) for their ability to inhibit bacterial interaction with both immobilised milk proteins and with the surface of the gastrointestinal epithelial cells.

5.2.3 Bacterial species

Three diarrhoea-causing human pathogens, *Campylobacter jejuni* NCTC11168, *Escherichia coli* O111:NM (ATCC 43887), *Salmonella typhimurium* (Group B) clinical isolate and a non-pathogenic bacterial strain, *Lactobacillus rhamnosus* (ATCC 53103) were used in this study. The growth conditions for these four human gastrointestinal bacteria are provided in Section 2.1.

5.2.4 HT-29 cell culture

HT-29 human colon carcinoma cell line (ATCC HTB-38) was used as a biological system to model the human gastrointestinal environment. HT-29 cells were grown in 10 ml Gibco® McCoy's 5A media (12330-031, Life Technologies, VIC, Australia), supplemented with 10 % (w/v) foetal calf serum and 100 µg/ml penicillin and streptomycin (15140, Invitrogen ™) in a Corning® 25 cm^2 cell culture flask (In Vitro Technologies, VIC, Australia) and incubated at 37 °C with 5 % carbon dioxide. After one week of culturing, the HT-29 cells were harvested

with trypsin/ethylenediaminetetraacetic acid (EDTA). The cells were collected either for membrane preparation (Section 5.2.8) or bacterial adherence and invasion assays (Section 5.2.5).

5.2.5 Bacterial binding and invasion assays

5.2.5.1 PVDF plate assay for measuring bacterial interaction with glycoproteins of bovine and human milk and the inhibition of binding by *N*-linked glycans from whey

The PVDF plate assay technique designed in Chapter 3 (Section 3.2.2) was used to determine the inhibition of bacterial binding to casein and whey fractions of bovine and human milk by *N*-linked glycans released from bovine and human whey proteins. A volume (50 µl) of whey or casein proteins from bovine and human milk (equating to representative casein and whey protein quantities in the volume equivalents in the milk from each source) was added to each well. The plate was incubated on a plate shaker at 400 rpm for 16 h at 4 °C. The liquid was removed and the wells were washed thrice with PBS. Each bacterial strain was stained with SYBR Green (Section 2.2) and resuspended in PBS to an OD_{600} of 1.5. Samples (50 µl) of each bacterial suspension were performed in triplicate and pelleted by centrifugation before resuspension in 50 µl of *N*-linked glycans released from the volume equivalents (50 µl) of 3.2 mg/ml bovine and human milk whey proteins (Section 5.2.2; i.e. equal to *N*-linked glycans released from approximately 160 µg of whey proteins). Bacteria were pre-incubated with the released *N*-linked glycans for 30 min prior to application of the bacterial-glycan mixture to each well containing either whey proteins or caseins of bovine and human milk. After 30 min incubation at RT in the dark on the plate shaker, the unbound bacteria were removed by washing four times in PBS. Fluorescence was measured by the FLUOstar GALAXY (BMG LABTECH) fluorescence microplate reader, where the filters were set at λ_{ex} = 485 nm and λ_{em} = 520 nm for SYBR Green fluorescence reading. Each plate assay was performed using three technical replicates. Results were compared to controls, in which bacteria were pre-incubated in PBS only (ie. no glycans) prior to binding to the milk proteins.

5.2.5.2 Cell-based assay for measuring bacterial adherence and invasion of HT-29 cells

Prior to harvesting the cultured HT-29 cells (Section 5.2.4), NUNC™ 24-well polystyrene flat-bottom plates (Thermo Fisher Scientific) were prepared with each well coated with 1 ml collagen I, rat tail (0.34 mg/ml; A1048301, Invitrogen ™) for 20 min at 37 °C in 5 % carbon dioxide. Each plate was then seeded with HT-29 cells at a concentration of 5×10^5 cells per well and the cells were grown in a confluent monolayer for 48 h at 37 °C with 5 % carbon

dioxide. HT-29 cell monolayers were washed three times with antibiotic free Gibco® McCoy's 5A media supplemented with 10 % (w/v) foetal calf serum to remove excess spent media. Each bacterial strain was applied to the HT-29 cells at a multiplicity of infection (MOI) of 100 i.e. there was approximately 5×10^7 bacteria applied to each well. The bacteria were incubated with the HT-29 cells for 3 h at 37 °C and 5 % carbon dioxide to allow bacterial adherence and invasion to occur.

Bacterial adherence and invasion assays were carried out in parallel using two separate plates with triplicate technical replicates on each plate. Both adherence and invasion assays were performed as previously described (Man *et al.* 2010) with slight modifications. After the 3 h incubation period of the HT-29 cell monolayers with the bacteria, the two plates were washed three times with antibiotic-free Gibco® McCoy's 5A media to remove unbound bacteria. The plate used for the invasion assay was also treated with 200 mg/ml of Gibco® gentamicin (Life Technologies) for 1 h at 37 °C with 5 % carbon dioxide to kill any bacteria that had bound to the exterior of the HT-29 cells. The cells were then washed three times with antibiotic free Gibco® McCoy's 5A media to remove traces of gentamicin solution and dead bacteria.

For both plates, the HT-29 cell monolayer was lysed with 0.5 ml 1% (v/v) Triton X-100 for 5 min at 37 °C with 5 % carbon dioxide to release either adhered or internalised (invaded) bacteria. The lysate solution was serially diluted for each bacterial strain. Each dilution was plated in quadruplicates on appropriate agar plates (either HBA or Nutrient agar) and plates were grown according to conditions as mentioned earlier (Section 2.1). Bacterial adherence was calculated by subtracting the number of internalised bacteria (invasion assay) from the total number of bacteria counted (adherence assay) and expressed as a relative percentage of the initial number of bacteria applied.

To assess the ability of the released *N*-linked glycans from bovine and human milk whey to inhibit bacterial adherence to and invasion of HT-29 cells, 50 µl of the bacterial suspensions (containing approximately 5×10^7 bacterial cells) were pelleted and resuspended in 50 µl of the *N*-linked glycans released from volume equivalents of 3.2 mg/ml bovine and human milk whey proteins, as described in Section 5.2.5.1. The bacteria were incubated with the *N*-linked glycans of bovine and human milk whey proteins for 30 min. Controls (without *N*-linked glycans) were also prepared, in which the bacteria were pre-incubated for 30 min with PBS only. The bacteria were then applied to the HT-29 cells. After three hours incubation, binding and invasion of the HT-29 cells by bacteria pre-incubated with the *N*-linked glycans was

compared to that of bacteria pre-incubated with PBS alone. The bacterial-glycan mixture was also plated onto appropriate agar plate in triplicate to check if the *N*-linked glycan solution of bovine and/or human milk can cause bacterial cell lysis. Bacterial colonies formed on the agar plate were counted to determine the number of viable cells. The statistical significance of the differences between the levels of adherence and invasion (mean ± SEM) of the HT-29 cells by bacteria pre-incubated with the *N*-linked glycans compared to that of bacteria pre-incubated with PBS alone (i.e. without *N*-linked glycans) was analysed separately for each bacterial species using two tailed unpaired Student's t-test with Microsoft Excel, where results were considered significant for $p<0.05$ (*), highly significant for $p<0.01$ (**) and exceedingly significant for $p<0.001$ (***).

5.2.6 Mass spectrometric analysis of *N*-linked glycans released from bovine and human milk whey proteins

A small volume of 2.5 µl of the released *N*-linked glycans from bovine and human milk whey glycoproteins (Section 5.2.2) were analysed by carbon LC-ESI-MS in the negative ion mode, using the conditions described in Chapters 2 and 3. To predict the possible glycan compositions on milk whey proteins, the calculated mono-isotopic experimental masses $[M-H]^{1-}$ were input to the GlycoMod program (http://web.expasy.org/glycomod/), with a mass tolerance set at ± 0.5 Da (see Section 2.5). The potential *N*-linked glycan structures corresponding to the monosaccharide compositions were predicted if possible by MS/MS analysis. The corresponding relative abundances of the most abundant *N*-linked glycans were determined by integration of the mass EICs. The percentage abundance of each structure was calculated by dividing by the sum of the abundances of the total *N*-linked glycans.

5.2.7 Monosaccharide analysis of released *N*-linked glycans from bovine and human milk whey proteins

Monosaccharide analysis was performed to quantitate the released *N*-linked glycans from 20 µg of bovine and human milk whey proteins. The released glycans were hydrolysed using 2 M trifluoroacetic acid at 100 °C for 4 h (neutral sugar analysis), 4 M hydrochloric acid at 100 °C for 6 h (amino sugar analysis) and 0.1 M trifluoroacetic acid at 80 °C for 40 min (acidic sugar analysis), according to the protocol in Section 3.2.2.7.

5.2.8 Membrane preparation of HT-29 cells prior to glycan analysis

The membrane proteins of HT-29 cells were prepared by PhD student Jenny Chik, as previously described (Lee *et al.* 2009) with slight modifications. Briefly, cell pellet containing

approximately 1 x 10^7 cells were resuspended with 2 ml of lysis buffer containing 50 mM Tris-hydrochloric acid, 100 mM sodium chloride, 1 mM EDTA and protease inhibitor (pH 7.4, Roche Diagnostics) and chilled on ice for 20 min. The cells were thoroughly homogenised with a Polytron homogenizer (Omni TH homogeniser, Omni International Inc, VA) on ice for 15 min. The homogenised HT-29 cells were centrifuged at 2000 × g for 20 min at 4 °C to remove cellular proteins. The supernatant was collected and diluted with 2 ml of Tris binding buffer (20 mM Tris-hydrochloric acid, 100 mM sodium chloride, pH 7.4) and proteins were sedimented by ultracentrifugation at 120000 × g for 80 min at 4 °C. The supernatant was discarded and 140 µl of Tris binding buffer was added to resuspend the pellet which comprised of a mixture of membrane proteins and cell membranes. A volume of 450 µl of Tris binding buffer containing 1 % (v/v) Triton X-114 was added to the suspended mixture and chilled on ice with intermittent pipetting for 10 min. The samples were heated at 37 °C for 20 min and phase partitioned by centrifugation at 1000 × g for 3 min. The upper aqueous phase was carefully collected and stored at -20 °C for further analysis, whereas the lower detergent phase containing membrane proteins were precipitated with 1 ml of ice-cold acetone and left overnight at -20 °C. Precipitated membrane proteins of HT-29 cells were pelleted by centrifugation at 1000 × g for 3 min and solubilised in 10 µl of 8 M urea for further use.

5.2.9 PNG*ase* F treatment of HT-29 cell membrane proteins to release *N*-linked glycans for mass spectrometry analysis

N-linked glycans were released from HT-29 cell membrane proteins as previously described (Jensen *et al.* 2012). Briefly, the resolubilised membrane proteins of HT-29 cells (Section 5.2.8) were immobilised by dot blotting onto an Immobilon-P PVDF membrane (0.45 µm, Millipore). A glycoprotein standard containing 10 µg of bovine fetuin (Sigma-Aldrich) was also blotted on to the same membrane as a control. The *N*-linked glycans were released with 3 U of PNG*ase* F (Roche Diagnostics) overnight at 37 °C. The released *N*-linked glycans of HT-29 membrane proteins were reduced and desalted according as described in Chapters 2 and 3. The glycan alditols were finally resuspended in 15 µl of Milli-Q water prior to mass spectrometry analysis as described in Section 5.2.6.

5.3 Results

5.3.1 Inhibition of bacterial binding to milk fractions by *N*-linked glycans from bovine and human milk whey proteins

The involvement of the full structures of the *N*-linked glycans of bovine and human milk whey proteins in the binding of the three gastrointestinal bacteria *E. coli*, *S. typhimurium* and

L. rhamnosus used previously, plus the semi-anaerobic pathogenic *C. jejuni*, was tested by determining the extent of inhibition of the bacterial adhesion to whey and casein by pre-incubation with the released milk whey protein *N*-linked glycans. The four bacterial strains were incubated with 50 µl of *N*-linked glycans released from 160 µg bovine and human milk whey proteins (Section 5.2.2) prior to measuring their binding to immobilised bovine and human casein and whey proteins on a PVDF plate. Binding inhibition by the *N*-linked glycans was calculated by comparison to the binding of control samples of bacteria which had been incubated in PBS alone.

Firstly, the binding of the four bacteria to bovine and human whey and casein proteins (without *N*-linked glycan inhibition) is presented in Figure 5.1, revealing similar trends for the three bacteria to those observed in Figure 4.7 (Chapter 4). In particular, the binding of all the gastrointestinal bacteria was lower to bovine milk whey proteins than to human milk whey proteins (prepared from volume equivalents of milk). Overall, *C. jejuni* resembled the binding potential of *S. typhimurium* and both of these pathogens adhered more to the milk proteins than *E. coli*.

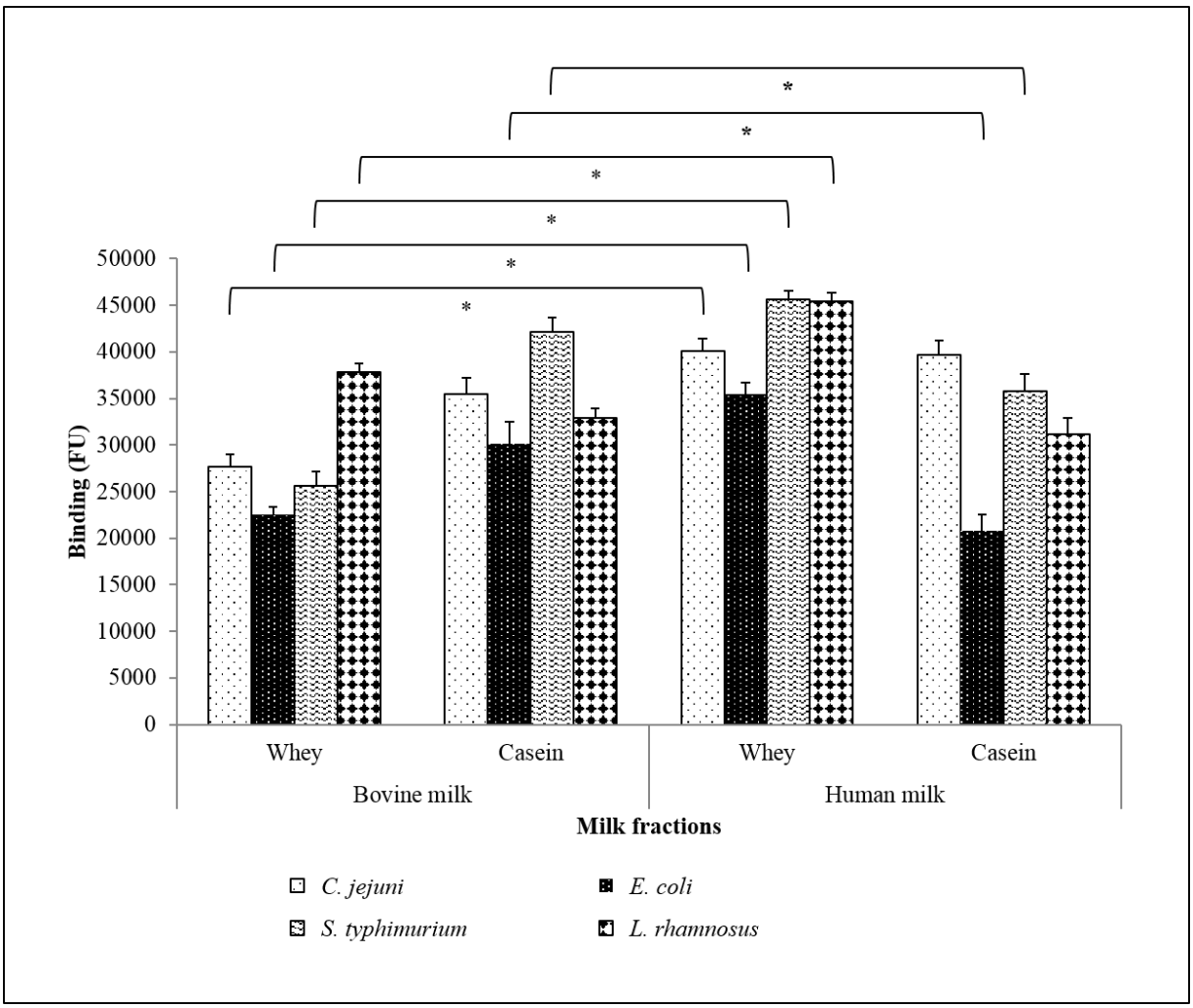


Fig. 5.1. The binding of four gastrointestinal bacteria to bovine and human milk whey and casein proteins extracted from 50 µl of bovine and human milk. These data represent the controls for the experiment shown in Figure 5.2, in which the bacteria were incubated for 30 min in *N*-linked glycans prior to binding to the milk proteins. Control samples were incubated for the same time in PBS alone. Data were corrected for the varied fluorescence uptake by the different bacteria and represent the mean ± SEM of three technical replicates. The binding of bacteria to human milk whey proteins was significantly (* = $p<0.05$) higher compared to bovine milk whey proteins for all four bacterial strains. In contrast, only *E. coli* and *S. typhimurium* binding was significantly ($p<0.05$) different between bovine and human milk casein proteins. Abbreviation: FU, fluorescence unit.

Pre-incubation of the bacteria in the *N*-linked glycans released from bovine and human milk whey proteins (Section 5.2.2) inhibited bacterial binding of all the strains to both bovine and human milk casein and whey proteins to some extent (Figure 5.2 and Table 5.1). This clearly indicated that *N*-linked glycans are at least in part involved in the binding of all four bacteria to both whey and casein of bovine and human milk glycoproteins.

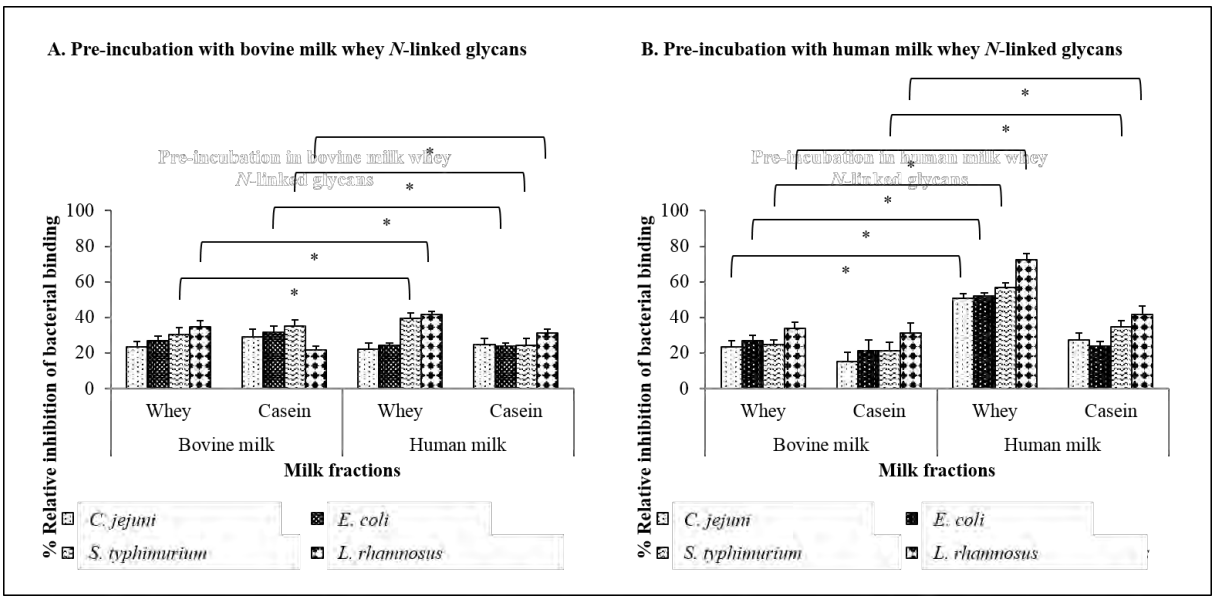


Fig. 5.2. Inhibition of bacterial binding to bovine and human milk whey and casein by pre-incubation of the bacteria in: A) *N*-linked glycans from bovine milk whey and B) *N*-linked glycans from human milk whey. The * denotes the inhibition of bacterial binding to bovine and human milk proteins extracted from whey and casein fraction was significantly ($p<0.05$) different.

As would be expected, the highest degree of inhibition (50 - 70 %) of the binding of all the gastrointestinal bacteria to human milk whey followed from pre-incubation with the human milk whey *N*-linked glycans (Figure 5.2 B). The binding of *L. rhamnosus* was inhibited most (72 % inhibition), indicating a strong dependence of this commensal bacterium which binds to *N*-linked glycans. Some inhibition by the *N*-linked human milk whey glycans was also observed for the binding of all bacteria to human milk casein, and to bovine milk whey and casein, but the degree of inhibition was only about half of the inhibition of binding to the human milk whey (Figure 5.2 B).

Pre-incubation of the bacteria in the *N*-linked glycans of bovine milk whey also resulted in a similar degree of inhibition of bacterial binding to bovine milk casein and whey fractions (Figure 5.2 A), as achieved by the *N*-linked glycans of human milk whey (20 - 30 %; Figure 5.2 B). However, less inhibition of bacterial binding to human milk whey and casein fractions were achieved by the bovine milk whey *N*-linked glycans than the human milk whey *N*-linked glycans.

Overall, the results of this experiment indicated predominantly that the *N*-linked glycans of both bovine and human milk are involved in bacterial binding since the glycans alone inhibited the binding to all the milk fractions. Also clear is that the adhesion is not entirely

glycan dependant. There were some similarities in the effects on the binding of the *N*-linked glycans from bovine and human milk whey, indicating that both may contain binding epitopes for both pathogenic and commensal human gastrointestinal bacteria. However, the *N*-linked glycans from human milk whey appear to have greater ability to bind to these human infective bacteria than *N*-linked glycans from bovine milk, a phenomenon that may be caused by the nature or abundance of the *N*-linked glycans in these milk fractions.

5.3.2 Comparison of the ability of human gastrointestinal bacteria to adhere and invade HT-29 cells

The previous section demonstrated that the *N*-linked glycan component of both bovine and human milk whey glycoproteins can bind to gastrointestinal bacteria. To test the hypothesis that these *N*-linked milk whey protein glycans can compete with the adhesion and invasion of the human intestinal cells by these bacteria, an assay using live gastrointestinal cells (HT-29 cell line) was established (Section 5.2.5.2).

Lactobacillus rhamnosus demonstrated the greatest capacity to bind to HT-29 human gastrointestinal cells, with 7.9 % of the initial inoculum of *L. rhamnosus* adhering to the HT-29 cells, compared to 6.1 % of *S. typhimurium*, 5.2 % of *C. jejuni* and 2.1 % of *E. coli* (Figure 5.3). These data suggest that the beneficial gut bacterium (probiotic) binds slightly better to gastrointestinal cells than the gastrointestinal pathogens. It also implies that the human gastrointestinal cells express bacterial binding epitopes on their cell surfaces that are recognised by all four bacteria strains to a differing extent.

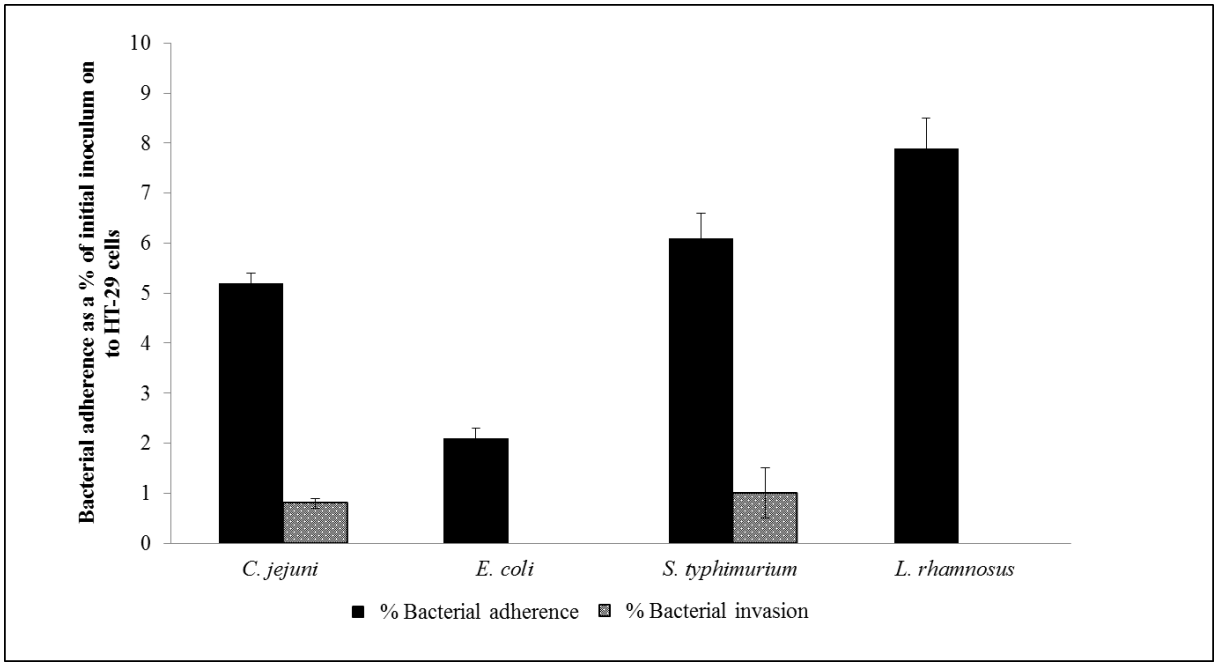


Fig. 5.3. Bacterial adherence and invasion of HT-29 human gastrointestinal epithelial cells. Results are expressed relative to the number of bacteria inoculated. Error bars represent the standard error of the mean for two independent experiments performed in triplicate.

Invasion of the HT-29 cells was only observed for *C. jejuni* (0.8 %) and *S. typhimurium* (1 %) (Figure 5.3). *E. coli* and *L. rhamnosus* did not invade the HT-29 cells, which was as expected since these are non-invasive species (Wilson *et al.* 2002).

5.3.3 Inhibition of bacterial adhesion and invasion of HT-29 human gastrointestinal cells by pre-incubation with *N*-linked glycans from bovine and human milk whey proteins

The assay was used to test the capacity of milk whey *N*-linked glycans to compete with the adhesion and invasion of the different bacteria into gastrointestinal cells. The ability of the gastrointestinal bacteria to adhere to and invade HT-29 cells after pre-incubation with *N*-linked glycans from bovine or human milk whey glycoproteins was compared to a control, in which bacteria were pre-incubated with PBS only (Figure 5.4).

Only 2 - 8 % of the applied bacteria (approximately 5×10^7 bacterial cells/well) adhered to the intestinal cells in the cell-based assay. However, the adherence of all four bacteria to HT-29 cells was significantly ($p<0.001$) inhibited by pre-incubation with released *N*-linked glycans from bovine or human milk whey proteins (Figure 5.4). The adherence of *C. jejuni*, *E. coli*, *S. typhimurium* and *L. rhamnosus* to HT-29 cells were inhibited by 23 %, 24 %, 46 %, and 51 %, respectively, when they were pre-incubated with 50 µl of *N*-linked glycans released from a volume equivalent of bovine milk whey proteins (160 µg) prior to binding to HT-29 cells. However, an even greater inhibition of bacterial binding to HT-29 cells was achieved by pre-incubation with 50 µl of released *N*-linked glycans from an equal quantity of human milk whey proteins with *C. jejuni* (54 %), *E. coli* (44 %), *S. typhimurium* (50 %) and *L. rhamnosus* (76 %). The glycans attached to human milk whey glycoproteins thus appear to act as better inhibitors of adherence of the gastrointestinal bacteria to human gastrointestinal cells than the glycans attached to bovine milk whey. Interestingly, it is noted that the binding of *L. rhamnosus* to the HT-29 cells was inhibited significantly ($p<0.001$) more by the whey glycans from both bovine and human milk than the pathogenic bacteria, with the greatest inhibition again achieved by the human milk whey *N*-linked glycans. The preferred binding of *Lactobacillus* to the milk glycans that is again shown here, may act as a mechanism for transport of these commensal bacteria to the gut.

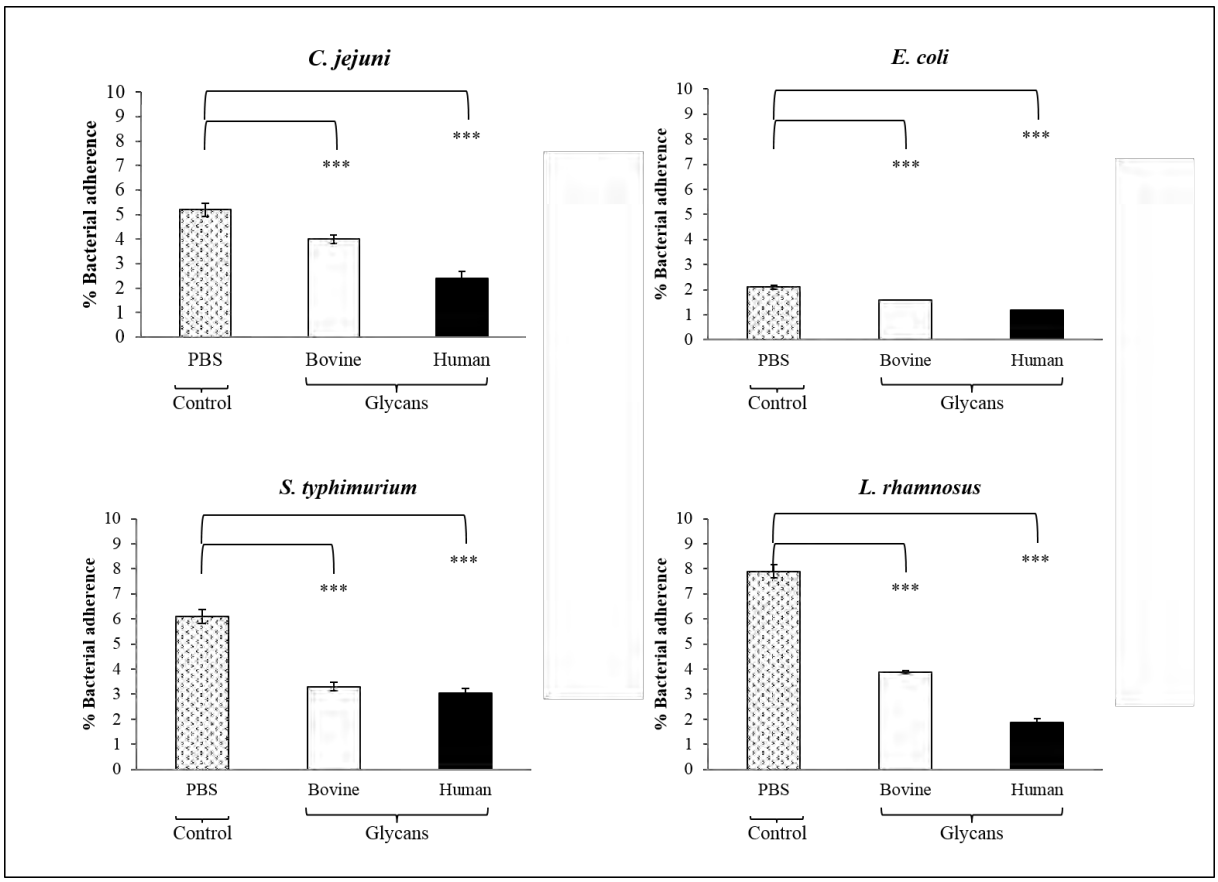


Fig. 5.4. The effect of pre-incubating bacteria with *N*-linked glycans from bovine and human milk whey proteins on subsequent adhesion of the bacteria to HT-29 human gastrointestinal epithelial cells. Adherence is expressed relative to the number of bacteria inoculated, and is compared to adherence of bacteria that were incubated in PBS alone (i.e. without *N*-linked glycans). Differences in binding were considered significant when $p<0.001$ (***).

Cell invasion assays were also carried out to determine whether the *N*-linked glycans of bovine and human milk glycoproteins affected bacterial invasion of, as well as the adhesion to, the HT-29 cells. As only *C. jejuni* and *S. typhimurium* were shown to invade HT-29 cells (Figure 5.5), only these strains were tested for the effect of *N*-linked glycans on their invasiveness. Although cell invasion is low (~1 % of the applied bacteria), highly significant ($p<0.001$) inhibition of invasion by both bacterial strains was observed by pre-incubation in both bovine and human milk whey *N*-linked glycans. Human milk whey *N*-linked glycans inhibited the invasion of *C. jejuni* by 53 %, significantly ($p<0.001$) more inhibition than achieved by bovine milk whey *N*-linked glycans (31 %). However, bovine milk whey *N*-linked glycans were slightly more effective in inhibiting the invasion of the cells by *S. typhimurium* (71 % inhibition) compared to human milk whey *N*-linked glycans (63 % inhibition).

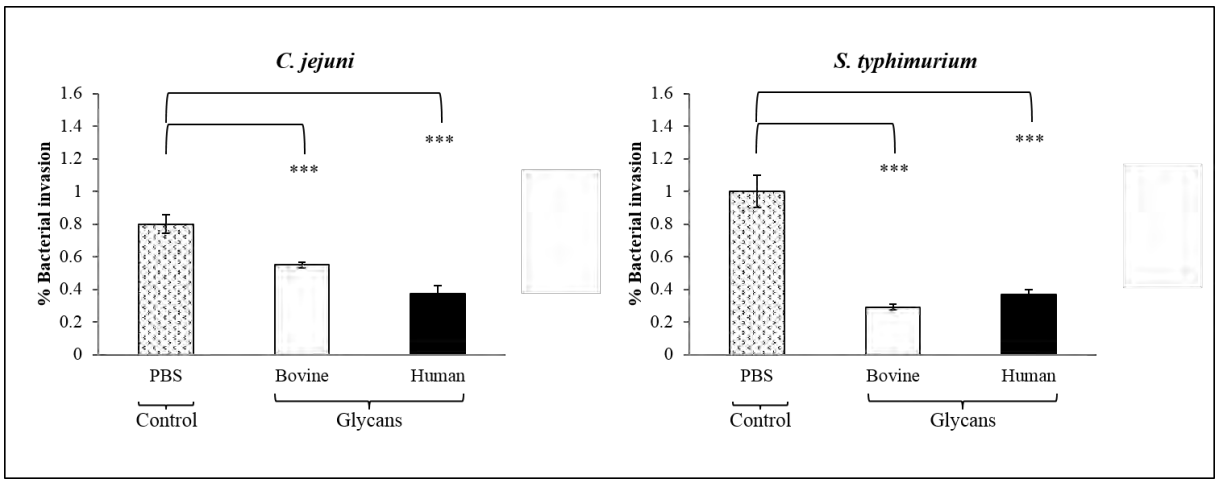


Fig. 5.5. The effect of pre-incubating bacteria with *N*-linked glycans from bovine and human milk whey proteins on subsequent invasion of the bacteria into HT-29 human gastrointestinal epithelial cells. Bacterial invasion of HT-29 cells is expressed relative to the number of bacteria inoculated, and is compared to the invasiveness of bacteria that were pre-incubated in PBS alone (i.e. without *N*-linked glycans). Differences in invasiveness were considered extremely significant when $p<0.001$ (***).

The above experiments revealed that the *N*-linked glycans of both bovine and human milk whey proteins have the ability to inhibit binding of human gastrointestinal bacteria to human gastrointestinal cells, as well as the invasion of the cells by invasive pathogens. The *N*-linked glycans from human milk whey appear to be, in general, more effective than the *N*-linked glycans from bovine milk whey in inhibiting bacterial binding (Figure 5.4) and invasion (Figure 5.5). This suggests that the glycans from the two mammalian whey fractions may present different binding epitopes to the bacteria; moreover, the binding epitopes presented by human whey glycans seem to be more effective decoys for pathogen attachment, possibly because they more closely resemble the glycan receptors for human pathogen binding to human gastrointestinal cells.

5.3.4 Monosaccharide analysis of released *N*-linked glycans from bovine and human milk whey proteins

A monosaccharide analysis was carried out on the *N*-linked glycans released from equal quantities (20 μg) of whey proteins from bovine and human milk to gauge overall differences in total glycan content and the relative proportions of different monosaccharide sugars in the *N*-linked glycans from each milk source. As shown in Table 5.1, a higher amount of total monosaccharides was obtained as *N*-linked glycans from human milk whey (124 μg of sugars/mg of protein) than from bovine milk whey (74 μg of sugars/mg of protein). Seven monosaccharides, galactose (Gal), *N*-acetylgalactosamine (GalNAc), *N*-acetylglucosamine (GlcNAc), mannose (Man), fucose (Fuc), *N*-acetylneuraminic acid (NeuAc) and *N*-

glycolylneuraminic acid (NeuGc) were detected in bovine milk whey. However, GalNAc and NeuGc residues were not detected in the *N*-linked glycans from human milk whey. The monosaccharides that make up the typical *N*-linked glycan core formed a substantial proportion of the total monosaccharides in both milk sources, with GlcNAc residues predominating in both bovine milk whey *N*-linked glycans (31.9 %) and human milk whey *N*-linked glycans (38.9 %), and mannose residues accounting for 25.4 % and 20.6 % of total monosaccharides respectively. Of particular note was the higher proportion of Gal in human milk whey *N*-linked glycans (35.4 %) than in bovine milk whey *N*-linked glycans (20.4 %), suggesting larger structures, as well as the high amount of GalNAc (11.9 %) in only bovine whey *N*-linked glycans, which may be explained by the reported observation of GalNAcβ1-4GlcNAc on the *N*-linked glycans of bovine milk glycoproteins (Van den Nieuwenhof *et al.* 1999). There was also a higher proportion of Fuc in human milk whey *N*-linked glycans (3.5 %) than in bovine milk whey *N*-linked glycans (2.1 %). Bovine milk whey *N*-linked glycans contained a higher proportion of NeuAc (6.8 %) than the human milk whey *N*-linked glycans (1.6 %).

Table 5.1. Monosaccharide analysis of total *N*-linked glycans released from bovine and human milk whey proteins as determined by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Percentages (%) are of total monosaccharide content (74 µg and 124 µg of sugars in 1 mg of bovine and human milk whey proteins, respectively)

Monosaccharide	**Gal**	**GalNAc**	**GlcNAc**	**Man**	**Fuc**	**NeuAc**	**NeuGc**	**Total**
Bovine milk *N*-linked glycans	20.4 %	11.9 %	31.9 %	25.4 %	2.1 %	6.8 %	1.5 %	100 %
Human milk *N*-linked glycans	35.4 %	ND	38.9 %	20.6 %	3.5 %	1.6 %	ND	100 %

Abbrevations: Fuc, fucose; Gal, galactose; Man, mannose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycolylneuraminic acid; ND, not detected; %, percent.

5.3.4 Mass spectrometric analysis of *N*-linked glycans released from bovine and human milk whey proteins and HT-29 cell membrane proteins

In order to investigate similarities and differences between the glycan epitopes presented to bacteria by each of the glycoprotein sources, global profiling of the enzymatically released *N*-linked glycans from bovine and human milk whey proteins, as well as the membrane proteins

from HT-29 gastrointestinal cells was carried out by LC-ESI-MS. Figure 5.7 shows the average mass spectra of the released *N*-linked glycans from these milk and cell line samples. As detailed in Table S4 (Supplementary Data), not all the *N*-linked glycan structures seen in the total milk protein analysis (N1 - N45) of Chapter 3 were seen, but 33 structures could be proposed from the mass spectra of the *N*-linked glycans from bovine milk whey glycoproteins and 29 structures on human milk whey glycoproteins, while 35 structures can be proposed for the *N*-linked glycans from the membrane proteins of the human intestinal HT-29 cells. Eleven (A1 - A11; Table S4, Supplementary Data) of these structures had not been previously identified from the total human milk glycan analysis described in Chapter 3, and were mainly bovine milk whey protein related. Table 5.2 presents the 18 common *N*-linked glycans (N3, A1, N6, N8, N9, N10, N12, N13, N15, N18, N19, N21, N22, N24, N26, N26, N33 and N35) released from bovine milk whey, human milk whey and membrane proteins of HT-29 human intestinal epithelial cells, where many were found to be high mannose, bisecting core-fucosylated and/or sialylated structures.

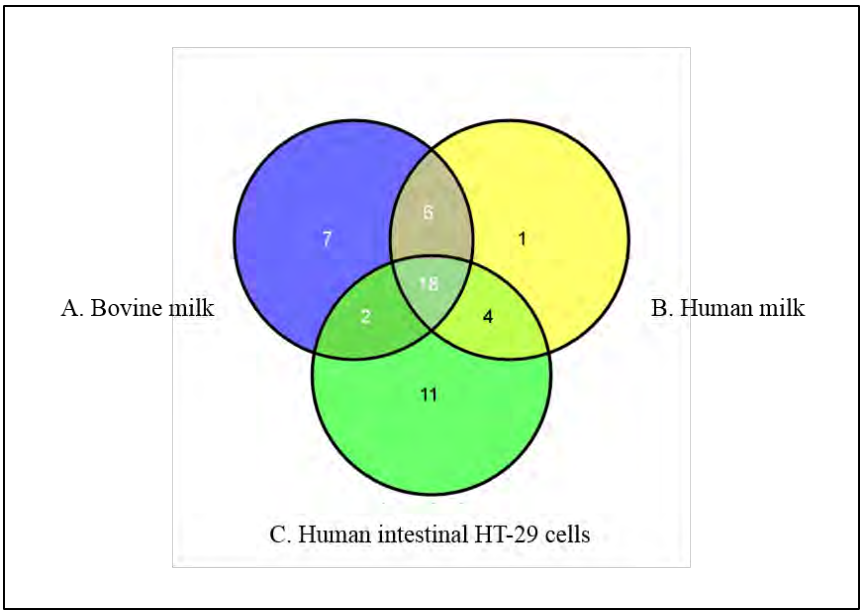
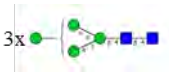
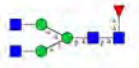
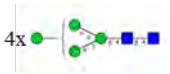
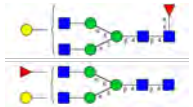
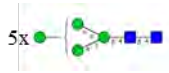
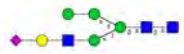
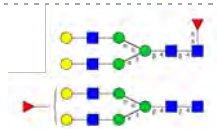
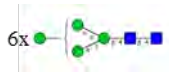
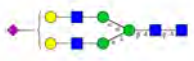
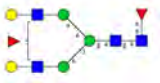
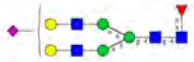
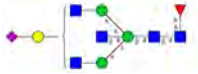
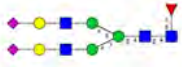
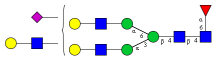


Fig. 5.6. Venn diagram indicating the number of proposed glycan structures that were unique or shared amongst the *N*-linked glycans released from (A) bovine milk whey; (B) human milk whey; and (C) membrane proteins of HT-29 human intestinal epithelial cells.

Table 5.2. The 18 common *N*-linked glycans released from bovine milk whey human milk whey and membrane proteins of HT-29 human intestinal epithelial cells.

S/ No.	Calculated experimental mass $[M-H]^{1-}$	Theoretical mass $[M-H]^{1-}$	Proposed *N*-linked glycan structure from this study	Relative abundance on bovine milk whey proteins (%)	Relative abundance on human milk whey proteins (%)	Relative abundance on HT-29 cell membrane proteins (%)
N 3	1397.6	1397.5		1.62	0.91[1]	3.57
*A 1	1463.6	1463.5		1.08[2]	0.41	0.38[4]
N 6	1559.6	1559.5		1.40[2]	0.50[1,2]	8.21[4]
N 8	1625.6	1625.6	 Possible Le	3.16[2]	1.90[2]	0.08
N 9	1641.8	1641.6		7.75[2]	1.06[1,2]	0.41
N 10	1666.8	1666.6		0.65	5.22[2]	0.34[4]

S/ No.	Calculated experimental mass $[M-H]^{1-}$	Theoretical mass $[M-H]^{1-}$	Proposed *N*-linked glycan structure from this study	Relative abundance on bovine milk whey proteins (%)	Relative abundance on human milk whey proteins (%)	Relative abundance on HT-29 cell membrane proteins (%)
N 12	1721.8	1721.6		6.28[2]	1.33[2]	20.51
N 13	1729.8	1729.6		1.98[2]	0.84[2]	1.98
N 15	1787.8	1787.6	 Possible Le	7.98[2]	7.85[1,2]	2.32
N 18	1883.8	1883.6		4.04[2]	2.12[2]	22.17
N 19	1891.8	1891.7		2.58[2,3]	0.88[2]	4.17
N 21	1932.6	1932.7		6.17[2,3]	7.71[1,2]	3.17

S/ No.	Calculated experimental mass $[M-H]^{1-}$	Theoretical mass $[M-H]^{1-}$	Proposed *N*-linked glycan structure from this study	Relative abundance on bovine milk whey proteins (%)	Relative abundance on human milk whey proteins (%)	Relative abundance on HT-29 cell membrane proteins (%)
N 22	1933.8	1933.7	 Le	0.26[2]	0.97[1,2]	2.05
N 24	2078.8	2078.7		3.04[2,3]	26.08[1,2,3]	5.64[4]
N 26	2120.0	2119.8		8.39[2,3]	1.15[2]	0.17
N 28	2224.0	2223.8		0.93[1]	7.39[1,2]	1.81
N 33	2370.0	2369.8		0.40	7.07[1,3]	2.14
N 35	2444.0	2443.9		0.54[3]	0.82	0.40

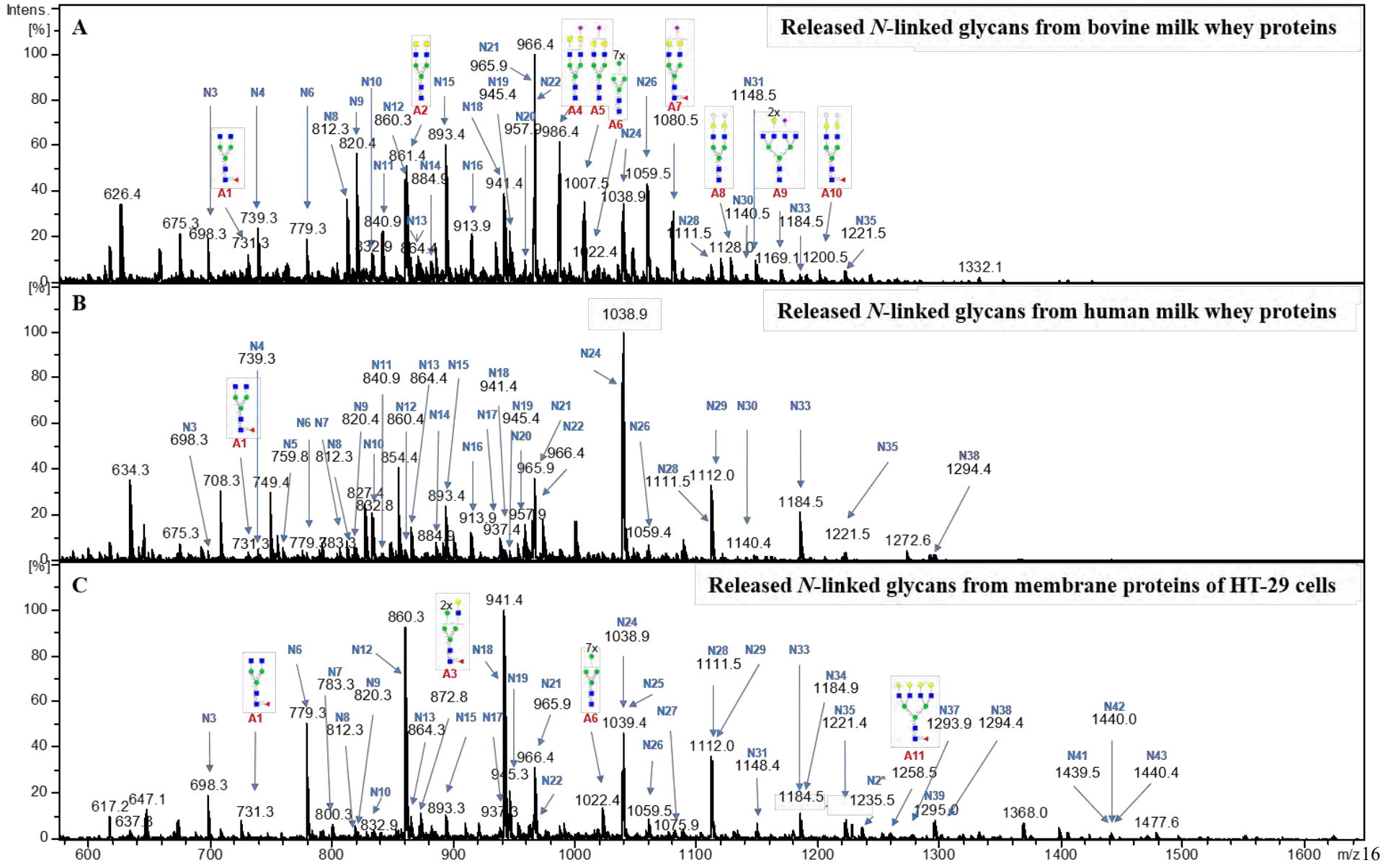

A
Released N-linked glycans from bovine milk whey proteins
B
Released N-linked glycans from human milk whey proteins
C
Released N-linked glycans from membrane proteins of HT-29 cells
Intens.
[%]
m/z

Fig. 5.7. The *N*-linked glycosylation profile (summed average mass spectra) of (A) bovine milk whey; (B) human milk whey; and (C) HT29 human gastrointestinal cells. Structures A1 - A11 were not found in the total human milk glycan analysis described in Chapter 3. Representative $[M-2H]^{2-}$ ions (* denotes (N2) with *m/z* $[1235.4]^{1-}$ is singly charged) corresponding to proposed structures of released *N*-linked glycans respectively as in Table S4, Supplementary Data.

The Venn diagram in Figure 5.6 displays the number of proposed glycan structures that were common or unique amongst the *N*-linked glycan samples from bovine and human milk whey and membrane proteins from HT-29 human intestinal cells. A total of 18 proposed *N*-linked glycan structures (N3, A1, N6, N8, N9, N10, N12, N13, N15, N18, N19, N21, N22, N24, N26, N28, N33 and N35) were common to the *N*-glycosylation profiles of all three samples (Figure 5.7; Table S4 in Supplementary Data,). Notably, two of the proposed glycan structures that were common only to human milk whey and the human HT-29 membrane proteins (but not identified from bovine whey) contained possible Le or SLe epitopes; the most abundant of these was N29, with a relative abundance of 11.4% and 8.3% in human milk whey and HT-29 membrane proteins, respectively. Two of the proposed bovine whey glycans that shared common structures with *N*-linked glycans from the HT-29 cells, but were not found amongst the human whey glycans were high mannose (A6) structure and bi-antennary sialylated (N31) structures.

Six structures (N4, N11, N14, N16, N20 and N30) were common to both mammalian milks but not found amongst the glycan structures proposed for HT-29 cells, comprising a variety of structures, three of which (N11, N16 and N30) feature bisecting *N*-acetylglucosamine (Table S4, Supplementary Data). Seven proposed complex type glycan structures (A2, A4, A5, A7, A8, A9 and A10) were unique to bovine milk whey proteins, with none of these structures previously identified from the *N*-linked glycans of total human milk proteins (Chapter 3). Some of these structures (A2, A4, A5 and A7) feature possible lacdiNAc (GalNAcβ1-4GlcNAc; Van den Nieuwenhof *et al.* 1999), which have been previously reported on bovine milk proteins (Sato *et al.* 1993) and bovine mammary epithelial cells (Nakata *et al.* 1993). Other glycan structures unique to bovine milk in this study include those containing *N*-glycolylneuraminic acid (NeuGc), (A8 and A10) and a tetra-antennary mono-sialylated structure (A9). Only one proposed glycan structure (N5) was identified as being unique to human milk whey, specifically a bisecting structure (N5, *m/z* $[759.8]^{2-}$), which was 1.6 % of total *N*-linked glycans. Eleven proposed structures were unique to HT-29 cells, including, albeit in trace amounts, of several tri- or tetra-antennary glycans containing possible Le/SLe epitopes (N39, N41, N42 and N43).

An analysis was carried out to compare the relative abundance of different glycan types (ie. high mannose, core fucose, terminal fucose, NeuAc/NeuGc) amongst the proposed *N*-linked glycan structures from bovine and human milk whey and HT-29 human gastrointestinal membrane proteins. As shown in Table 5.2, the human milk whey proteins analysed in this

study appear to carry more fucosylated *N*-linked glycans, comprising core- (62.2 - 72 %) and terminal- fucosylated (12.8 - 22.5 %) structures compared to bovine milk whey proteins (25.2 - 36.4 % and 0.3 - 11.4 %, respectively). In terms of the sialylated *N*-linked glycans, these structures represented 46.5 % and 72.5 % of the proposed total *N*-linked glycans from bovine and human milk whey, respectively. A small percentage of NeuGc (0.7 % of total *N*-linked glycans) was only found in the proposed glycan structures of the bovine milk whey glycoproteins. Of particular note, there was a high proportion of high mannose structures proposed from the HT-29 human gastrointestinal membrane proteins (57.6 % of total *N*-linked glycans; Table 5.2). In particular, N12 was a proposed high mannose structure and most abundant on HT-29 cell membrane proteins (20.5 % of total *N*-linked glycans; see Table S4 in Supplementary Data). This structure is also proposed as an *N*-linked glycan of bovine and human milk whey, although at lower levels of relative abundance (6.3 % and 1.3 %, respectively; see Table S4 in Supplementary Data).

Table 5.2. Relative abundance of *N*-linked glycan types from bovine and human milk whey, and HT-29 human gastrointestinal epithelial cells

	Relative abundance (%)				
***N*-linked glycans from**	**High Man**	**Core Fuc**	**Terminal Fuc**	**NeuAc**	**NeuGc**
Bovine milk whey proteins	14.9	25.2 - 36.4*	0.3 - 11.4*	46.5	0.7
Human milk whey proteins	4.9	62.2 - 72*	12.8 - 22.5*	72.5	Not detected
HT-29 membrane proteins	57.6	27.4 – 29.8*	15.5 - 17.9*	31.3	Not detected

* includes two proposed structures (N8 and N15) that could carry either a core or terminal fucose.

5.4 Discussion

Identification of carbohydrate-based ligands of pathogenic bacteria has been defined as the key step necessary for controlling microbial infections (Day *et al.* 2009; Gilboa-Garber *et al.* 1994; Lloyd *et al.* 2007; Scharfman *et al.* 1999; Sharon 2008; Yajima *et al.* 2008). In the

present study, two approaches were employed to investigate the involvement of *N*-linked glycans displayed on the milk glycoproteins in binding to the bacteria and inhibiting the interaction with the surface glycans displayed on the intestinal cells. Since the milk whey proteins carry the majority of the *N*-linked glycans, the whey was separated from the lipid and casein fractions.

The equivalent volume of human milk whey had about 1.6 times more protein attached glycans based on monosaccharide content than the bovine milk whey. All the intestinal bacteria bound more strongly ($p<0.05$) to the human whey proteins than to the bovine whey proteins. The involvement of the glycan component of the whey proteins was further substantiated by the observation that the free glycans released from both bovine and human whey proteins were able to effectively inhibit all the intestinal bacteria from binding to human and bovine milk whey.

As previously mentioned (Section 1.2, Chapter 1), the adhesion of bacteria to host cells is the first step in infection. To mimic the intestinal lining of the gut, HT-29 human colon epithelial cells were used as an *in vitro* model to study the involvement of glycans on proteins in milk in the adhesion and invasion of bacteria to gastrointestinal cells. Many studies have employed the use of cultured human colon cell lines, such as Caco-2 (Coppa *et al.* 2006; Liu B *et al.* 2012) and T84 (Crane *et al.* 1994; Wine *et al.* 2008), as well as a human intestinal cell line INT 407 (Konkel *et al.* 1992a) because they can form a polarised monolayer, making them a suitable model for investigating the nature of host-pathogen interactions during an infection. Other benefits of using *in vitro* cell culture methods are their protocols have been well-established (Crane *et al.* 1994; Friis *et al.* 2005; Konkel *et al.* 1992b; McCormick 2003; Russell and Blake 1994) and the availability of cultured human epithelial colon cell lines in the American Type Culture Collection (ATCC).

Lactobacillus rhamnosus, which is a commensal bacterium of the human gastrointestinal tract (probiotic) was the most adhesive bacteria to HT-29 cells compared to the tested pathogenic bacteria, *C. jejuni, E. coli* and *S. typhimurium*. Other studies have reported that the adherence of *Lactobacillus* species to HT-29 cells was inhibited by methyl-alpha-D-mannoside, suggesting that mannose provides a binding epitope for the commensal bacterium on the gastrointestinal epithelial cells (Adlerberth *et al.* 1996; Ahrné *et al.* 1998). Interestingly, in this study, *Lactobacillus* adherence was shown to be significantly more inhibited by released *N*-linked glycans from bovine and human milk whey glycoproteins than *C. jejuni*, *E. coli* and

S. typhimurium. This study is the first evidence of *Lactobacillus* binding to intestinal cells being influenced by milk glycans suggesting that milk may also be interacting with the binding of beneficial bacteria in the gut.

Escherichia coli was shown to adhere the least effectively to HT-29 cells as compared to the other pathogenic bacteria *C. jejuni* and *S. typhimurium* and there is varied evidence that *N*-linked glycans mediate this binding. Most *E. coli* are reported to colonise the gastrointestinal tract of human infants (Kaper *et al.* 2004) and evidence suggests that they bind to high mannose structures (Cavallone *et al.* 2004; Liu B *et al.* 2012). On the other hand, *Salmonella* species have been shown to bind to terminal α-D-mannose and the trisaccharide Galβ1-4(Fucα1-3)GlcNAc, also known as the Le^x blood group residues on host glycoproteins (Chessa *et al.* 2009; Duncan *et al.* 2005; Kisiela *et al.* 2005). In addition, the adherence of *S. typhimurium* to Caco-2 cells was demonstrated to be inhibited by pre-incubation of the monolayer with peanut agglutinin (PNA) that recognises specifically the Galβ1-3GalNAc epitopes (Giannasca *et al.* 1996). Further, *C. jejuni* has been shown to bind to a broad range of structures, including fucosylated, terminal galactose, mannose and sialylated structures (Avril *et al.* 2006; Day *et al.* 2009, Lane *et al.* 2012b).

The *N*-linked glycans released from bovine and human milk whey glycoproteins clearly inhibited bacterial binding to the human intestinal cells in a bacterial species-specific mode. From this assay, it is speculated that the mechanism underlying the binding of these bacteria to milk glycoproteins varies among the bacterial species due to the differences in their preferred binding epitopes. Particularly, the bacteria may bind selectively and differentially to high mannose, bisecting core-fucosylated or sialylated structures. Structural analysis showed the presence of all these epitopes on the complex *N*-linked glycans of both the bovine and human whey protein glycans and on the membrane proteins of the intestinal cells. The *N*-linked glycans from milk whey glycoproteins contained complex, fucosylated and sialylated structures, which are important bacterial binding epitopes for pathogen recognition, and can compete for the similar structures displayed on the HT-29 cells. The proportion of fucosylated complex glycans in this study is similar to that reported by Dallas *et al.* (2011) and Nswosu *et al.* (2012), in which there is a higher fucosylation of human milk whey proteins. However, in this study higher sialylation (72.5 % of total *N*-linked glycans) occurs on the human whey *N*-linked glycans compared to that on the bovine milk proteins (46.5 % of total *N*-linked glycans), perhaps again due to the individual variation shown by a single bovine donor. The released glycans from bovine and human milk whey in this study effectively ($p<0.001$)

inhibited the adhesion of all bacterial strains to the intestinal cells; with human milk *N*-linked glycans being generally more effective than the bovine milk *N*-linked glycans.

The invasion assays confirmed that the particular strain of *C. jejuni* and *S. typhimurium* used in this study are both invasive. The *C. jejuni* NCTC11168 used is less invasive than *C. jejuni* ATCC 43431(TGH 9011) and 81-176 (Lane *et al.* 2012a; Poly *et al.* 2004). The mechanism of invasion by *C. jejuni* and *S. typhimurium* is well-documented, in which these pathogens bind via their flagella to host membrane receptors and gain entry into intestinal cells by inducing rearrangement of the network of actin filaments (Hu and Kopecko 1999; Hu *et al.* 2008; Misselwitz *et al.* 2012; Unsworth *et al.* 2004). *Escherichia coli* O111:NM did not invade the HT-29 cells at all, as the EPEC strain that was used in this study is a non-motile (NM) diarrheagenic pathogen, which is unable to invade and infect the host cells (Nataro and Kaper 1998) and thus would not be affected in this aspect by the surface glycoproteins. *Lactobacillus* species are not pathogens of the human gastrointestinal mucosa (Fernández *et al.* 2003; Servin 2004) and this was confirmed by their non-invasion of HT-29 cells. Where the bacteria were invasive, such as *C. jejuni* and *S. typhimurium,* the human milk protein glycans again had a greater inhibitory effect on the invasive potential than the bovine milk whey protein glycans. It is therefore evident that the *N*-linked glycans on the milk whey proteins have a protective role in inhibiting both the bacterial adherence and invasion of human specific intestinal bacteria in the gastrointestinal tract

The membrane *N*-linked protein glycans of HT-29 cells have been described (Vercoutter-Edouart *et al.* 2008), and this study showed about the same major abundance of high mannose structures (57.6 % of total *N*-linked glycans compared with 51.7 % in the previous report). However, there was a different distribution of the complex *N*-linked glycans in both studies. Vercoutter-Edouart *et al.* (2008) found a high content of highly branched glycans comprising tri-and tetra-antennary structures (over 46 % of total *N*-linked glycans) in the HT-29 cells by positive MALDI-MS. Among the 35 structures proposed in this study, only six *N*-linked glycan masses (A1, N6, N10, N24, N29 and A11) corresponded to those previously reported. This may be because Vercoutter-Edouart *et al.* (2008) used a differentiating subclone of HT-29 cells (HT-29-18 C1), which developed enterocyte-like properties after 20 days of growth. In contrast, the HT-29 cells used in this work are known to remain undifferentiated when grown in the presence of glucose (Wils *et al.* 1993), as had been the case here (Section 5.2.4).

The *N*-glycosylation profile of the HT-29 membrane proteins used in this study was different

from that of the *N*-linked bovine and human milk whey glycoproteins, but contained some of the same structures. Although there was a significant difference of the *N*-glycosylation specific structures between the secreted milk and cell surface glycoproteins, both bovine and human milk whey proteins contained core-fucosylated *N*-linked glycans, while the human intestinal cells had more terminal fucosylaton, with both the human milk and the human epithelial cells having Lewis epitopes, known receptors for bacterial adhesion, in common. A high abundance of sialylated structures, another well-described bacterial lectin receptor, was seen across all three samples. Hence, it is clear from this study that there are variations in glycan compositions between bovine and human milk and that the host cells are expressing a different, yet similar, array of bacterial binding glycan epitopes as those milk glycans attached to mammalian milk protein sources.

Chapter 6. Summary and future directions

The ability of bovine and human milk glycoproteins to bind to gastrointestinal bacteria, and thus potentially inhibit pathogen adhesion to the human gastrointestinal tract, was investigated in this study. The development of an efficient 96-well PVDF plate assay to measure bacterial binding to milk protein fractions enabled assessment of the contribution of different components of bovine and human milk to bacterial adhesion, as well as establishing the involvement of the glycans, and in particular sialic acid, carried by the milk proteins in the interactions. In addition, a study of the potential of *N*-linked glycans released from bovine and human milk whey glycoproteins to competitively inhibit the bacterial adhesion to and invasion of the intestinal epithelial cell surface was performed by an *in vitro* assay using a human colon epithelial cell line as a model system of the human gastrointestinal tract. Mass spectrometry was used to analyse the global glycan profiles of bovine and human milk proteins and their whey fractions, as well as the global glycan profile of human intestinal cells; in this way, similarities and differences in the glycan profiles could be determined, enabling speculation on the mechanisms of protection by the milk glycoproteins against pathogen adherence to the gastrointestinal tract. The main findings of this work are summarised in this chapter.

6.1 Development of methods for assessing bacterial binding to conjugated milk glycans

Two methods were trialled to assess the potential of gastrointestinal bacteria to bind to glycan epitopes on human milk glycoproteins. The first method was a glycan microarray technology developed by the Institute for Glycomics at Griffith University, comprising synthetic glycan structures (including those on milk glycoproteins) that were covalently immobilised onto activated glass slides. Although this method has been routinely used to measure the binding of purified protein, antibodies, lectins and viruses to the immobilised glycans, it was not found to be a suitable technique for measuring whole bacterial binding to the glycans due to non-specific binding and lack of reproducibility.

The second successful method involved a plate assay in which milk proteins were immobilised onto PVDF membranes in a 96-well plate. The presented glycans on the proteins bound to the PVDF membrane through hydrophobic interactions were probed with fluorescently-labelled bacteria and bacterial binding quantified using a fluorescent plate reader. Human gastrointestinal and oral bacteria bound to human milk proteins but not to the unglycosylated BSA, suggesting the importance of the conjugated milk glycans as binding

epitopes. All the gastrointestinal bacteria tested, including the non-pathogenic, (probiotic) *Lactobacillus rhamnosus*, and the pathogenic *Campylobacter jejuni*, *Salmonella typhimurium* clinical isolate and *Escherichia coli* bound to different extents to the human milk proteins. The oral streptococci species, *Streptococcus gordonii* and *S. mutans* bound less efficiently to the total human milk proteins than gastrointestinal bacteria. Interestingly, *S. gordonii*, which is an initial coloniser of enamel bound more efficiently than *S. mutans*, a plaque-forming bacterium that aggregates later on the biofilm layer. These findings indicated that many bacteria that inhabit human epithelial surfaces bind to human milk proteins, including both pathogens and commensals, and the extent of binding varies between species.

A rather unexpected outcome of this work was that the commensals, *L. rhamnosus* and *S. gordonii*, appeared to bind to the human milk proteins slightly better than the pathogenic bacteria. These results seem to suggest that human milk proteins may not only act as decoy receptors for potential pathogens to reduce their colonisation in the human body, as hypothesised in Chapter 1, but also bind to commensals. It is possible that the binding of commensals to human milk might facilitate their regular entry to the body from a healthy mother, as well as ensuring a healthy cycling of the commensal microbiome in the infant GIT that promotes microbial genetic diversity. In addition, the results suggest that there are other mechanisms that favour adhesion and colonisation of commensals in the gastrointestinal tract of infants, which were untapped by this *in vitro* work.

6.2 Sialic acid involvement in bacterial binding to human milk proteins

Glycan involvement in bacterial binding to the human milk proteins, and specifically the contribution of sialic acid was investigated by incubating the two streptococci species in free sialic acid prior to binding to human milk proteins, and by washing pre-bound bacteria with free sialic acid. The results from both approaches showed a significantly reduced binding of *S. gordonii* to the human milk proteins, but the binding of *S. mutans* was not reduced. This suggested that the binding of *S. gordonii* to the human milk proteins was at least partially to the sialic acid moieties on the conjugated milk glycans, but the closely related species, *S. mutans* appeared to use different binding epitopes. The involvement of sialic acid in the binding of gastrointestinal and oral bacteria to total human milk proteins was verified by the enzymatic removal of terminal sialic acid residues on the human milk glycoproteins. The neuraminidase treatment significantly ($p<0.05$) reduced the binding of all the bacterial strains to the human milk proteins, with a greater effect observed for the gastrointestinal bacteria than the oral streptococci.

Mass spectrometry was used to confirm that the neuraminidase treatment of the milk proteins had removed the terminal sialic residues for the bacterial binding assay. Terminal sialic acids were substantially reduced in the mass spectra after desialylation. The mass spectrometry also enabled a global glycan profiling of the human milk glycoproteins, which were found to consist of mainly sialylated bi-antennary *N*-linked glycans and *O*-linked glycans with mono-fucosylated and mono-sialylated core 2 structures. Many of these structures were consistent with those of human milk proteins reported in the literature.

6.3 Comparison of the bacterial binding of bovine and human milk proteins

Bacterial binding to bovine and human milk proteins were measured to compare the potential of the two types of milk to prevent pathogen adhesion to the human gut and subsequent diarrhoeal disease. To help determine which of the various components of the milk contributed to bacterial adherence, several techniques were explored to fractionate the milk proteins prior to application in the bacterial binding assay. The most successful technique involved separation of the insoluble casein from the soluble whey proteins by ultracentrifugation with pH adjustment. The whey was then further fractionated by a size exclusion column fitted to an FPLC system.

The pathogenic *S. typhimurium* and the non-pathogenic *L. rhamnosus* bound slightly more efficiently to the total human milk proteins than the total bovine milk proteins, prepared from volume equivalent of each type of milk, despite the higher protein content of bovine milk. Fractionation of the milk into its whey and casein components helped to elucidate the contribution of the different milk proteins to bacterial adhesion. The binding efficiencies of all three gastrointestinal bacteria to the human milk whey, which was similar in protein content in the two milks, were significantly ($p<0.05$) higher than to bovine milk whey proteins and the casein fractions of either type of milk. This was substantiated, by competitive inhibition studies, as being contributed to by the glycan component of the human milk whey proteins. This indicated that the binding efficiencies of the bacteria to the bovine and human milk proteins was not simply a function of the amount of proteins in each type of milk and that other factors, such as different glycan profiles, may be involved.

Further fractionation of the whey proteins of bovine and human milk by gel filtration enabled further study as to the contribution of different whey proteins to bacterial adhesion. The gastrointestinal bacteria were found to bind to some extent to the proteins from all three fractions of bovine and human milk whey (including the high molecular weight

immunoglobulins, mid-molecular weight range whey proteins such as lactoferrin (particularly in human milk) and low molecular weight whey proteins, such as α-lactalbumin and β-lactoglobulin (bovine specific), suggesting that numerous milk whey proteins contribute to pathogen adhesion. Preference of bacterial binding to any particular whey fraction appeared to be species specific.

6.4 Glycan involvement in bacterial adherence to bovine and human milk proteins

Sialic acid was found to be integral to the adhesion of *E coli*, *S. typhimurium* and *L. rhamnosus* to bovine and human milk proteins. Bacterial binding to both types of mammalian milks was competitively inhibited by sialic acid as shown by washing of the pre-bound bacteria with free sialic acid, by pre-incubation of the bacteria with free sialic acid prior to binding to the milk proteins and by desialylation of the immobilised bovine and human milk glycoproteins prior to the adhesion assay. Notably, the inhibition of bacterial binding to the human milk whey and casein fractions by the sialic acid treatments was greater than that observed for the bovine milk whey and casein fractions.

To further examine the involvement of the protein conjugated glycans of bovine and human milk proteins in bacterial adhesion, *N*-linked glycans were released from the milk whey proteins and their ability to competitively inhibit bacterial binding to the protein fractions assessed. The whey *N*-linked glycans from both types of milk significantly inhibited bacterial binding to both the whey and casein milk protein fractions. This provided evidence for specific involvement of the protein conjugated glycans in bacterial adherence to the milk glycoproteins. Furthermore, pre-incubation of the gastrointestinal bacteria with released *N*-linked glycans from human milk whey proteins reduced the bacterial binding to human milk whey proteins significantly ($p<0.05$) more effectively than the *N*-linked glycans from bovine milk whey proteins, suggesting that the conjugated glycans of human milk whey have a greater influence on bacterial adhesion than that of cow's milk.

6.5 Glycan involvement in bacterial adherence to human gastrointestinal cells

An *in vitro* assay using a human colon adenocarcinoma cell line (HT-29) was used to provide a model of the epithelial lining of the human gastrointestinal tract. The *N*-linked glycans released from both bovine and human milk whey significantly ($p<0.001$) inhibited the adhesion of *C. jejuni*, *E. coli, S. typhimurium* and *L. rhamnosus* to the HT-29 cells. The *N*-linked glycans of human milk whey inhibited the binding of all the gastrointestinal bacteria to the human colon cells more than the *N*-linked glycans of bovine milk whey.

This study also investigated whether incubation of bacteria in whey glycans could inhibit potentially invasive pathogens from penetrating the human colon cells. Milk glycan involvement in pathogen invasion of host cells has received little attention in past research. The two potentially invasive gastrointestinal pathogens used in this work, *C. jejuni* and *S. typhimurium*, were significantly ($p<0.001$) inhibited from invading the HT-29 cells by pre-incubation in the *N*-linked glycans of both bovine and human whey proteins. The invasion of *C. jejuni* into the colon cells was inhibited more by the *N*-linked glycans of human milk whey than the *N*-linked glycans of bovine milk whey, whereas the invasion of *S. typhimurium* into the colon cells was inhibited slightly more by the *N*-linked glycans of bovine milk.

The results of the cell-based assay suggested the *N*-linked glycans attached to whey proteins in both bovine and human milk may contribute to the inhibition of bacterial adherence to, and invasion of, the human gastrointestinal tract. The differences in the relative abilities of the *N*-linked glycans from equal quantities of bovine and human milk whey proteins to inhibit bacterial adherence and invasion to the colon cells suggested that the number or type of *N*-linked glycans contributed by the two types of milk whey fractions are different. Interestingly, it appeared that the *N*-linked glycans of human milk whey have a higher potential to act as decoys for pathogen attachment than the *N*-linked glycans of bovine milk whey.

6.6 Comparison of the *N*-linked glycan structures on human and bovine milk whey and human gastrointestinal cell membrane proteins

Mass spectrometry was used to compare the *N*-linked glycan structures of bovine and human milk whey with those of the HT-29 human colon epithelial cells. Similarities and differences between the profiles contribute to an understanding of the relative abilities of the bovine and human milk whey glycans to competitively inhibit bacterial adhesion and invasion of the human colon cells. From a total of 49 proposed glycan structures, 18 were common to the *N*-glycosylation profiles of bovine and human milk whey and the colon epithelial cells. The bovine milk whey proteins contained seven unique complex type structures, which were absent in human milk whey and the HT-29 cells. These unique *N*-linked glycans on bovine milk whey glycoproteins were predicted to contain lacdiNAc and NeuGc-containing structures, which have previously been reported on bovine milk proteins.

Notably, two of the glycan structures that were common to human milk whey and the human colon cells, but not the bovine milk whey, contained possible Lewis or sialyl Lewis glycan epitopes. Together these Lewis or sialyl Lewis structures represented approximately 12 % and

9 % of the *N*-linked glycans from the human whey and colon cells, respectively. The adherence of human pathogens to Lewis epitopes on human epithelial cell surfaces has been well-documented. Hence, it is possible that the greater ability of the *N*-linked glycans of human milk whey compared to *N*-linked glycans of bovine milk whey to inhibit bacterial adhesion to the human colon epithelial cells in this work may be at least be partly attributable to the presence of the Lewis epitopes in human milk whey proteins, and their absence in the bovine milk whey proteins.

Monosaccharide analysis revealed a higher abundance of total sugars attached to human milk whey proteins than bovine milk whey, with a higher proportion of fucose residues in human milk whey *N*-linked glycans than bovine milk *N*-linked glycans, and conversely, a higher proportion of sialic acid in bovine milk whey *N*-linked glycans than human milk *N*-linked glycans. As a result of this analysis, and the global profiling of the glycan structures attached to the bovine and human milk whey proteins, it is clearly evident that a different range and quantity of glycan epitopes are presented to bacteria by the two types of milk. Moreover, human milk glycoproteins may act as better decoys to prevent pathogen attachment to the human gastrointestinal tract because they present more glycan epitopes that could mimic pathogen attachment sites on human epithelial cell surfaces than bovine milk glycoproteins.

6.7 Conclusions

Strong evidence has arisen from this work to support the original hypothesis that the conjugated glycans on milk glycoproteins have a potential role in protecting the infant GIT against pathogen adhesion. In addition, commensal bacteria were also found to bind to the milk glycans, suggesting that the mechanisms promoting a healthy GIT are complex and may involve additional factors that await further exploration. The extent of pathogenic and commensal bacterial binding to milk glycoproteins was shown to be dependent on the type and abundance of glycan epitopes and that these structures had the capacity to inhibit the adhesion and invasion of pathogenic bacteria to intestinal cells. In particular, the terminal sialic acids and *N*-linked glycans on milk proteins were shown to be directly involved in this sugar-pathogen interaction and human milk glycoproteins had a greater potential for protection of the infant against bacterial infection than bovine milk glycoproteins.

6.8 Future directions

This study suggests that the complex sialylated, fucosylated *N*-linked glycan structures attached to the proteins in human milk offer protection against bacterial infection of the infant

gastrointestinal tract and that this protection is superior to that offered by breast milk substitutes based on cow's milk.

There remains much work to be done to determine the detail of the exact oligosaccharide (sub)structures, which are responsible for this interaction and the differences in these structures that may account for the different extent of binding of the various types of gastrointestinal bacteria.

To achieve this end, further work would involve such approaches as:

1. Altering the glycosylation of the milk glycoproteins with exoglycosidases and testing the effect of changing the binding epitope on the adhesion of the bacteria, thus determining the exact substructure of the glycan epitopes in milk.
2. Investigating the role of the *O*-linked glycans in milk, particularly of the mucins of the milk fat globule membrane, on bacterial adhesion.
3. Testing the binding of milk glycoproteins to a wider range of bacterial pathogens that are isolated from people with intestinal disease, and to more commensal gut bacteria.
4. Using different intestinal cells and animal model systems to observe any difference in protection by the milk glycans against gastrointestinal infection.
5. Measuring the effect of breast milk on the protection against infection by bacteria over lactation time of both human and cow's milk.
6. Designing molecular mimics based on the knowledge of the glycan binding epitopes in human milk to test as inhibitors of bacterial binding, with the potential for use as a milk additive or nutritional supplement.
7. Investigating differences in glycan-mediated bacterial adhesion in milk from mothers of different origins, blood types, and disease states.
8. Determining other complex factors that could be involved in colonisation by the commensals rather than the pathogens in the healthy gastrointestinal tract.

References

1. Abe T, Horiuchi K, Kikuchi H, Aritsuka T, Takata Y, Fukushi E, Fukushi Y, Kawabata J, Ueno K, Onodera S, Shiomi N. 2012. Structural confirmation of oligosaccharides newly isolated from sugar beet molasses. *Chem Cent J.* 6:89. doi: 10.1186/1752-153X-6-89.
2. Acord J, Maskell J, Sefton A. 2005. A rapid microplate method for quantifying inhibition of bacterial adhesion to eukaryotic cells. *J Microbiol Methods.* 60:55-62.
3. Ahrné S, Nobaek S, Jeppsson B, Adlerberth I, Wold AE, Molin G. 1998. The normal *Lactobacillus* flora of healthy human rectal and oral mucosa. *J Appl Microbiol.* 85:88-94.
4. Aldredge DL, Geronimo MR, Hua S, Nwosu CC, Lebrilla CB, Barile D. 2013. Annotation and structural elucidation of bovine milk oligosaccharides and determination of novel fucosylated structures. *Glycobiology*. 23:664-676. doi: 10.1093/glycob/cwt007.
5. Adlerberth I, Ahrne S, Johansson ML, Molin G, Hanson LA, Wold AE. 1996. A mannose-specific adherence mechanism in *Lactobacillus plantarum* conferring binding to the human colonic cell line HT-29. *Appl Environ Microbiol.* 62:2244-2251.
6. Alves JG, Figueiroa JN, Meneses J, Alves GV. 2012. Breastfeeding protects against type 1 diabetes mellitus: a case-sibling study. *Breastfeed Med.* 7:25-28. doi: 10.1089/bfm.2011.0009.
7. Amano J, Osanai M, Orita T, Sugahara D, Osumi K. 2009. Structural determination by negative-ion MALDI-QIT-TOFMSn after pyrene derivatization of variously fucosylated oligosaccharides with branched decaose cores from human milk. *Glycobiology*. 19:601-614. doi: 10.1093/glycob/cwp026.
8. An HJ, Froehlich JW, Lebrilla CB. 2009. Determination of glycosylation sites and site-specific heterogeneity in glycoproteins. *Curr Opin Chem Biol.* 13:421-426. doi: 10.1016/j.cbpa.2009.07.022.
9. An HJ, Lebrilla CB. 2011. Structure elucidation of native *N*- and *O*-linked glycans by tandem mass spectrometry (tutorial). *Mass Spectrom Rev.* 30:560-578. doi: 10.1002/mas.20283.
10. Andersson B, Porras O, Hanson LA, Lagergård T, Svanborg-Edén C. 1986. Inhibition of attachment of *Streptococcus pneumoniae* and *Haemophilus influenzae* by human milk and receptor oligosaccharides. *J Infect Dis.* 153:232-

237.

11. Aniansson G, Alm B, Andersson B, Håkansson A, Larsson P, Nylén O, Peterson H, Rigenér P, Svanborg M, Sabharwal H, Svanborg C. 1994. A prospective cohort study on breast-feeding and otitis media in Swedish infants. *Pediatr Infect Dis J.* 13:183-188.
12. Aniansson G, Andersson B, Lindstedt R, Svanborg C. 1990. Anti-adhesive activity of human casein against *Streptococcus pneumoniae* and *Haemophilus influenzae*. *Microb Pathog.* 8:315-323.
13. Apweiler R, Hermjakob H, Sharon N. 1999. On the frequency of protein glycosylation, as deduced from analysis of the SWISS-PROT database. *Biochim Biophys Acta.* 1473:4-8.
14. Arifeen S, Black RE, Antelman G, Baqui A, Caulfield L, Becker S. 2001. Exclusive breastfeeding reduces acute respiratory infection and diarrhoea deaths among infants in Dhaka slums. *Pediatrics.* 108:E67.
15. Ashkenazi S, May L, LaRocco M, Lopez EL, Cleary TG. 1991. The effect of postnatal age on the adherence of enterohemorrhagic *Escherichia coli* to rabbit intestinal cells. *Pediatr Res.* 29:14-19.
16. Avril T, Wagner ER, Willison HJ, Crocker PR. 2006. Sialic acid-binding immunoglobulin-like lectin 7 mediates selective recognition of sialylated glycans expressed on *Campylobacter jejuni* lipooligosaccharides. *Infect Immun.* 74:4133-4141.
17. Bao Y, Chen C, Newburg DS. 2013. Quantification of neutral human milk oligosaccharides by graphitic carbon high-performance liquid chromatography with tandem mass spectrometry. *Anal Biochem*. 433:28-35. doi: 10.1016/j.ab.2012.10.003.
18. Bao Y, Zhu L, Newburg DS. 2007. Simultaneous quantification of sialyloligosaccharides from human milk by capillary electrophoresis. *Anal Biochem.* 370:206-214.
19. Barboza M, Pinzon J, Wickramasinghe S, Froehlich JW, Moeller I, Smilowitz JT, Ruhaak LR, Huang J, Lönnerdal B, German JB, Medrano JF, Weimer BC, Lebrilla CB. 2012. Glycosylation of human milk lactoferrin exhibits dynamic changes during early lactation enhancing its role in pathogenic bacteria-host interactions. *Mol Cell Proteomics.* 11:M111.015248. doi: 10.1074/mcp.M111.015248.

20. Beachy JC, Goldman D, Czech MP. 1981. Lectins activate lymphocyte pyruvate dehydrogenase by a mechanism sensitive to protease inhibitors. *Proc Natl Acad Sci U S A.* 78:6256-6260.

21. Becker PM, Widjaja-Greefkes HC, van Wikselaar PG. 2010. Inhibition of binding of the AB5-type enterotoxins LT-I and cholera toxin to ganglioside GM_1 by galactose-rich dietary components. *Foodborne Pathog Dis.* 7:225-233. doi: 10.1089/fpd.2009.0387.

22. Ben Bacha A, Abid I. 2013. Secretory phospholipase A2 in dromedary tears: a host defense against staphylococci and other gram-positive bacteria. *Appl Biochem Biotechnol.* 169:1858-1869. doi: 10.1007/s12010-013-0113-6.

23. Bener A, Denic S, Galadari S. 2001. Longer breast-feeding and protection against childhood leukaemia and lymphomas. *Eur J Cancer.* 37:234-238.

24. Bergström E, Hernell O, Persson LA, Vessby B. 1995. Serum lipid values in adolescents are related to family history, infant feeding, and physical growth. *Atherosclerosis.* 117:1-13.

25. Bessler HC, de Oliveira IR, Giugliano LG. 2006. Human milk glycoproteins inhibit the adherence of *Salmonella typhimurium* to HeLa cells. *Microbiol Immunol.* 50:877-882.

26. Blixt O, Head S, Mondala T, Scanlan C, Huflejt ME, Alvarez R, Bryan MC, Fazio F, Calarese D, Stevens J, Razi N, Stevens DJ, Skehel JJ, van Die I, Burton DR, Wilson IA, Cummings R, Bovin N, Wong CH, Paulson JC. 2004. Printed covalent glycan array for ligand profiling of diverse glycan binding proteins. *Proc Natl Acad Sci U S A.* 101:17033-17038.

27. Boccolini CS, Carvalho ML, Oliveira MI, Boccolini Pde M. 2011. Breastfeeding can prevent hospitalization for pneumonia among children under 1 year old. *J Pediatr (Rio J).* 87:399-404. doi: 10.2223/JPED.2136.

28. Bode L. 2009. Human milk oligosaccharides: prebiotics and beyond. *Nutr Rev.* 67:S183-S191. doi: 10.1111/j.1753-4887.2009.00239.x.

29. Bode L. 2012. Human milk oligosaccharides: every baby needs a sugar mama. *Glycobiology.* 22:1147-1162. doi: 10.1093/glycob/cws074.

30. Bode L, Kunz C, Muhly-Reinholz M, Mayer K, Seeger W, Rudloff S. 2004a. Inhibition of monocyte, lymphocyte, and neutrophil adhesion to endothelial cells by human milk oligosaccharides. *Thromb Haemost.* 92:1402-1410.

31. Bode L, Rudloff S, Kunz C, Strobel S, Klein N. 2004b. Human milk oligosaccharides reduce platelet-neutrophil complex formation leading to a

decrease in neutrophil beta 2 integrin expression. *J Leukoc Biol.* 76:820-826.

32. Borén T, Falk P, Roth KA, Larson G, Normark S. 1993. Attachment of *Helicobacter pylori* to human gastric epithelium mediated by blood group antigens. *Science.* 262:1892-1895.

33. Bosch JA, Veerman EC, Turkenburg M, Hartog K, Bolscher JG, Nieuw Amerongen AV. 2003. A rapid solid-phase fluorimetric assay for measuring bacterial adherence, using DNA-binding stains. *J Microbiol Methods.* 53:51-56.

34. Bramaud C, Aimar P, Daufin G. 1997. Whey protein fractionation: isoelectric precipitation of alpha-lactalbumin under gentle heat treatment. *Biotechnol Bioeng*. 56:391-397. doi: 10.1002/(SICI)1097-0290(19971120)56:4<391::AID-BIT5>3.0.CO;2-J.

35. Brassart D, Woltz A, Golliard M, Neeser JR. 1991. *In vitro* inhibition of adhesion of *Candida albicans* clinical isolates to human buccal epithelial cells by Fuc alpha 1-2Gal beta-bearing complex carbohydrates. *Infect Immun.* 59:1605-1613.

36. Brockhausen I, Schachter H, Stanley P. 2009. *O*-GalNAc Glycans. In: Varki A, Cummings RD, Esko JD, Freeze HH, Stanley P, Bertozzi CR, Hart GW, Etzler ME, editors. *Essentials of Glycobiology*. 2nd ed. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press. p. 115-127.

37. Cannon RD, Nand AK, Jenkinson HF. 1995. Adherence of *Candida albicans* to human salivary components adsorbed to hydroxylapatite. *Microbiology.* 141:213-219.

38. Caron E, Crepin VF, Simpson N, Knutton S, Garmendia J, Frankel G. 2006. Subversion of actin dynamics by EPEC and EHEC. *Curr Opin Microbiol.* 9:40-45.

39. Catzel D, Lalevski H, Marquis CP, Gray PP, Van Dyk D, Mahler SM. 2003. Purification of recombinant human growth hormone from CHO cell culture supernatant by Gradiflow preparative electrophoresis technology. *Protein Expr Purif.* 32:126-134.

40. Cavaletto M, Giuffrida MG, Conti A. 2008. Milk fat globule membrane components - a proteomic approach. *Adv Exp Med Biol.* 606:129-141. doi: 10.1007/978-0-387-74087-4_4.

41. Cavallone D, Malagolini N, Monti A, Wu XR, Serafini-Cessi F. 2004. Variation of high mannose chains of Tamm-Horsfall glycoprotein confers differential binding to type 1-fimbriated *Escherichia coli*. *J Biol Chem.* 2791:216-222.

42. Cederlund A, Kai-Larsen Y, Printz G, Yoshio H, Alvelius G, Lagercrantzm H, Strömberg R, Jörnvall H, Gudmundssonm GH, Agerberth B. 2013. Lactose in human breast milk an inducer of innate immunity with implications for a role in intestinal homeostasis. *PLoS One.* 8:e53876. doi: 10.1371/journal.pone.0053876.

43. César JA, Victora CG, Barros FC, Santos IS, Flores JA. 1999. Impact of breast feeding on admission for pneumonia during postneonatal period in Brazil: nested case-control study. *BMJ.* 318:1316-1320.

44. Chalmers NI, Palmer RJ Jr, Cisar JO, Kolenbrander PE. 2008. Characterization of a *Streptococcus* sp.-Veillonella sp. community micromanipulated from dental plaque. *J Bacteriol.* 190:8145-8154. doi: 10.1128/JB.00983-08.

45. Chantry CJ, Howard CR, Auinger P. 2006. Full breastfeeding duration and associated decrease in respiratory tract infection in US children. *Pediatrics.* 117:425-432.

46. Chapman DJ. 2011. New evidence: exclusive breastfeeding and reduced sudden infant death syndrome risk. *J Hum Lact.* 27:404-405. doi: 10.1177/0890334411420059.

47. Chatchatee P, Järvinen KM, Bardina L, Vila L, Beyer K, Sampson HA. 2001. Identification of IgE and IgG binding epitopes on beta- and kappa-casein in cow's milk allergic patients. *Clin Exp Allergy.* 31:1256-1262.

48. Chaturvedi P, Warren CD, Altaye M, Morrow AL, Ruiz-Palacios G, Pickering LK, Newburg DS. 2001a. Fucosylated human milk oligosaccharides vary between individuals and over the course of lactation. *Glycobiology.* 11:365-372.

49. Chaturvedi P, Warren CD, Buescher CR, Pickering LK, Newburg DS. 2001b. Survival of human milk oligosaccharides in the intestine of infants. *Adv Exp Med Biol.* 501:315-323.

50. Chessa D, Winter MG, Jakomin M, Bäumler AJ. 2009. *Salmonella enterica* serotype Typhimurium Std fimbriae bind terminal alpha(1,2)fucose residues in the cecal mucosa. *Mol Microbiol.* 71:864-875. doi: 10.1111/j.1365-2958.2008.06566.x.

51. Cheung GL, Thomas TM, Rylatt DB. 2003. Purification of antibody Fab and F(ab')2 fragments using Gradiflow technology. *Protein Expr Purif.* 32:135-140.

52. Chichlowski M, German JB, Lebrilla CB, Mills DA. 2011. The influence of milk oligosaccharides on microbiota of infants: opportunities for formulas. *Annu Rev Food Sci Technol.* 2:331-351. doi: 10.1146/annurev-food-022510-133743.

53. Cleary J, Lai LC, Shaw RK, Straatman-Iwanowska A, Donnenberg MS, Frankel

G, Knutton S. 2004. Enteropathogenic *Escherichia coli* (EPEC) adhesion to intestinal epithelial cells: role of bundle-forming pili (BFP), EspA filaments and intimin. *Microbiology.* 150(Pt 3):527-538.

54. Clemens JD, Sack DA, Harris JR, Khan MR, Chakraborty J, Chowdhury S, Rao MR, van Loon FP, Stanton BF, Yunus M. 1990. Breast feeding and the risk of severe cholera in rural Bangladeshi children. *Am J Epidemiol.* 131:400-411.

55. Coppa GV, Gabrielli O, Giorgi P, Catassi C, Montanari MP, Varaldo PE, Nichols BL. 1990. Preliminary study of breastfeeding and bacterial adhesion to uroepithelial cells. *Lancet.* 335:569-571.

56. Coppa GV, Pierani P, Zampini L, Carloni I, Carlucci A, Gabrielli O. 1999. Oligosaccharides in human milk during different phases of lactation. *Acta Paediatr Suppl.* 88:89-94.

57. Coppa GV, Zampini L, Galeazzi T, Facinelli B, Ferrante L, Capretti R, Orazio G. 2006. Human milk oligosaccharides inhibit the adhesion to Caco-2 cells of diarrhoeal pathogens: *Escherichia coli, Vibrio cholerae*, and *Salmonella fyris*. *Pediatr Res.* 59:377-382.

58. Cooper CA, Gasteiger E, Packer NH. 2001. GlycoMod - a software tool for determining glycosylation compositions from mass spectrometric data. *Proteomics*. 1:340-349.

59. Cooper C.A., Gasteiger E., Packer N. 2003. Predicting Glycan Composition from Experimental Mass Using GlycoMod. In: Conn PM, editor. *Handbook of Proteomic Methods*. Totowa (NJ): Humana Press. p. 225-232.

60. Crane JK, Azar SS, Stam A, Newburg DS. 1994. Oligosaccharides from human milk block binding and activity of the *Escherichia coli* heat-stable enterotoxin (STa) in T84 intestinal cells. *J Nutr.* 124:2358-2364.

61. Cravioto A, Tello A, Villafán H, Ruiz J, del Vedovo S, Neeser JR. 1991. Inhibition of localized adhesion of enteropathogenic *Escherichia coli* to HEp-2 cells by immunoglobulin and oligosaccharide fractions of human colostrum and breast milk. *J Infect Dis.* 163:1247-1255.

62. Crittenden RG, Bennett LE. 2005. Cow's milk allergy: a complex disorder. *J Am Coll Nutr*. 24:582S-591S.

63. Cruz JR, Gil L, Cano F, Caceres P, Pareja G. 1988. Breast milk anti-*Escherichia coli* heat-labile toxin IgA antibodies protect against toxin-induced infantile diarrhea. *Acta Paediatr Scand.* 77:658-662.

64. Cushing AH, Samet JM, Lambert WE, Skipper BJ, Hunt WC, Young SA,

McLaren LC. 1998. Breastfeeding reduces risk of respiratory illness in infants. *Am J Epidemiol.* 147:863-870.

65. Dallas DC, Martin WF, Hua S, German JB. 2012. Automated glycopeptide analysis--review of current state and future directions. *Brief Bioinform.* 14:361-374. doi: 10.1093/bib/bbs045.

66. Dallas DC, Martin WF, Strum JS, Zivkovic AM, Smilowitz JT, Underwood MA, Affolter M, Lebrilla CB, German JB. 2011. *N*-linked glycan profiling of mature human milk by high-performance microfluidic chip liquid chromatography time-of-flight tandem mass spectrometry. *J Agric Food Chem.* 59:4255-4263. doi: 10.1021/jf104681p.

67. Dallas SD, Rolfe RD. 1998. Binding of *Clostridium difficile* toxin A to human milk secretory component. *J Med Microbiol.* 47:879-888.

68. Danielsson Niemi L, Hernell O, Johansson I. 2009. Human milk compounds inhibiting adhesion of mutans streptococci to host ligand-coated hydroxyapatite *in vitro*. *Caries Res.* 43:171-178. doi: 10.1159/000213888.

69. Davidson B, Meinzen-Derr JK, Wagner CL, Newburg DS, Morrow AL. 2004. Fucosylated oligosaccharides in human milk in relation to gestational age and stage of lactation. *Adv Exp Med Biol.* 554:427-430.

70. Davis MK, Savitz DA, Graubard BI. 1988. Infant feeding and childhood cancer. Lancet. 2:365-368.

71. Day CJ, Semchenko EA, Korolik V. 2012. Glycoconjugates play a key role in *Campylobacter jejuni* Infection: interactions between host and pathogen. *Front Cell Infect Microbiol.* 2:9. doi: 10.3389/fcimb.2012.00009.

72. Day CJ, Tiralongo J, Hartnell RD, Logue CA, Wilson JC, von Itzstein M, Korolik V. 2009. Differential carbohydrate recognition by *Campylobacter jejuni* strain 11168: influences of temperature and growth conditions. *PLoS One.* 4:e4927. doi: 10.1371/journal.pone.0004927.

73. de Araújo AN, Giugliano LG. 2001. Lactoferrin and free secretory component of human milk inhibit the adhesion of enteropathogenic *Escherichia coli* to HeLa cells. *BMC Microbiol.* 1:25.

74. Dickson RC. 1998. Sphingolipid functions in *Saccharomyces cerevisiae*: comparison to mammals. *Annu Rev Biochem.* 67:27-48.

75. Domann PJ, Pardos-Pardos AC, Fernandes DL, Spencer DI, Radcliffe CM, Royle L, Dwek RA, Rudd PM. 2007. Separation-based glycoprofiling approaches using fluorescent labels. *Proteomics.* 7:70-76.

76. Downham MA, Scott R, Sims DG, Webb JK, Gardner PS. 1976. Breast-feeding protects against respiratory syncytial virus infections. *Br Med J.* 2:274-276.

77. Duffy LC, Faden H, Wasielewski R, Wolf J, Krystofik D. 1997. Exclusive breastfeeding protects against bacterial colonization and day care exposure to otitis media. *Pediatrics.* 100:E7.

78. Duijts L, Ramadhani MK, Moll HA. 2009. Breastfeeding protects against infectious diseases during infancy in industrialized countries. A systematic review. *Matern Child Nutr.* 5:199-210. doi: 10.1111/j.1740-8709.2008.00176.x.

79. Duncan B, Ey J, Holberg CJ, Wright AL, Martinez FD, Taussig LM. 1993. Exclusive breast-feeding for at least 4 months protects against otitis media. *Pediatrics.* 91:867-872.

80. Duncan MJ, Mann EL, Cohen MS, Ofek I, Sharon N, Abraham SN. 2005. The distinct binding specificities exhibited by enterobacterial type 1 fimbriae are determined by their fimbrial shafts. *J Biol Chem.* 280:37707-37716.

81. Ebrahim GJ. 1998. Breastmilk nucleotides. *J Trop Pediatr.* 44:318-319.

82. El-Sayed MM, Chase HA. 2010. Purification of the two major proteins from whey concentrate using a cation-exchange selective adsorption process. *Biotechnol Prog.* 26:192-199. doi: 10.1002/btpr.316.

83. Elliott EJ, Dalby-Payne JR. 2004. 2. Acute infectious diarrhoea and dehydration in children. *Med J Aust.* 181:565-570.

84. Engfer MB, Stahl B, Finke B, Sawatzki G, Daniel H. 2000. Human milk oligosaccharides are resistant to enzymatic hydrolysis in the upper gastrointestinal tract. *Am J Clin Nutr.* 71:1589-1596.

85. Everest-Dass AV, Jin D, Thaysen-Andersen M, Nevalainen H, Kolarich D, Packer NH. 2012. Comparative structural analysis of the glycosylation of salivary and buccal cell proteins: innate protection against infection by *Candida albicans*. *Glycobiology.* 22:1465-1479. doi: 10.1093/glycob/cws112.

86. Fábián TK, Hermann P, Beck A, Fejérdy P, Fábián G. 2012. Salivary defense proteins: their network and role in innate and acquired oral immunity. *Int J Mol Sci.* 13:4295-4320. doi: 10.3390/ijms13044295.

87. Falk P, Roth KA, Borén T, Westblom TU, Gordon JI, Normark S. 1993. An *in vitro* adherence assay reveals that *Helicobacter pylori* exhibits cell lineage-specific tropism in the human gastric epithelium. *Proc Natl Acad Sci U S A.* 90:2035-2039.

88. Fälth-Magnusson K, Franzén L, Jansson G, Laurin P, Stenhammar L. 1996. Infant

feeding history shows distinct differences between Swedish celiac and reference children. *Pediatr Allergy Immunol.* 7:1-5.

89. Fazio F, Bryan MC, Blixt O, Paulson JC, Wong CH. 2002. Synthesis of sugar arrays in microtiter plate. *J Am Chem Soc.* 124:14397-1402.

90. Feachem RG, Koblinsky MA. 1984. Interventions for the control of diarrhoeal diseases among young children: promotion of breast-feeding. *Bull World Health Organ.* 62:271-291.

91. Fernández MF, Boris S, Barbés C. 2003. Probiotic properties of human lactobacilli strains to be used in the gastrointestinal tract. *J Appl Microbiol.* 94:449-455.

92. Ferrer E, Alegría A, Farré R, Clemente G, Calvo C. 2005. Fluorescence, browning index, and color in infant formulas during storage. *J Agric Food Chem.* 53:4911-4917.

93. Fiat AM, Jollès J, Aubert JP, Loucheux-Lefebvre MH, Jollès P. 1980. Localisation and importance of the sugar part of human casein. *Eur J Biochem.* 111:333-339.

94. Fischer Walker CL, Perin J, Aryee MJ, Boschi-Pinto C, Black RE. 2010. Diarrhoea incidence in low- and middle-income countries in 1990 and 2010: a systematic review. *BMC Public Health.* 12:220. doi: 10.1186/1471-2458-12-220.

95. Fisk CM, Crozier SR, Inskip HM, Godfrey KM, Cooper C, Roberts GC, Robinson SM; Southampton Women's Survey Study Group. 2011. Breastfeeding and reported morbidity during infancy: findings from the Southampton Women's Survey. *Matern Child Nutr.* 7:61-70. doi: 10.1111/j.1740-8709.2010.00241.x.

96. Ford RP, Taylor BJ, Mitchell EA, Enright SA, Stewart AW, Becroft DM, Scragg R, Hassall IB, Barry DM, Allen EM, Roberts AP. 1993. Breastfeeding and the risk of sudden infant death syndrome. *Int J Epidemiol.* 22:885-890.

97. Fort P, Lanes R, Dahlem S, Recker B, Weyman-Daum M, Pugliese M, Lifshitz F. 1986. Breast feeding and insulin-dependent diabetes mellitus in children. *J Am Coll Nutr.* 5:439-441.

98. Fortunato D, Giuffrida MG, Cavaletto M, Garoffo LP, Dellavalle G, Napolitano L, Giunta C, Fabris C, Bertino E, Coscia A, Conti A. 2003. Structural proteome of human colostral fat globule membrane proteins. *Proteomics*. 3:897-905.

99. Franklin RM. 1989. The ocular secretory immune system: a review. *Curr Eye Res.* 8:599-606.

100. Friis LM, Pin C, Pearson BM, Wells JM. 2005. *In vitro* cell culture methods for investigating *Campylobacter* invasion mechanisms. *J Microbiol Methods.* 61:145-160.

101. Froehlich JW, Dodds ED, Barboza M, McJimpsey EL, Seipert RR, Francis J, An HJ, Freeman S, German JB, Lebrilla CB. 2010. Glycoprotein expression in human milk during lactation. *J Agric Food Chem.* 58:6440-6448. doi: 10.1021/jf100112x.

102. Fu D, O'Neill RA. 1995. Monosaccharide composition analysis of oligosaccharides and glycoproteins by high-performance liquid chromatography. *Anal Biochem.* 227:377-384.

103. Fukui S, Feizi T, Galustian C, Lawson AM, Chai W. 2002. Oligosaccharide microarrays for high-throughput detection and specificity assignments of carbohydrate-protein interactions. *Nat Biotechnol.* 20:1011-1017.

104. Fuller ME, Streger SH, Rothmel RK, Mailloux BJ, Hall JA, Onstott TC, Fredrickson JK, Balkwill DL, DeFlaun MF. 2000. Development of a vital fluorescent staining method for monitoring bacterial transport in subsurface environments. *Appl Environ Microbiol*. 66:4486-4496.

105. Gabrielli O, Zampini L, Galeazzi T, Padella L, Santoro L, Peila C, Giuliani F, Bertino E, Fabris C, Coppa GV. 2011. Preterm milk oligosaccharides during the first month of lactation. *Pediatrics.* 128:e1520-e1531. doi: 10.1542/peds.2011-1206.

106. Gagnon M, Zihler Berner A, Chervet N, Chassard C, Lacroix C. 2013. Comparison of the Caco-2, HT-29 and the mucus-secreting HT29-MTX intestinal cell models to investigate *Salmonella* adhesion and invasion. *J Microbiol Methods.* 94:274-279. doi: 10.1016/j.mimet.2013.06.027.

107. Gallegos KM, Conrady DG, Karve SS, Gunasekera TS, Herr AB, Weiss AA. 2012. Shiga toxin binding to glycolipids and glycans. *PLoS One.* 7:e30368. doi: 10.1371/journal.pone.0030368.

108. Garofalo R. 2010. Cytokines in human milk. *J Pediatr.* 156(2 Suppl):S36-40. doi: 10.1016/j.jpeds.2009.11.019.

109. Gee SC, Bate IM, Thomas TM, Rylatt DB. 2003. The purification of IgY from chicken egg yolk by preparative electrophoresis. *Protein Expr Purif.* 30:151-155.

110. Gillin FD, Reiner DS, Wang CS. 1983a. Human milk kills parasitic intestinal protozoa. *Science.* 221:1290-1292.

111. Georgi G, Bartke N, Wiens F, Stahl B. 2013. Functional glycans and glycoconjugates in human milk. *Am J Clin Nutr.* 98:578S-585S. doi: 10.3945/ajcn.112.039065.

112. Giannasca KT, Giannasca PJ, Neutra MR. 1996. Adherence of *Salmonella typhimurium* to Caco-2 cells: identification of a glycoconjugate receptor. *Infect Immun.* 64:135-145.

113. Gibbons RJ, Qureshi JV. 1979. Inhibition of adsorption of *Streptococcus mutans* strains to saliva-treated hydroxyapatite by galactose and certain amines. *Infect Immun.* 26:1214-1217.

114. Gilboa-Garber N, Sudakevitz D, Sheffi M, Sela R, Levene C. 1994. PA-I and PA-II lectin interactions with the ABO(H) and P blood group glycosphingolipid antigens may contribute to the broad spectrum adherence of *Pseudomonas aeruginosa* to human tissues in secondary infections. *Glycoconj J.* 11:414-417.

115. Gillin FD, Reiner DS, Wang CS. 1983b. Killing of *Giardia lamblia* trophozoites by normal human milk. *J Cell Biochem.* 23:47-56.

116. Girón JA, Torres AG, Freer E, Kaper JB. 2002. The flagella of enteropathogenic *Escherichia coli* mediate adherence to epithelial cells. *Mol Microbiol.* 44:361-379.

117. Giugliano LG, Ribeiro ST, Vainstein MH, Ulhoa CJ. 1995. Free secretory component and lactoferrin of human milk inhibit the adhesion of enterotoxigenic *Escherichia coli. J Med Microbiol.* 42:3-9.

118. Glass RI, Svennerholm AM, Stoll BJ, Khan MR, Hossain KM, Huq MI, Holmgren J. 1983. Protection against cholera in breast-fed children by antibodies in breast milk. *N Engl J Med.* 308:1389-1392.

119. Gnoth MJ, Kunz C, Kinne-Saffran E, Rudloff S. 2000. Human milk oligosaccharides are minimally digested *in vitro*. *J Nutr.* 130:3014-3020.

120. Goldman AS, Garza C, Nichols BL, Goldblum RM. 1982. Immunologic factors in human milk during the first year of lactation. *J Pediatr.* 100:563-567.

121. Gomez HF, Ochoa TJ, Carlin LG, Cleary TG. 2003. Human lactoferrin impairs virulence of *Shigella flexneri*. *J Infect Dis.* 187:87-95.

122. Gopal PK, Gill HS. 2000. Oligosaccharides and glycoconjugates in bovine milk and colostrum. *Br J Nutr.* 84:S69-S74.

123. Gouveri E, Papanas N, Hatzitolios AI, Maltezos E. 2011. Breastfeeding and diabetes. *Curr Diabetes Rev.* 7:135-142.

124. Green ED, Adelt G, Baenziger JU, Wilson S, Van Halbeek H. 1988. The asparagine-linked oligosaccharides on bovine fetuin. Structural analysis of *N*-

glycanase-released oligosaccharides by 500-megahertz 1H NMR spectroscopy. *J Biol Chem.* 263:18253-18268.

125. Groer M, Davis M, Steele K. 2004. Associations between human milk sIgA and maternal immune, infectious, endocrine, and stress variables. *J Hum Lact.* 20:153-158.

126. Gross G, van der Meulen J, Snel J, van der Meer R, Kleerebezem M, Niewold TA, Hulst MM, Smits MA. 2008. Mannose-specific interaction of *Lactobacillus plantarum* with porcine jejunal epithelium. *FEMS Immunol Med Microbiol.* 54:215-223. doi: 10.1111/j.1574-695X.2008.00466.x.

127. Grufferman S. 1998. Methodologic approaches to studying environmental factors in childhood cancer. *Environ Health Perspect.* 106:881-886.

128. Grulee C, Sanford H, Schwartz J. 1934. Breast and artificially fed infants. *JAMA.* 104:1986-1988.

129. Grzymajlo K, Kuzminska-Bajor M, Jaworski J, Dobryszycki P, Ugorski M. 2010. The high-adhesive properties of the FimH adhesin of *Salmonella enterica* serovar Enteritidis are determined by a single F118S substitution. *Microbiology.* 156:1738-1748. doi: 10.1099/mic.0.039206-0.

130. Guarner F, Malagelada JR. 2003. Gut flora in health and disease. *Lancet.* 361:512-519.

131. Guttman A, Chen FT, Evangelista RA. 1996. Separation of 1-aminopyrene-3,6,8-trisulfonate-labeled asparagine-linked fetuin glycans by capillary gel electrophoresis. *Electrophoresis*. 17:412-417.

132. Hajishengallis G, Nikolova E, Russell MW. 1992. Inhibition of *Streptococcus mutans* adherence to saliva-coated hydroxyapatite by human secretory immunoglobulin A (S-IgA) antibodies to cell surface protein antigen I/II: reversal by IgA1 protease cleavage. *Infect Immun.* 60:5057-5064.

133. Hakkarainen J, Toivanen M, Leinonen A, Frängsmyr L, Strömberg N, Lapinjoki S, Nassif X, Tikkanen-Kaukanen C. 2005. Human and bovine milk oligosaccharides inhibit *Neisseria meningitidis* pili attachment *in vitro*. *J Nutr.* 135:2445-2448.

134. Hale TW, Hartmann, P.E. 2007. Mammary gland: past, present, and future. In; *Textbook of human lactation*. 1st ed. Amarillo (TX): Hale Publishing.

135. Hamosh M. 1998. Protective function of proteins and lipids in human milk. *Biol Neonate.* 74:163-176.

136. Hanisch FG, Peter-Katalinic J, Egge H, Dabrowski U, Uhlenbruck G. 1990. Structures of acidic *O*-linked polylactosaminoglycans on human skim milk mucins. *Glycoconj J.* 7:525-543.

137. Hanson LA. 2004. Protective effects of breastfeeding against urinary tract infection. *Acta Paediatr.* 93:154-156.

138. Harazono A, Kobayashi T, Kawasaki N, Itoh S, Tada M, Hashii N, Ishii A, Yanagihara S, Yagi Y, Koga A, Tsuda Y, Kimura M, Sakita M, Kitamura S, Yamaguchi H, Mimura H, Murata Y, Hamazume Y, Sato T, Natsuka S, Kakehi K, Kinoshita M, Watanabe S, Yamaguchi T. 2011. A comparative study of monosaccharide composition analysis as a carbohydrate test for biopharmaceuticals. *Biologicals.* 39:171-180. doi: 10.1016/j.biologicals.2011.04.002.

139. Harmsen MC, Swart PJ, de Béthune MP, Pauwels R, De Clercq E, The TH, Meijer DK. 1995. Antiviral effects of plasma and milk proteins: lactoferrin shows potent activity against both human immunodeficiency virus and human cytomegalovirus replication *in vitro*. *J Infect Dis.* 172:380-388.

140. Haslam SM, North SJ, Dell A. 2006. Mass spectrometric analysis of *N*- and *O*-glycosylation of tissues and cells. *Curr Opin Struct Bio.* 16:584-591.

141. Hauck FR, Thompson JM, Tanabe KO, Moon RY, Vennemann MM. 2011. Breastfeeding and reduced risk of sudden infant death syndrome: a meta-analysis. *Pediatrics.* 128:103-110. doi: 10.1542/peds.2010-3000.

142. Hayase T, Rice KG, Dziegielewska KM, Kuhlenschmidt M, Reilly T, Lee YC. Comparison of *N*-glycosides of fetuins from different species and human alpha 2-HS-glycoprotein. *Biochemistry*. 31:4915-421.

143. Heimburg-Molinaro J, Tappert M, Song X, Lasanajak Y, Air G, Smith DF, Cummings RD. 2012. Probing virus-glycan interactions using glycan microarrays. *Methods Mol Biol.* 808:251-267. doi: 10.1007/978-1-61779-373-8_18.

144. Heine WE, Klein PD, Reeds PJ. 1991. The importance of alpha-lactalbumin in infant nutrition. *J Nutr.* 121:277-283.

145. Herrera-Insua I, Gomez HF, Diaz-Gonzalez VA, Chaturvedi P, Newburg DS, Cleary TG. 2001. Human milk lipids bind Shiga toxin. *Adv Exp Med Biol.* 501:333-339.

146. Hester SN, Chen X, Li M, Monaco MH, Comstock SS, Kuhlenschmidt TB, Kuhlenschmidt MS, Donovan SM. 2013. Human milk oligosaccharides inhibit

rotavirus infectivity *in vitro* and in acutely infected piglets. *Br J Nutr.* 110:1233-42. doi: 10.1017/S0007114513000391.

147. Holmgren J, Svennerholm AM, Lindblad M. 1983. Receptor-like glycocompounds in human milk that inhibit classical and El Tor *Vibrio cholerae* cell adherence (hemagglutination). *Infect Immun.* 39:147-154.

148. Horvath ZS, Corthals GL, Wrigley CW, Margolis J. 1994. Multifunctional apparatus for electrokinetic processing of proteins. *Electrophoresis.* 15:968-971.

149. Houseman BT, Mrksich M. 2002. Carbohydrate arrays for the evaluation of protein binding and enzymatic modification. *Chem Biol.* 9:443-454.

150. Howie PW, Forsyth JS, Ogston SA, Clark A, Florey CD. 1990. Protective effect of breast feeding against infection. *BMJ.* 300:11-16.

151. Hu L, Kopecko DJ. 1999. *Campylobacter jejuni* 81-176 associates with microtubules and dynein during invasion of human intestinal cells. *Infect Immun.* 67:4171-4182.

152. Hu L, Tall BD, Curtis SK, Kopecko DJ. 2008. Enhanced microscopic definition of *Campylobacter jejuni* 81-176 adherence to, invasion of, translocation across, and exocytosis from polarized human intestinal Caco-2 cells. *Infect Immun.* 76:5294-5304. doi: 10.1128/IAI.01408-07.

153. Huang IN, Okawara T, Watanabe M, Kawai Y, Kitazawa H, Ohnuma S, Shibata C, Horii A, Kimura K, Taketomo N, Xiao JZ, Iwatsuki K, Saito T. 2013. New screening methods for probiotics with adhesion properties to sialic acid and sulphate residues in human colonic mucin using the Biacore assay. *J Appl Microbiol.* 114:854-60. doi: 10.1111/jam.12063.

154. Hurley WL, Grieve RC, Magura CE, Hegarty HM, Zou S. 1993. Electrophoretic comparisons of lactoferrin from bovine mammary secretions, milk neutrophils, and human milk. *J Dairy Sci.* 76:377-387.

155. Hurley WL, Rejman JJ. 1993. Bovine lactoferrin in involuting mammary tissue. *Cell Biol Int.* 17:283-289.

156. Idota T, Kawakami H. 1995. Inhibitory effects of milk gangliosides on the adhesion of *Escherichia coli* to human intestinal carcinoma cells. *Biosci Biotechnol Biochem.* 59:69-72.

157. Idota T, Kawakami H, Murakami Y, Sugawara M. 1995a. Inhibition of cholera toxin by human milk fractions and sialyllactose. *Biosci Biotechnol Biochem.* 59:417-419.

158. Iskarpatyoti JA, Morse EA, McClung RP, Ikizler M, Wetzel JD, Contractor N,

Dermody TS. 2012. Serotype-specific differences in inhibition of reovirus infectivity by human-milk glycans are determined by viral attachment protein σ1. *Virology.* 433:489-497. doi: 10.1016/j.virol.2012.08.036.

159. Iyengar SR, Walker WA. 2012. Immune factors in breast milk and the development of atopic disease. *J Pediatr Gastroenterol Nutr.* 55:641-647. doi: 10.1097/MPG.0b013e3182617a9d.

160. Jackson AD. 2001. Airway goblet-cell mucus secretion. *Trends Pharmacol Sci.* 22:39-45.

161. Jakobsson I, Lindberg T, Benediktsson B, Hansson BG. 1985. Dietary bovine beta-lactoglobulin is transferred to human milk. *Acta Paediatr Scand.*74:342-345.

162. Jensen PH, Karlsson NG, Kolarich D, Packer NH. 2012. Structural analysis of *N*- and *O*-glycans released from glycoproteins. *Nat Protoc.* 7:1299-1310. doi: 10.1038/nprot.2012.063.

163. Jensen RG. 2002. The composition of bovine milk lipids: January 1995 to December 2000. *J Dairy Sci.* 85:295-350.

164. Jensen RG, Clark RM, Ferris AM. 1980. Composition of the lipids in human milk: a review. *Lipids.* 15:345-355.

165. Jenssen H, Sandvik K, Andersen JH, Hancock RE, Gutteberg TJ. 2008. Inhibition of HSV cell-to-cell spread by lactoferrin and lactoferricin. *Antiviral Res.* 79:192-198. doi: 10.1016/j.antiviral.2008.03.004.

166. Jiang X, Huang P, Zhong W, Tan M, Farkas T, Morrow AL, Newburg DS, Ruiz-Palacios GM, Pickering LK. 2004. Human milk contains elements that block binding of noroviruses to human histo-blood group antigens in saliva. *J Infect Dis.* 190:1850-1859.

167. Johnson PH, Donald AS, Feeney J, Watkins WM. 1992. Reassessment of the acceptor specificity and general properties of the Lewis blood-group gene associated alpha-3/4-fucosyltransferase purified from human milk. *Glycoconj J.* 9:251-264.

168. Johnson PH, Watkins WM. 1992. Purification of the Lewis blood-group gene associated alpha-3/4-fucosyltransferase from human milk: an enzyme transferring fucose primarily to type 1 and lactose-based oligosaccharide chains. *Glycoconj J.* 9:241-249.

169. Jones GW, Isaacson RE. 1983. Proteinaceous bacterial adhesins and their receptors. *Crit Rev Microbiol.* 10:229-260.

170. Kalidas C, Joshi L, Batt C. 2001. Characterization of glycosylated variants of beta-lactoglobulin expressed in *Pichia pastoris*. *Protein Eng.* 143:201-207.

171. Kaliner MA. 1992. Human nasal host defense and sinusitis. *J Allergy Clin Immunol.* 90:424-430.

172. Kaper JB, Nataro JP, Mobley HL. 2004. Pathogenic *Escherichia coli*. *Nat Rev Microbiol.* 2:123-140.

173. Karlsson NG, Schulz BL, Packer NH. 2004a. Structural determination of neutral *O*-linked oligosaccharide alditols by negative ion LC-electrospray-MSn. *J Am Soc Mass Spectrom.* 15:659-672.

174. Karlsson NG, Wilson NL, Wirth HJ, Dawes P, Joshi H, Packer NH. 2004b. Negative ion graphitised carbon nano-liquid chromatography/mass spectrometry increases sensitivity for glycoprotein oligosaccharide analysis. *Rapid Commun Mass Spectrom*. 18:2282-2292.

175. Keenan TW, Patton S. 1995. The structure of milk: Implications for sampling and storage. In: Jensen RG, editor. *Handbook of Milk Composition*. San Diego (CA): Academic Press. p. 5-50

176. Kelly CG, Younson JS. 2000. Anti-adhesive strategies in the prevention of infectious disease at mucosal surfaces. *Expert Opin Investig Drugs.* 9:1711-1721.

177. Kelley L. 2012. Increasing the consumption of breast milk in low-birth-weight infants: can it have an impact on necrotizing enterocolitis? *Adv Neonatal Care.* 12:267-272. doi: 10.1097/ANC.0b013e3182613bff.

178. Kisiela D, Sapeta A, Kuczkowski M, Stefaniak T, Wieliczko A, Ugorski M. (2005) Characterization of FimH adhesins expressed by *Salmonella enterica* serovar Gallinarum biovars Gallinarum and Pullorum: reconstitution of mannose-binding properties by single amino acid substitution. *Infect Immun.* 73:6187-6190.

179. Klein MI, Bergel E, Gibbons L, Coviello S, Bauer G, Benitez A, Serra ME, Delgado MF, Melendi GA, Rodríguez S, Kleeberger SR, Polack FP. 2008. Differential gender response to respiratory infections and to the protective effect of breast milk in preterm infants. *Pediatrics.* 121:e1510-e1516. doi: 10.1542/peds.2007-1757.

180. Kobata A. 2010. Structures and application of oligosaccharides in human milk. *Proc Jpn Acad Ser B Phys Biol Sci.* 86:731-747.

181. Kobata, A. 2013. Exo- and endoglycosidases revisited. *Proc Jpn Acad Ser B Phys Biol Sci.* 89:97-117.

182. Kolarich D, Jensen PH, Altmann F, Packer NH. 2012. Determination of site-specific glycan heterogeneity on glycoproteins. *Nat Protoc.* 7:1285-1298. doi: 10.1038/nprot.2012.062.

183. Koletzko S, Sherman P, Corey M, Griffiths A, Smith C. 1989. Role of infant feeding practices in development of Crohn's disease in childhood. BMJ. 298:1617-1618.

184. Konkel ME, Corwin MD, Joens LA, Cieplak W. 1992a. Factors that influence the interaction of *Campylobacter jejuni* with cultured mammalian cells. *J Med Microbiol.* 37:30-37.

185. Konkel ME, Hayes SF, Joens LA, Cieplak W Jr. 1992b. Characteristics of the internalization and intracellular survival of *Campylobacter jejuni* in human epithelial cell cultures. *Microb Pathog.* 13:357-370.

186. Kotloff KL, Nataro JP, Blackwelder WC, Nasrin D, Farag TH, Panchalingam S, Wu Y, Sow SO, Sur D, Breiman RF, Faruque AS, Zaidi AK, Saha D, Alonso PL, Tamboura B, Sanogo D, Onwuchekwa U, Manna B, Ramamurthy T, Kanungo S, Ochieng JB, Omore R, Oundo JO, Hossain A, Das SK, Ahmed S, Qureshi S,Quadri F, Adegbola RA, Antonio M, Hossain MJ, Akinsola A, Mandomando I, Nhampossa T, Acácio S, Biswas K, O'Reilly CE, Mintz ED, Berkeley LY, Muhsen K, Sommerfelt H, Robins-Browne RM, Levine MM. 2013. Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. *Lancet.* 382:209-222. doi: 10.1016/S0140-6736(13)60844-2.

187. Kramer MS, Chalmers B, Hodnett ED, Sevkovskaya Z, Dzikovich I, Shapiro S, Collet JP, Vanilovich I, Mezen I, Ducruet T, Shishko G, Zubovich V, Mknuik D, Gluchanina E, Dombrovskiy V, Ustinovitch A, Kot T, Bogdanovich N, Ovchinikova L, Helsing E; PROBIT Study Group (Promotion of Breastfeeding Intervention Trial). 2001. Promotion of Breastfeeding Intervention Trial (PROBIT): a randomized trial in the Republic of Belarus. *JAMA.* 285:413-420.

188. Kreth J, Zhang Y, Herzberg MC. 2008. Streptococcal antagonism in oral biofilms: *Streptococcus sanguinis* and *Streptococcus gordonii* interference with *Streptococcus mutans*. *J Bacteriol.* 190:4632-4640. doi: 10.1128/JB.00276-08.

189. Kulkarni AD, Rudolph FB, Van Buren CT. 1994. The role of dietary sources of nucleotides in immune function: a review. *J Nutr.* 124(8 Suppl):1442S-1446S.

190. Kumazaki T, Yoshida A. 1984. Biochemical evidence that secretor gene, Se, is a structural gene encoding a specific fucosyltransferase. *Proc Natl Acad Sci U S A.*

81:4193-4197.

191. Kunz C, Lönnerdal B. 1989. Human milk proteins: separation of whey proteins and their analysis by polyacrylamide gel electrophoresis, fast protein liquid chromatography (FPLC) gel filtration, and anion-exchange chromatography. *Am J Clin Nutr.* 49:464-470.

192. Kunz C, Lönnerdal B. 1993. Protein composition of rhesus monkey milk: comparison to human milk. *Comp Biochem Physiol Comp Physiol.* 104:793-797.

193. Kunz C, Rodriguez-Palmero M, Koletzko B, Jensen R. 1999a. Nutritional and biochemical properties of human milk, Part I: General aspects, proteins, and carbohydrates. *Clin Perinatol.* 26:307-333.

194. Kunz C, Rudloff S. 1993. Biological functions of oligosaccharides in human milk. *Acta Paediatr.* 82:903-912.

195. Kunz C, Rudloff S, Baier W, Klein N, Strobel S. 2000. Oligosaccharides in human milk: structural, functional, and metabolic aspects. *Annu Rev Nutr.* 20:699-722.

196. Kunz C, Rudloff S, Schad W, Braun D. 1999b. Lactose-derived oligosaccharides in the milk of elephants: comparison with human milk. *Br J Nutr.* 82:391-399.

197. Kuramitsu HK, Wang BY. 2006. Virulence properties of cariogenic bacteria. BMC Oral Health. 6:S11.

198. Kvistgaard AS, Pallesen LT, Arias CF, López S, Petersen TE, Heegaard CW, Rasmussen JT. 2004. Inhibitory effects of human and bovine milk constituents on rotavirus infections. *J Dairy Sci.* 87:4088-4096.

199. Laegreid A, Kolstø Otnaess AB. 1987. Trace amounts of ganglioside GM_1 in human milk inhibit enterotoxins from *Vibrio cholerae* and *Escherichia coli*. *Life Sci.* 40:55-62.

200. Lamberti LM, Fischer Walker CL, Noiman A, Victora C, Black RE. 2011. Breastfeeding and the risk for diarrhea morbidity and mortality. *BMC Public Health.* 11:S15. doi: 10.1186/1471-2458-11-S3-S15.

201. Lane JA, Mariño K, Naughton J, Kavanaugh D, Clyne M, Carrington SD, Hickey RM. 2012a. Anti-infective bovine colostrum oligosaccharides: *Campylobacter jejuni* as a case study. *Int J Food Microbiol.* 157:182-188. doi: 10.1016/j.ijfoodmicro.2012.04.027.

202. Lane JA, Mariño K, Rudd PM, Carrington SD, Slattery H, Hickey RM. 2012b. Methodologies for screening of bacteria-carbohydrate interactions: anti-adhesive milk oligosaccharides as a case study. *J Microbiol Methods.* 90:53-59.

203. Ledeboer NA, Frye JG, McClelland M, Jones BD. 2006. *Salmonella enterica*

serovar Typhimurium requires the Lpf, Pef, and Tafi fimbriae for biofilm formation on HEp-2 tissue culture cells and chicken intestinal epithelium. *Infect Immun.* 74:3156-3169. doi: 10.1016/j.mimet.2012.03.017.

204. Lee A, Kolarich D, Haynes PA, Jensen PH, Baker MS, Packer NH. 2009. Rat liver membrane glycoproteome: enrichment by phase partitioning and glycoprotein capture. *J Proteome Res.* 8:770-781. doi: 10.1021/pr800910w.

205. Leppänen A, Cummings RD. 2010. Fluorescence-based solid-phase assays to study glycan-binding protein interactions with glycoconjugates. *Methods Enzymol.* 478:241-264. doi: 10.1016/S0076-6879(10)78012-5.

206. Levine MJ, Herzberg MC, Levine MS, Ellison SA, Stinson MW, Li HC, van Dyke T. 1978. Specificity of salivary-bacterial interactions: role of terminal sialic acid residues in the interaction of salivary glycoproteins with *Streptococcus sanguis* and *Streptococcus mutans*. *Infect Immun*. 19:107-115.

207. Lewallen DM, Siler D, Iyer SS. 2009. Factors affecting protein-glycan specificity: effect of spacers and incubation time. *Chembiochem*. 10:1486-1489. doi: 10.1002/cbic.200900211.

208. Liao Y, Alvarado R, Phinney B, Lönnerdal B. 2011. Proteomic characterization of specific minor proteins in the human milk casein fraction. *J Proteome Res.* 10:5409-5415. doi: 10.1021/pr200660t.

209. Ligtenberg AJ, Veerman EC, Nieuw Amerongen AV. 2000. A role for Lewis a antigens on salivary agglutinin in binding to *Streptococcus mutans*. *Antonie Van Leeuwenhoek.* 77:21-30.

210. Liu B, Yu Z, Chen C, Kling DE, Newburg DS. 2012. Human milk mucin 1 and mucin 4 inhibit *Salmonella enterica* serovar Typhimurium invasion of human intestinal epithelial cells *in vitro*. *J Nutr.* 142:1504-1509. doi: 10.3945/jn.111.155614.

211. Liu L, Johnson HL, Cousens S, Perin J, Scott S, Lawn JE, Rudan I, Campbell H, Cibulskis R, Li M, Mathers C, Black RE; Child Health Epidemiology Reference Group of WHO and UNICEF. 2012. Global, regional, and national causes of child mortality: an updated systematic analysis for 2010 with time trends since 2000. *Lancet.* 379:2151-2161. doi: 10.1016/S0140-6736(12)60560-1.

212. Logan RP, Robins A, Turner GA, Cockayne A, Borriello SP, Hawkey CJ. 1998. A novel flow cytometric assay for quantitating adherence of *Helicobacter pylori* to gastric epithelial cells. *J Immunol Methods.* 213:19-30.

213. Lloyd DH, Viac J, Werling D, Rème CA, Gatto H. 2007. Role of sugars in surface microbe-host interactions and immune reaction modulation. *Vet Dermatol.* 18:197-204.

214. Lonardi E, Balog CI, Deelder AM, Wuhrer M. 2010. Natural glycan microarrays. *Expert Rev Proteomics.* 7:761-774. doi: 10.1586/epr.10.41.

215. Lönnerdal B. 2003. Nutritional and physiologic significance of human milk proteins. *Am J Clin Nutr.* 77:1537S-1543S.

216. Lönnerdal B. 2008. Personalizing nutrient intakes of formula-fed infants: breast milk as a model. *Nestle Nutr Workshop Ser Pediatr Program.* 62:189-198. doi: 10.1159/000146272.

217. Lönnerdal B, Lien EL. 2003. Nutritional and physiologic significance of alpha-lactalbumin in infants. *Nutr Rev.* 61:295-305.

218. López Álvarez MJ. 2007. Proteins in human milk. *Breastfeed Rev.* 15:5-16.

219. Lucas A, Cole TJ. 1990. Breast milk and neonatal necrotising enterocolitis. *Lancet.* 336:1519-23.

220. Ly L, Wasinger VC. 2010. Mass and charge selective protein fractionation for the differential analysis of T-cell and CD34+ stem cell proteins from cord blood. *J Proteomics.* 73:571-578. doi: 10.1016/j.jprot.2009.09.003.

221. Mahdavi J, Sondén B, Hurtig M, Olfat FO, Forsberg L, Roche N, Angstrom J, Larsson T, Teneberg S, Karlsson KA, Altraja S, Wadström T, Kersulyte D, Berg DE, Dubois A, Petersson C, Magnusson KE, Norberg T, Lindh F, Lundskog BB, Arnqvist A, Hammarström L, Borén T. 2002. *Helicobacter pylori* SabA adhesin in persistent infection and chronic inflammation. *Science.* 297:573-578.

222. Man SM, Kaakoush NO, Leach ST, Nahidi L, Lu HK, Norman J, Day AS, Zhang L, Mitchell HM. 2010. Host attachment, invasion, and stimulation of proinflammatory cytokines by *Campylobacter concisus* and other non-*Campylobacter jejuni Campylobacter* species. *J Infect Dis.* 202:1855-1865. doi: 10.1086/657316.

223. Manimala JC, Roach TA, Li Z, Gildersleeve JC. 2007. High-throughput carbohydrate microarray profiling of 27 antibodies demonstrates widespread specificity problems. *Glycobiology*. 17:17C-23C.

224. Marchetti M, Superti F, Ammendolia MG, Rossi P, Valenti P, Seganti L. 1999. Inhibition of poliovirus type 1 infection by iron-, manganese- and zinc-saturated lactoferrin. *Med Microbiol Immunol.* 187:199-204.

225. Mårild S, Jodal U, Hanson LA. 1990. Breastfeeding and urinary-tract infection. *Lancet.* 336:942.

226. Martin RM, Gunnell D, Owen CG, Smith GD. 2005. Breast-feeding and childhood cancer: A systematic review with metaanalysis. *Int J Cancer.* 117:1020-1031.

227. Matsumoto A, Yoshima H, Takasaki S, Kobata A. 1982. Structural study of the sugar chains of human lactoferrin: finding of four novel complex-type asparagine-linked sugar chains. *J Biochem.* 91:143-155.

228. McArthur WP, Rhodin NR, Seifert TB, Oli MW, Robinette RA, Demuth DR, Brady LJ. 2007. Characterization of epitopes recognized by anti-*Streptococcus mutans* P1 monoclonal antibodies. *FEMS Immunol Med Microbiol.* 50:342-353.

229. McCormick BA. 2003. The use of transepithelial models to examine host-pathogen interactions. *Curr Opin Microbiol.* 6:77-81.

230. Mechref Y, Chen P, Novotny MV. 1999. Structural characterization of the *N*-linked oligosaccharides in bile salt-stimulated lipase originated from human breast milk. *Glycobiology*. 9:227-234.

231. Miller JF. 1966. Immunity in the foetus and the new-born. *Br Med Bull.* 22:21-26.

232. Misselwitz B, Barrett N, Kreibich S, Vonaesch P, Andritschke D, Rout S, Weidner K, Sormaz M, Songhet P, Horvath P, Chabria M, Vogel V, Spori DM, Jenny P, Hardt WD. 2012. Near surface swimming of *Salmonella Typhimurium* explains target-site selection and cooperative invasion. *PLoS Pathog.* 8:e1002810. doi: 10.1371/journal.ppat.1002810.

233. Moffatt DC, Ilnyckyj A, Bernstein CN. 2009. A population-based study of breastfeeding in inflammatory bowel disease: initiation, duration, and effect on disease in the postpartum period. *Am J Gastroenterol.* 104:2517-2523. doi: 10.1038/ajg.2009.362.

234. Moran AP, Gupta A, Joshi L. 2011. Sweet-talk: role of host glycosylation in bacterial pathogenesis of the gastrointestinal tract. *Gut.* 60:1412-1425. doi: 10.1136/gut.2010.212704.

235. Morrow AL, Rangel JM. 2004. Human milk protection against infectious diarrhoea: implications for prevention and clinical care. *Semin Pediatr Infect Dis.* 15:221-228.

236. Morrow AL, Ruiz-Palacios GM, Altaye M, Jiang X, Guerrero ML, Meinzen-Derr JK, Farkas T, Chaturvedi P, Pickering LK, Newburg DS. 2004. Human milk oligosaccharide blood group epitopes and innate immune protection against campylobacter and calicivirus diarrhoea in breastfed infants. *Adv Exp Med Biol.*

554:443-446.

237. Morrow AL, Ruiz-Palacios GM, Jiang X, Newburg DS. 2005. Human-milk glycans that inhibit pathogen binding protect breast-feeding infants against infectious diarrhoea. *J Nutr.* 135:1304-1307.

238. Morelle W, Michalski JC. 2005. Glycomics and mass spectrometry. *Curr Pharm Des.* 11:2615-2645.

239. Murray PA, Prakobphol A, Lee T, Hoover CI, Fisher SJ. 1992. Adherence of oral streptococci to salivary glycoproteins. *Infect Immun.* 60:31-38.

240. Mwiru RS, Spiegelman D, Duggan C, Peterson K, Liu E, Msamanga G, Aboud S, Fawzi WW. 2011. Relationship of exclusive breast-feeding to infections and growth of Tanzanian children born to HIV-infected women. *Public Health Nutr.* 14:1251-1258. doi: 10.1017/S136898001000306X.

241. Mysore JV, Wigginton T, Simon PM, Zopf D, Heman-Ackah LM, Dubois A. 1999. Treatment of *Helicobacter pylori* infection in rhesus monkeys using a novel antiadhesion compound. *Gastroenterology.* 117:1316-1325.

242. Naarding MA, Dirac AM, Ludwig IS, Speijer D, Lindquist S, Vestman EL, Stax MJ, Geijtenbeek TB, Pollakis G, Hernell O, Paxton WA. 2006. Bile salt-stimulated lipase from human milk binds DC-SIGN and inhibits human immunodeficiency virus type 1 transfer to CD4+ T cells. *Antimicrob Agents Chemother.* 50:3367-3374.

243. Naarding MA, Ludwig IS, Groot F, Berkhout B, Geijtenbeek TB, Pollakis G, Paxton WA. 2005. Lewis X component in human milk binds DC-SIGN and inhibits HIV-1 transfer to CD4+ T lymphocytes. *J Clin Invest.* 115:3256-3264.

244. Nakano T, Sugawara M, Kawakami H. 2001. Sialic acid in human milk: composition and functions. *Acta Paediatr Taiwan.* 42:11-17.

245. Nakao T, Kon K, Ando S, Hirabayashi Y. 1991. A NeuGc-containing trisialoganglioside of bovine brain. *Biochim Biophys Acta*. 1086:305-309.

246. Nataro JP, Kaper JB. 1998. Diarrhoeagenic *Escherichia coli*. *Clin Microbiol Rev.* 11:142-201.

247. Nakata N, Furukawa K, Greenwalt DE, Sato T, Kobata A. 1993. Structural study of the sugar chains of CD36 purified from bovine mammary epithelial cells: occurrence of novel hybrid-type sugar chains containing the Neu5Ac alpha 2-->6GalNAc beta 1-->4GlcNAc and the Man alpha 1-->2Man alpha 1-->3Man alpha 1-->6Man groups. *Biochemistry.* 32:4369-4383.

248. Nataro JP, Kaper JB. 1998. Diarrheagenic *Escherichia coli*. *Clin Microbiol Rev.* 11:142-201.

249. Newburg DS. 1997. Do the binding properties of oligosaccharides in milk protect human infants from gastrointestinal bacteria? *J Nutr.* 127:980S-984S.

250. Newburg DS. 1999. Human milk glycoconjugates that inhibit pathogens. *Curr Med Chem.* 6:117-127.

251. Newburg DS. 2005. Innate immunity and human milk. *J Nutr.* 135:1308-1312.

252. Newburg DS. 2009. Neonatal protection by an innate immune system of human milk consisting of oligosaccharides and glycans. *J Anim Sci.* 87:26-34. doi: 10.2527/jas.2008-1347.

253. Newburg DS. 1996. Oligosaccharides and glycoconjugates in human milk: their role in host defense. *J Mammary Gland Biol Neoplasia.* 1:271-283.

254. Newburg DS, Ashkenazi S, Cleary TG. 1992. Human milk contains the Shiga toxin and Shiga-like toxin receptor glycolipid Gb_3. *J Infect Dis.* 166:832-836.

255. Newburg DS, Chaturvedi P. 1992. Neutral glycolipids of human and bovine milk. *Lipids.* 27:923-927.

256. Newburg DS, Chaturvedi P. 1997. Sulfated glycolipids in human milk. *Pediatr Res.* 41:224-224.

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

258. Newburg DS, Peterson JA, Ruiz-Palacios GM, Matson DO, Morrow AL, Shults J, Guerrero ML, Chaturvedi P, Newburg SO, Scallan CD, Taylor MR, Ceriani RL, Pickering LK. 1998. Role of human-milk lactadherin in protection against symptomatic rotavirus infection. *Lancet.* 351:1160-1164.

259. Newburg DS, Pickering LK, McCluer RH, Cleary TG. 1990. Fucosylated oligosaccharides of human milk protect suckling mice from heat-stabile enterotoxin of *Escherichia coli*. *J Infect Dis.* 162:1075-1080.

260. Newburg DS, Ruiz-Palacios GM, Altaye M, Chaturvedi P, Meinzen-Derr J, Guerrero Mde L, Morrow AL. 2004. Innate protection conferred by fucosylated oligosaccharides of human milk against diarrhoea in breastfed infants. *Glycobiology.* 14:253-263.

261. Newburg DS, Ruiz-Palacios GM, Morrow AL. Human milk glycans protect infants against enteric pathogens. 2005. *Annu Rev Nutr.* 25:37-58.

262. Newburg DS, Shen Z, Warren CD. 2000. Quantitative analysis of human milk

oligosaccharides by capillary electrophoresis. *Adv Exp Med Biol.* 478:381-382.

263. Nickerson CA, Goodwin TJ, Terlonge J, Ott CM, Buchanan KL, Uicker WC, Emami K, LeBlanc CL, Ramamurthy R, Clarke MS, Vanderburg CR, Hammond T, Pierson DL. 2001. Three-dimensional tissue assemblies: novel models for the study of *Salmonella enterica* serovar Typhimurium pathogenesis. *Infect Immun.* 69:7106-7120.

264. Nimrichter L, Gargir A, Gortler M, Altstock RT, Shtevi A, Weisshaus O, Fire E, Dotan N, Schnaar RL. 2004. Intact cell adhesion to glycan microarrays. *Glycobiology*. 14:197-203.

265. Ninonuevo MR, Park Y, Yin H, Zhang J, Ward RE, Clowers BH, German JB, Freeman SL, Killeen K, Grimm R, Lebrilla CB. 2006. A strategy for annotating the human milk glycome. *J Agric Food Chem.* 54:7471-7480.

266. Nowak JA, Jain NK, Stinson MW, Merrick JM. 1986. Interaction of bovine erythrocyte *N*-glycolylneuraminic acid-containing gangliosides and glycoproteins with a human Hanganutziu-Deicher serum. *Mol Immunol.* 23:693-700.

267. Nwosu CC, Aldredge DL, Lee H, Lerno LA, Zivkovic AM, German JB, Lebrilla CB. 2012. Comparison of the human and bovine milk *N*-glycome via high-performance microfluidic chip liquid chromatography and tandem mass spectrometry. *J Proteome Res.* 11:2912-2924. doi: 10.1021/pr300008u.

268. Oddy WH. 2001. Breastfeeding protects against illness and infection in infants and children: a review of the evidence. *Breastfeed Rev.* 9:11-18.

269. Ogra SS, Ogra PL. 1978. Immunologic aspects of human colostrum and milk. I. Distribution characteristics and concentrations of immunoglobulins at different times after the onset of lactation. *J Pediatr.* 92:546-549.

270. Oho T, Bikker FJ, Nieuw Amerongen AV, Groenink J. 2004. A peptide domain of bovine milk lactoferrin inhibits the interaction between streptococcal surface protein antigen and a salivary agglutinin peptide domain. *Infect Immun.* 72:6181-6184.

271. O'Reilly CE, Jaron P, Ochieng B, Nyaguara A, Tate JE, Parsons MB, Bopp CA, Williams KA, Vinjé J, Blanton E, Wannemuehler KA, Vulule J, Laserson KF, Breiman RF, Feikin DR, Widdowson MA, Mintz E. 2012. Risk factors for death among children less than 5 years old hospitalized with diarrhoea in rural western Kenya, 2005-2007: a cohort study. *PLoS Med.* 9:e1001256. doi: 10.1371/journal.pmed.1001256.

272. Otnaess AB, Laegreid A, Ertresvåg K. 1983. Inhibition of enterotoxin from

Escherichia coli and *Vibrio cholerae* by gangliosides from human milk. *Infect Immun.* 40:563-569.

273. Owen CG, Whincup PH, Cook DG. 2011. Breast-feeding and cardiovascular risk factors and outcomes in later life: evidence from epidemiological studies. *Proc Nutr Soc.* 70:478-484. doi: 10.1017/S0029665111000590.

274. Oyelaran O, Gildersleeve JC. 2009. Glycan arrays: recent advances and future challenges. *Curr Opin Chem Biol.* 13:406-413. doi: 10.1016/j.cbpa.2009.06.021.

275. Oyelaran O, Li Q, Farnsworth D, Gildersleeve JC. 2009. Microarrays with varying carbohydrate density reveal distinct subpopulations of serum antibodies. J Proteome Res. 8:3529-3538. doi: 10.1021/pr9002245.

276. Pabst M, Bondili JS, Stadlmann J, Mach L, Altmann F. 2007. Mass + retention time = structure: a strategy for the analysis of *N*-glycans by carbon LC-ESI-MS and its application to fibrin *N*-glycans. *Anal Chem.* 79:5051-5017.

277. Packer NH, Lawson MA, Jardine DR, Redmond JW. 1998. A general approach to desalting oligosaccharides released from glycoproteins. *Glycoconj J.* 15:737-747.

278. Palma GD, Capilla A, Nova E, Castillejo G, Varea V, Pozo T, Garrote JA, Polanco I, López A, Ribes-Koninckx C, Marcos A, García-Novo MD, Calvo C, Ortigosa L, Peña-Quintana L, Palau F, Sanz Y. 2012. Influence of milk-feeding type and genetic risk of developing coeliac disease on intestinal microbiota of infants: the PROFICEL study. *PLoS One.* 7:e30791. doi: 10.1371/journal.pone.0030791.

279. Pan XL, Izumi T. 2000. Variation of the ganglioside compositions of human milk, cow's milk and infant formulas. *Early Hum Dev.* 57:25-31.

280. Pandit SA, Scott HL. 2007. Atomistic and coarse-grained computer simulations of raft-like lipid mixtures. *Methods Mol Biol.* 398:283-302. doi: 10.1007/978-1-59745-513-8_19.

281. Panwar H, Calderwood D, Grant IR, Grover S, Green BD. 2014. *Lactobacillus* strains isolated from infant faeces possess potent inhibitory activity against intestinal alpha- and beta-glucosidases suggesting anti-diabetic potential. *Eur J Nutr.* [Epub ahead of print]. doi: 10.1007/s00394-013-0649-9.

282. Park MK, Briles DE, Nahm MH. 2000. A latex bead-based flow cytometric immunoassay capable of simultaneous typing of multiple pneumococcal serotypes (Multibead assay). *Clin Diagn Lab Immunol.* 7:486-489.

283. Parker P, Sando L, Pearson R, Kongsuwan K, Tellam RL, Smith S. 2010. Bovine Muc1 inhibits binding of enteric bacteria to Caco-2 cells. *Glycoconj J.* 27:89-97. doi: 10.1007/s10719-009-9269-2.

284. Pawson T. 1995. Protein modules and signalling networks. *Nature.* 373:573-580.

285. Penders J, Vink C, Driessen C, London N, Thijs C, Stobberingh EE. 2005. Quantification of Bifidobacterium spp., *Escherichia coli* and *Clostridium difficile* in faecal samples of breast-fed and formula-fed infants by real-time PCR. *FEMS Microbiol Lett.* 243:141-147.

286. Peters U, Schneeweiss S, Trautwein EA, Erbersdobler HF. 2001. A case-control study of the effect of infant feeding on celiac disease. *Ann Nutr Metab.* 45:135-142.

287. Peterson JA, Scallan CD, Ceriani RL, Hamosh M. 2001. Structural and functional aspects of three major glycoproteins of the human milk fat globule membrane. *Adv Exp Med Biol.* 501:179-187.

288. Peterson JA, Hamosh M, Scallan CD, Ceriani RL, Henderson TR, Mehta NR, Armand M, Hamosh P. 1998. Milk fat globule glycoproteins in human milk and in gastric aspirates of mother's milk-fed preterm infants. *Pediatr Res.* 44:499-506.

289. Picariello G, Ferranti P, Mamone G, Roepstorff P, Addeo F. 2008. Identification of *N*-linked glycoproteins in human milk by hydrophilic interaction liquid chromatography and mass spectrometry. *Proteomics.* 8:3833-3847. doi: 10.1002/pmic.200701057.

290. Picciano MF. 2001. Nutrient composition of human milk. *Pediatr Clin North Am.* 48:53-67.

291. Pierce-Crétel A, Decottignies JP, Wieruszeski JM, Strecker G, Montreuil J, Spik G. 1989. Primary structure of twenty three neutral and monosialylated oligosaccharides *O*-glycosidically linked to the human secretory immunoglobulin A hinge region determined by a combination of permethylation analysis and 400-MHz 1H-NMR spectroscopy. *Eur J Biochem.* 182:457-476.

292. Pisacane A, Graziano L, Mazzarella G, Scarpellino B, Zona G. 1992. Breastfeeding and urinary tract infection. *J Pediatr.* 120:87-89.

293. Pisacane A, Graziano L, Zona G. 1990. Breastfeeding and urinary tract infection. *Lancet.* 336:50.

294. Pisacane A, Graziano L, Zona G, Granata G, Dolezalova H, Cafiero M, Coppola A, Scarpellino B, Ummarino M, Mazzarella G. 1994. Breast feeding and acute lower respiratory infection. *Acta Paediatr.* 83:714-718.

295. Plummer C, Douglas CW. 2006. Relationship between the ability of oral streptococci to interact with platelet glycoprotein Ibalpha and with the salivary low-molecular-weight mucin, MG2. *FEMS Immunol Med Microbiol.* 48:390-399.

296. Poly F, Threadgill D, Stintzi A. 2004. Identification of *Campylobacter jejuni* ATCC 43431-specific genes by whole microbial genome comparisons. *J Bacteriol.* 186:4781-4795.

297. Portelli J, Gordon A, May JT. 1998. Effect of compounds with antibacterial activities in human milk on respiratory syncytial virus and cytomegalovirus *in vitro*. *J Med Microbiol.* 47:1015-1018.

298. Potapenko IO, Haakensen VD, Lüders T, Helland A, Bukholm I, Sørlie T, Kristensen VN, Lingjaerde OC, Børresen-Dale AL. 2010. Glycan gene expression signatures in normal and malignant breast tissue; possible role in diagnosis and progression. *Mol Oncol.* 4:98-118. doi: 10.1016/j.molonc.2009.12.001.

299. Prakobphol A, Burdsal CA, Fisher SJ. 1995. Quantifying the strength of bacterial adhesive interactions with salivary glycoproteins. *J Dent Res.* 74:1212-1218.

300. Puente R, García-Pardo LA, Hueso P. 1992. Gangliosides in bovine milk. Changes in content and distribution of individual ganglioside levels during lactation. *Biol Chem Hoppe Seyler.* 373:283-288.

301. Qureshi K, Mølbak K, Sandström A, Kofoed PE, Rodrigues A, Dias F, Aaby P, Svennerholm AM. 2006. Breast milk reduces the risk of illness in children of mothers with cholera: observations from an epidemic of cholera in Guinea-Bissau. *Pediatr Infect Dis J.* 25:1163-1166.

302. Räihä NC. 1985. Nutritional proteins in milk and the protein requirement of normal infants. *Pediatrics.* 75:136-141.

303. Rieg S, Steffen H, Seeber S, Humeny A, Kalbacher H, Dietz K, Garbe C, Schittek B. 2005. Deficiency of dermcidin-derived antimicrobial peptides in sweat of patients with atopic dermatitis correlates with an impaired innate defense of human skin in vivo. *J Immunol.* 174:8003-8010.

304. Robbe C, Capon C, Coddeville B, Michalski JC. 2004. Structural diversity and specific distribution of *O*-glycans in normal human mucins along the intestinal tract. *Biochem.* 384:307-16.

305. Rodriguez-Palmero M, Koletzko B, Kunz C, Jensen R. 1999. Nutritional and biochemical properties of human milk: II. Lipids, micronutrients, and bioactive factors. *Clin Perinatol.* 26:335-359.

306. Rueda R. 2007. The role of dietary gangliosides on immunity and the prevention of infection. *Br J Nutr.* 98:S68-S73.

307. Ruíz-Palacios GM, Cervantes LE, Ramos P, Chavez-Munguia B, Newburg DS. 2003. *Campylobacter jejuni* binds intestinal H(O) antigen (Fuc alpha 1, 2Gal beta

1, 4GlcNAc), and fucosyloligosaccharides of human milk inhibit its binding and infection. *J Biol Chem.* 278:14112-1420.

308. Russell RG, Blake DC Jr. 1994. Cell association and invasion of Caco-2 cells by *Campylobacter jejuni*. *Infect Immun.* 62:3773-3779.

309. Ruvoën-Clouet N, Mas E, Marionneau S, Guillon P, Lombardo D, Le Pendu J. 2006. Bile-salt-stimulated lipase and mucins from milk of 'secretor' mothers inhibit the binding of Norwalk virus capsids to their carbohydrate ligands. *Biochem J.* 393:627-634.

310. Saarinen UM, Kajosaari M. 1995. Breastfeeding as prophylaxis against atopic disease: prospective follow-up study until 17 years old. *Lancet.* 346:1065-1069.

311. Saeland E, de Jong MA, Nabatov AA, Kalay H, Geijtenbeek TB, van Kooyk Y. 2009. MUC1 in human milk blocks transmission of human immunodeficiency virus from dendritic cells to T cells. *Mol Immunol.* 46:2309-2316. doi: 10.1016/j.molimm.2009.03.025.

312. Sagi D, Kienz P, Denecke J, Marquardt T, Peter-Katalinić J. 2005. Glycoproteomics of *N*-glycosylation by in-gel deglycosylation and matrix-assisted laser desorption/ionisation-time of flight mass spectrometry mapping: application to congenital disorders of glycosylation. *Proteomics*. 5:2689-2701.

313. Saito T, Itoh T, Adachi S. 1987. Chemical structure of three neutral trisaccharides isolated in free form from bovine colostrum. *Carbohydr Res.* 165:43-51.

314. Sakarya S, Oncü S. 2003. Bacterial adhesins and the role of sialic acid in bacterial adhesion. *Med Sci Monit.* 9:RA76-82.

315. Salcedo J, Barbera R, Matencio E, Alegría A, Lagarda MJ. 2013. Gangliosides and sialic acid effects upon newborn pathogenic bacteria adhesion: An *in vitro* study. *Food Chem.* 136:726-734. doi: 10.1016/j.foodchem.2012.08.078.

316. Samyn-Petit B, Gruber V, Flahaut C, Wajda-Dubos JP, Farrer S, Pons A, Desmaizieres G, Slomianny MC, Theisen M, Delannoy P. 2001. *N*-glycosylation potential of maize: the human lactoferrin used as a model. *Glycoconj J*. 18:519-527.

317. Sánchez-Juanes F, Alonso JM, Zancada L, Hueso P. 2009. Glycosphingolipids from bovine milk and milk fat globule membranes: a comparative study. Adhesion to enterotoxigenic *Escherichia coli* strains. *Biol Chem.* 390:31-40. doi: 10.1515/BC.2009.003.

318. Sato T, Furukawa K, Greenwalt DE, Kobata A. 1993. Most bovine milk fat globule membrane glycoproteins contain asparagine-linked sugar chains with GalNAc beta 1-->4GlcNAc groups. *J Biochem.* 114:890-900.

319. Scallan E, Majowicz SE, Hall G, Banerjee A, Bowman CL, Daly L, Jones T, Kirk MD, Fitzgerald M, Angulo FJ. 2005. Prevalence of diarrhoea in the community in Australia, Canada, Ireland, and the United States. *Int J Epidemiol.* 34:454-460.

320. Scharfman A, Degroote S, Beau J, Lamblin G, Roussel P, Mazurier J. 1999. *Pseudomonas aeruginosa* binds to neoglycoconjugates bearing mucin carbohydrate determinants and predominantly to sialyl-Lewis x conjugates. *Glycobiology.* 9:757-764.

321. Scheiffele P, Füllekrug J. 2000. Glycosylation and protein transport. *Essays Biochem.* 36:27-35.

322. Schroten H, Hanisch FG, Plogmann R, Hacker J, Uhlenbruck G, Nobis-Bosch R, Wahn V. 1992a. Inhibition of adhesion of S-fimbriated *Escherichia coli* to buccal epithelial cells by human milk fat globule membrane components: a novel aspect of the protective function of mucins in the nonimmunoglobulin fraction. *Infect Immun.* 60:2893-2899.

323. Schroten H, Lethen A, Hanisch FG, Plogmann R, Hacker J, Nobis-Bosch R, Wahn V. 1992b. Inhibition of adhesion of S-fimbriated *Escherichia coli* to epithelial cells by meconium and feces of breast-fed and formula-fed newborns: mucins are the major inhibitory component. *J Pediatr Gastroenterol Nutr.* 15:150-158.

324. Schroten H, Plogmann R, Hanisch FG, Hacker J, Nobis-Bosch R, Wahn V. 1993. Inhibition of adhesion of S-fimbriated *E. coli* to buccal epithelial cells by human skim milk is predominantly mediated by mucins and depends on the period of lactation. *Acta Paediatr.* 82:6-11.

325. Schroten H, Stapper C, Plogmann R, Köhler H, Hacker J, Hanisch FG. 1998. Fab-independent antiadhesion effects of secretory immunoglobulin A on S-fimbriated *Escherichia coli* are mediated by sialyloligosaccharides. *Infect Immun.* 66:3971-3973.

326. Schulz BL, Oxley D, Packer NH, Karlsson NG. 2002. Identification of two highly sialylated human tear-fluid DMBT1 isoforms: the major high-molecular-mass glycoproteins in human tears. *Biochem J.* 366:511-520.

327. Schüpbach P, Neeser JR, Golliard M, Rouvet M, Guggenheim B. 1996. Incorporation of caseinoglycomacropeptide and caseinophosphopeptide into the

salivary pellicle inhibits adherence of mutans streptococci. *J Dent Res.* 75:1779-1788.

328. Schwarz M, Spector L, Gargir A, Shtevi A, Gortler M, Altstock RT, Dukler AA, Dotan N. 2003. A new kind of carbohydrate array, its use for profiling antiglycan antibodies, and the discovery of a novel human cellulose-binding antibody. *Glycobiology*. 13:749-754.

329. Schwertmann A, Schroten H, Hacker J, Kunz C. 1999. S-fimbriae from *Escherichia coli* bind to soluble glycoproteins from human milk. *J Pediatr Gastroenterol Nutr.* 28:257-263.

330. Servin AL. 2004. Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. *FEMS Microbiol Rev.* 28:405-440.

331. Séverin S, Wenshui X. 2005. Milk biologically active components as nutraceuticals: review. *Crit Rev Food Sci Nutr.* 45:645-656.

332. Sharon N. 2008. Lectins: past, present and future. *Biochem Soc Trans.* 36:1457-1460. doi: 10.1042/BST0361457.

333. Shu XO, Linet MS, Steinbuch M, Wen WQ, Buckley JD, Neglia JP, Potter JD, Reaman GH, Robison LL. 1999. Breast-feeding and risk of childhood acute leukemia. *J Natl Cancer Inst.* 91:1765-1772.

334. Silfverdal SA, Bodin L, Hugosson S, Garpenholt O, Werner B, Esbjörner E, Lindquist B, Olcén P. 1997. Protective effect of breastfeeding on invasive *Haemophilus influenzae* infection: a case-control study in Swedish preschool children. *Int J Epidemiol.* 26:443-450.

335. Silfverdal SA, Bodin L, Olcén P. 1999. Protective effect of breastfeeding: an ecologic study of *Haemophilus influenzae* meningitis and breastfeeding in a Swedish population. *Int J Epidemiol.* 28:152-156.

336. Silfverdal SA, Bodin L, Ulanova M, Hahn-Zoric M, Hanson LA, Olcen P. 2002. Long term enhancement of the IgG2 antibody response to *Haemophilus influenzae* type b by breast-feeding. *Pediatr Infect Dis J.* 21:816-821.

337. Simon PM, Goode PL, Mobasseri A, Zopf D. 1997. Inhibition of *Helicobacter pylori* binding to gastrointestinal epithelial cells by sialic acid-containing oligosaccharides. *Infect Immun.* 65:750-757.

338. Smolenski G, Haines S, Kwan FY, Bond J, Farr V, Davis SR, Stelwagen K, Wheeler TT. 2007. Characterisation of host defence proteins in milk using a proteomic approach. *J Proteome Res.* 6:207-215.

339. Song X, Lasanajak Y, Xia B, Smith DF, Cummings RD. 2009. Fluorescent glycosylamidesproduced by microscale derivatization of free glycans for natural glycan microarrays. *ACS Chem Biol.* 4:741-750. doi: 10.1021/cb900067h.

340. Sorva R, Mäkinen-Kiljunen S, Juntunen-Backman K. 1994. Beta-lactoglobulin secretion in human milk varies widely after cow's milk ingestion in mothers of infants with cow's milk allergy. *J Allergy Clin Immunol*. 93:787-792.

341. Srikanth CV, McCormick BA. 2008. Interactions of the intestinal epithelium with the pathogen and the indigenous microbiota: a three-way crosstalk. *Interdiscip Perspect Infect Dis.* 2008:626827. doi: 10.1155/2008/626827.

342. Stahl B, Thurl S, Henker J, Siegel M, Finke B, Sawatzki G. 2001. Detection of four human milk groups with respect to Lewis-blood-group-dependent oligosaccharides by serologic and chromatographic analysis. *Adv Exp Med Biol.* 501:299-306.

343. Stepans MB, Wilhelm SL, Hertzog M, Rodehorst TK, Blaney S, Clemens B, Polak JJ, Newburg DS. 2006. Early consumption of human milk oligosaccharides is inversely related to subsequent risk of respiratory and enteric disease in infants. *Breastfeed Med.* 1:207-215.

344. Strand TA, Sharma PR, Gjessing HK, Ulak M, Chandyo RK, Adhikari RK, Sommerfelt H. 2012. Risk factors for extended duration of acute diarrhea in young children. PLoS One. 7:e36436. doi: 10.1371/journal.pone.0036436.

345. Strömqvist M, Falk P, Bergström S, Hansson L, Lönnerdal B, Normark S, Hernell O. 1995. Human milk kappa-casein and inhibition of *Helicobacter pylori* adhesion to human gastric mucosa. *J Pediatr Gastroenterol Nutr.* 21:288-296.

346. Sundekilde UK, Barile D, Meyrand M, Poulsen NA, Larsen LB, Lebrilla CB, German JB, Bertram HC. 2012. Natural variability in bovine milk oligosaccharides from Danish Jersey and Holstein-Friesian breeds. *J Agric Food Chem.* 60:6188-6196. doi: 10.1021/jf300015j.

347. Sung MA, Chen HA, Matthews S. 2001a. Sequential assignment and secondary structure of the triple-labelled carbohydrate-binding domain of papG from uropathogenic *E. coli*. *J Biomol NMR.* 19:197-198.

348. Sung MA, Fleming K, Chen HA, Matthews S. 2001b.The solution structure of PapGII from uropathogenic *Escherichia coli* and its recognition of glycolipid receptors. *EMBO Rep.* 2:621-627.

349. Superti F, Ammendolia MG, Valenti P, Seganti L. 1997. Antirotaviral activity of milk proteins: lactoferrin prevents rotavirus infection in the enterocyte-like cell

line HT-29. *Med Microbiol Immunol.* 186:83-91.

350. Suzuki Y, Ito T, Suzuki T, Holland RE Jr, Chambers TM, Kiso M, Ishida H, Kawaoka Y. 2000. Sialic acid species as a determinant of the host range of influenza A viruses. *J Virol.* 74:11825-11831.

351. Swart PJ, Kuipers ME, Smit C, Pauwels R, deBéthune MP, de Clercq E, Meijer DK, Huisman JG. 1996. Antiviral effects of milk proteins: acylation results in polyanionic compounds with potent activity against human immunodeficiency virus types 1 and 2 *in vitro*. *AIDS Res Hum Retroviruses.* 12:769-775.

352. Takamizawa K, Iwamori M, Mutai M, Nagai Y. 1986. Selective changes in gangliosides of human milk during lactation: a molecular indicator for the period of lactation. *Biochim Biophs Acta.* 870:73-77.

353. Tao N, DePeters EJ, Freeman S, German JB, Grimm R, Lebrilla CB. 2008. Bovine milk glycome. *J Dairy Sci.* 91:3768-3778. doi: 10.3168/jds.2008-1305.

354. Tao N, DePeters EJ, German JB, Grimm R, Lebrilla CB. 2009. Variations in bovine milk oligosaccharides during early and middle lactation stages analyzed by high-performance liquid chromatography-chip/mass spectrometry. *J Dairy Sci.* 92:2991-3001. doi: 10.3168/jds.2008-1642.

355. Tao SC, Li Y, Zhou J, Qian J, Schnaar RL, Zhang Y, Goldstein IJ, Zhu H, Schneck JP. 2008. Lectin microarrays identify cell-specific and functionally significant cell surface glycan markers. *Glycobiology.* 18:761-769. doi: 10.1093/glycob/cwn063.

356. Tarrant RC, Sheridan-Pereira M, Younger KM, Kearney JM. 2012. The positive role of breastfeeding on infant health during the first 6 weeks: findings from a prospective observational study based on maternal reports. *Ir Med J.* 105:75-78.

357. Tenovuo J, Lehtone OP, Aaltonen AS, Vilja P, Tuohimaa P. 1986. Antimicrobial factors in whole saliva of human infants. *Infect Immun.* 51:49-53.

358. Tesh VL, O'Brien AD. 1991. The pathogenic mechanisms of Shiga toxin and the Shiga-like toxins. *Mol Microbiol.* 5:1817-1822.

359. Thurl S, Henker J, Siegel M, Tovar K, Sawatzki G. 1997. Detection of four human milk groups with respect to Lewis blood group dependent oligosaccharides. *Glycoconj J.* 14:795-799.

360. Thurl S, Munzert M, Henker J, Boehm G, Müller-Werner B, Jelinek J, Stahl B. 2010. Variation of human milk oligosaccharides in relation to milk groups and lactational periods. *Br J Nutr.* 104:1261-1271. doi: 10.1017/S0007114510002072.

361. Townsend RR, Hardy MR, Wong TC, Lee YC. 1986. Binding of N-linked bovine fetuin glycopeptides to isolated rabbit hepatocytes: Gal/GalNAc hepatic lectin

discrimination between Gal beta(1,4)GlcNAc and Gal beta(1,3)GlcNAc in a triantennary structure. *Biochemistry*. 25:5716-5725.

362. Ubesie AC, Ibeziako NS, Ndiokwelu CI, Uzoka CM, Nwafor CA. 2012. Under-five protein energy malnutrition admitted at the University of Nigeria Teaching Hospital, Enugu: a 10 year retrospective review. *Nutr J.* 11:43. doi:10.1186/1475-2891-11-43.

363. Unsworth KE, Way M, McNiven M, Machesky L, Holden DW. 2004. Analysis of the mechanisms of *Salmonella*-induced actin assembly during invasion of host cells and intracellular replication. *Cell Microbiol.* 6:1041-1055.

364. Urashima T, Taufik E, Fukuda K, Asakuma S. 2013. Recent advances in studies on milk oligosaccharides of cows and other domestic farm animals. *Biosci Biotechnol Biochem.* 77:455-466.

365. Vacca-Smith AM, Van Wuyckhuyse BC, Tabak LA, Bowen WH. 1994. The effect of milk and casein proteins on the adherence of *Streptococcus mutans* to saliva-coated hydroxyapatite. *Arch Oral Biol.* 39:1063-1069.

366. Van den Nieuwenhof IM, Schiphorst WE, Van Die I, Van den Eijnden DH. 1999. Bovine mammary gland UDP-GalNAc:GlcNAcbeta-R beta1-->4-N-acetylgalactosaminyltransferase is glycoprotein hormone nonspecific and shows interaction with alpha-lactalbumin. *Glycobiology.* 92:115-123.

367. Varki A, Cummings RD, Esko JD, Freeze HH, Stanley P, Bertozzi CR, Hart GW, Etzler ME. 2009a. In: *Essentials of Glycobiology*. 2nd ed. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press.

368. Varki_A, Cummings RD, Esko JD, Freeze HH, Stanley P, Marth JD, Bertozzi CR, Hart GW, Etzler ME. 2009b. Symbol nomenclature for glycan representation. *Proteomics*. 9:5398-5399. doi: 10.1002/pmic.200900708.

369. Vercoutter-Edouart AS, Slomianny MC, Dekeyzer-Beseme O, Haeuw JF, Michalski JC. 2008. Glycoproteomics and glycomics investigation of membrane *N*-glycosylproteins from human colon carcinoma cells. *Proteomics.* 8:3236-356. doi: 10.1002/pmic.200800151.

370. Vesterlund S, Paltta J, Karp M, Ouwehand AC. 2005. Measurement of bacterial adhesion-*in vitro* evaluation of different methods. *J Microbiol Methods.* 60:225-233.

371. Vimal DB, Khullar M, Gupta S, Ganguly NK. 2000. Intestinal mucins: the binding sites for *Salmonella typhimurium*. *Mol Cell Biochem.* 204:107-117.

372. Viverge D, Grimmonprez L, Cassanas G, Bardet L, Solere M. 1990a. Discriminant

carbohydrate components of human milk according to donor secretor types. *J Pediatr Gastroenterol Nutr.* 11(3):365-370.

373. Viverge D, Grimmonprez L, Cassanas G, Bardet L, Solere M. 1990b. Variations in oligosaccharides and lactose in human milk during the first week of lactation. *J Pediatr Gastroenterol Nutr.* 11:361-364.

374. Wada A, Hasegawa M, Wong PF, Shirai E, Shirai N, Tan LJ, Llanes R, Hojo H, Yamasaki E, Ichinose A, Ichinose Y, Senba M. 2010. Direct binding of gangliosides to *Helicobacter pylori* vacuolating cytotoxin (VacA) neutralizes its toxin activity. *Glycobiology.* 20:668-678. doi: 10.1093/glycob/cwq014.

375. Wal JM. 1998. Cow's milk allergens. Allergy. 53:1013-1022.

376. Walker WA. 2000. Role of nutrients and bacterial colonization in the development of intestinal host defense. *J Pediatr Gastroenterol Nutr.* 30:S2-S7.

377. Wang D, Coscoy L, Zylberberg M, Avila PC, Boushey HA, Ganem D, DeRisi JL. 2002. Microarray-based detection and genotyping of viral pathogens. *Proc Natl Acad Sci U S A.* 99:15687-15692.

378. Wasinger VC, Locke VL, Raftery MJ, Larance M, Rothemund D, Liew A, Bate I, Guilhaus M. 2005. Two-dimensional liquid chromatography/tandem mass spectrometry analysis of Gradiflow fractionated native human plasma. *Proteomics.* 5:3397-3401.

379. Wernersson J, Danielsson Niemi L, Einarson S, Hernell O, Johansson I. 2006. Effects of human milk on adhesion of *Streptococcus mutans* to saliva-coated hydroxyapatite *in vitro*. *Caries Res.* 40: 412-417.

380. Wessel D, Flügge UI. 1984. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Anal Biochem.* 138:141-143.

381. WHO Collaborative Study Team on the Role of Breastfeeding on the Prevention of Infant Mortality. 2000. Effect of breastfeeding on infant and child mortality due to infectious diseases in less developed countries: a pooled analysis. *Lancet.* 355:451-455.

382. Williams DB. 2006. Beyond lectins: the calnexin/calreticulin chaperone system of the endoplasmic reticulum. *J Cell Sci.* 119:615-623.

383. Wils P, Legrain S, Frenois E, Scherman D. 1993. HT29-18-C1 intestinal cells: a new model for studying the epithelial transport of drugs. *Biochim Biophys Acta*. 1177:134-138.

384. Wilson JW, Schurr MJ, LeBlanc CL, Ramamurthy R, Buchanan KL, Nickerson CA. 2002. Mechanisms of bacterial pathogenicity. *Postgrad Med J.* 78:216-224.

385. Wilson NL, Robinson LJ, Donnet A, Bovetto L, Packer NH, Karlsson NG. 2008. Glycoproteomics of milk: differences in sugar epitopes on human and bovine milk fat globule membranes. *J Proteome Res.* 7:3687-3696. doi: 10.1021/pr700793k.

386. Wine E, Chan VL, Sherman PM. 2008. *Campylobacter jejuni* mediated disruption of polarized epithelial monolayers is cell-type specific, time dependent, and correlates with bacterial invasion. *Pediatr Res.* 64:599-60. doi: 10.1203/PDR.0b013e31818702b9.

387. Wold AE, Mestecky J, Tomana M, Kobata A, Ohbayashi H, Endo T, Edén CS. 1990. Secretory immunoglobulin A carries oligosaccharide receptors for *Escherichia coli* type 1 fimbrial lectin. Infect Immun. 58:3073-3077.

388. World Health Organization. 2009. Diarrhoeal diseases. Retrieved on September 27, 2012. From http://www.who.int/mediacentre/factsheets/fs330/en/.

389. World Health Organization. 1990. Readings on diarrhoea. Student Manual. (WHO/CDD/SER/90,13). World. Health Organization, Geneva, Switzerland.

390. World Health Organization, UNICEF. 2011. Global strategy for infant and young child feeding. Geneva: World Health Organization. Retrieved on September 27, 2012. From http://www.who.int/nutrition/publications/infantfeeding/9241562218/en/.

391. Wu S, Tao N, German JB, Grimm R, Lebrilla CB. 2010. Development of an annotated library of neutral human milk oligosaccharides. *J Proteome Res.* 9:4138-4151. doi: 10.1021/pr100362f.

392. Wu S, Grimm R, German JB, Lebrilla CB. 2011. Annotation and structural analysis of sialylated human milk oligosaccharides. *J Proteome Res.* 10:856-868. doi: 10.1021/pr101006u.

393. Yajima A, Urano-Tashiro Y, Shimazu K, Takashima E, Takahashi Y, Konishi K. 2008. Hsa, an adhesin of *Streptococcus gordonii* DL1, binds to alpha2-3-linked sialic acid on glycophorin A of the erythrocyte membrane. *Microbiol Immunol.* 52:69-77. doi: 10.1111/j.1348-0421.2008.00015.x.

394. Yamauchi K, Azuma N, Kobayashi H, Kaminogawa S. 1981. Isolation and properties of human kappa-casein. *J Biochem.* 90:1005-1012.

395. Yang B, Chuang H, Chen RF. 2012. Protection from viral infections by human milk oligosaccharides: direct blockade and indirect modulation of intestinal ecology and immune reactions. *Open Glycosci.* 5:19-25.

396. Yolken RH, Peterson JA, Vonderfecht SL, Fouts ET, Midthun K, Newburg DS. 1992. Human milk mucin inhibits rotavirus replication and prevents experimental gastroenteritis. *J Clin Invest.* 90:1984-1991.

397. Young J, Watson K, Ellis L, Raven L. 2012. Responding to evidence: breastfeed baby if you can--the sixth public health recommendation to reduce the risk of sudden and unexpected death in infancy. *Breastfeed Rev.* 20:7-15.

398. Young KT, Davis LM, Dirita VJ. 2007. *Campylobacter jejuni*: molecular biology and pathogenesis. *Nat Rev Microbiol.* 5:665-679.

399. Yu ZT, Chen C, Kling DE, Liu B, McCoy JM, Merighi M, Heidtman M, Newburg DS. 2013. The principal fucosylated oligosaccharides of human milk exhibit prebiotic properties on cultured infant microbiota. *Glycobiology.* 23:169-177. doi: 10.1093/glycob/cws138.

400. Zanetta JP, Breckenridge WC, Vincendon G. 1972. Analysis of monosaccharides by gas-liquid chromatography of the *O*-methyl glycosides as trifluoroacetate derivatives. Application to glycoproteins and glycolipids. *J Chromatogr.* 69:291-304.

401. Zhao Z, Liu J, Wasinger VC, Malouf T, Nguyen-Khuong T, Walsh B, Willcox MD. 2010. Tear lipocalin is the predominant phosphoprotein in human tear fluid. *Exp Eye Res.* 90:344-349. doi: 10.1016/j.exer.2009.11.013.

402. Zivkovic AM, German JB, Lebrilla CB, Mills DA. 2011. Human milk glycobiome and its impact on the infant gastrointestinal microbiota. *Proc Natl Acad Sci U S A.* 108(Suppl 1):4653-4658. doi: 10.1073/pnas.1000083107.

403. Zopf D, Roth S. 1996. Oligosaccharide anti-infective agents. *Lancet.* 347:1017-1021.

Appendix A (pages 212-213) removed from Open Access version as they may contain sensitive/confidential content

Appendix B (pages 214-227) of this thesis have been removed as they contain published material. Please refer to the following citation for details of the article contained in these pages.

Peterson, R., Cheah, W. Y., Grinyer, J., & Packer, N. (2013). Glycoconjugates in human milk: Protecting infants from disease. *Glycobiology*, *23*(12), 1425-1438.

DOI: 10.1093/glycob/cwt072

Appendix C (pages 228) of this thesis has been removed as it contains published material. Please refer to the following citation for details of the article contained in this page.

Everest-Dass A, Cheah WY, Nguyen-Khuong T, Kautto L, Petersen R, Lisacek F, Kolarich D, Campbell MP, Packer NH. 2013. Enabling glycoanalysis with informatics: discovery of glycans involved in infection. In proceeding of: GLYCO 22 XXII International Symposium on Glycoconjugates. *Glycoconj J.* 30:281–461.

DOI: 10.1007/s10719-013-9474-x

Appendix D (pages 229) of this thesis has been removed as it contains published material. Please refer to the following citation for details of the article contained in this page.

Grinyer J, Cheah WY, Kolarich D, Packer N. 2011. Differences in pathogen-sugar interactions between human and bovine milk.

https://milkgenomics.org/presentation/differences-in-pathogen-sugar-interactions-between-human-and-bovine-milk-glycoproteins/

Appendix E (pages 230) of this thesis has been removed as it contains published material. Please refer to the following citation for details of the article contained in this page.

Grinyer J., Cheah W. Y., Kolarich D. & Packer N. (2011). Protein-sugar interactions between secreted fluids and pathogens as a protective mechanism. In *The First International Conference on the Glycobiology of Human Milk Oligosaccharides : book of abstracts*, p.29

284: Glycomics of human breast milk: an antimicrobial defence mechanism

D. Kolarich, P.H. Jensen, W.Y. Cheah, J. Grinyer, N. Packer; Department of Chemistry and Biomolecular Sciences, Macquarie University, Sydney/AU
daniel.kolarich@mpikg.mpg.de

Human milk contains a highly potent mixture of protective agents such as free sugars, antibodies and anti-microbial proteins constituting an innate immune system, whereby the mother protects her infant from enteric and other diseases. Human milk free sugars have been reported to inhibit microbial pathogenesis in various in vivo, ex vivo and in vitro experimental systems however the changes which occur to specific sugar structures attached to glycoproteins in milk over the course of lactation have not been investigated. Glycoproteins are contained in the soluble fraction of milk as well as in the milk fat globule membrane (MFGM) which surrounds and solubilises the lipid. The oligosaccharide structures expressed on these proteins were found to be blood group independent of the individual, so that it is possible that the protection offered by these sugars in milk is common to all women. The N- and O-linked glycan phenotype, both of the total glycoprotein complement as well as of specific glycoproteins, of milk from five different individuals during the course of infant breastfeeding will be presented. The structures of those protein-attached glycans which are recognized by human gut pathogens will be described. These oligosaccharide moieties present on the milk glycoproteins, with homology to epithelial mucus cell surface pathogen receptors in the stomach and intestine, may inhibit infection by competitively binding with the pathogens and clearing them from the infant gut. This knowledge offers the opportunity to design glycoconjugates that may be added to infant milk formula to help protect infants from endemic pathogens.

XXI International Symposium on Glycoconjugates. Glycoconj J 28, 197–369 (2011).
https://doi.org/10.1007/s10719-011-9334-5

283: Body fluids: sweet protection against infection?

N. Packer[1], L. Kautto[1], J. Grinyer[1], T. Nguyen-Khuong[1], W.Y. Cheah[1], A.V.E. Dass[1], M.T. Andersen[1], M. Willcox[2], D. Kolarich[3]; [1]Chemistry and Biomolecular Sciences, Macquarie University, Sydney/AU, [2]Brien Holden Vision Institute, Sydney/AU, [3]Max Planck Institute of Colloids and Interfaces, Berlin/DE
nicki.packer@mq.edu.au

We show that the human innate immune system has evolved to use the infection mechanism of pathogens to its own advantage by using the same protein-sugar interactions to bind the pathogen to glycoproteins in various human biological fluids. Once bound, the pathogens can then be expelled physically from the body.

In particular, using carbon LC-ESI-MS/MS, we show that breast milk has a different oligosaccharide composition to cow's milk and that the sugar epitopes exposed on proteins in human breast milk serve to bind specific bacterial pathogens that might otherwise infect the infant gut. A similar phenomenon occurs in eyes in which the tear film of the eye protects this crucial exposed surface from pathogen infection. The eye has a complex tear film comprising glycoproteins which display specific sugar epitopes to the environment and the structures of these provide a protective mechanism against the bacterial species known to infect the eye. In addition, buccal epithelial cells lining the oral cavity are sites of interaction of oral pathogens. These interactions involve adherence between the glycans of the host cells and the carbohydrate binding proteins of the pathogen. We show that there is an innate immune mechanism provided by the glycoproteins in the flow of saliva that constantly bathes the epithelial surface of the mouth, which competes with pathogen adhesion to the cells. Exploration of the molecular mechanisms operating in the protection of the epithelial surfaces by the secreted body fluids opens up the possibility of new preventative therapies using molecular mimics.

XXI International Symposium on Glycoconjugates. Glycoconj J 28, 197–369 (2011).
https://doi.org/10.1007/s10719-011-9334-5

Supplementary Data

Table S1. Full glycan library on the array were sourced from two companies. Glycans sourced from A) Dextra Laboratories (Reading, UK).

Spacer form of saccharide	Common name
Fucα-sp3	L-α-Fuc
Galα-sp3	α-Gal
Galβ-sp3	β-Gal
GalNAcα-sp0	TnSer
GalNAcα-sp3	T_n
GalNAcβ-sp3	β-GalNAc
Glcα-sp3	α-Glc
Glcβ-sp3	β-Glc
GlcNAcβ-sp3	β-GlcNAc
GlcN(Gc)β-sp4	β-GlcN(Gc)
$HOCH_2(HOCH)_4CH_2NH_2$	Amino-glucitol
Manα-sp3	α-Man
Manβ-sp4	β-Man
ManNAcβ-sp4	β-ManAc
Rhaα-sp3	L-α-Rha
GlcNAcβ-sp4	β-GlcNAc
3-O-Su-Galβ-sp3	3-O-Su-β-Gal
3-O-Su-GalNAcα-sp3	3-O-Su-β-GalNac
6-O-Su-GlcNAcβ-sp3	6-O-Su-β-GlcNAc
GlcAα-sp3	α-glucoronic acid
GlcAβ-sp3	β-glucoronic acid
6-H_2PO_3Glcβ-sp4	β-Glc6P
6-H_2PO_3Manα-sp3	α-Man6P
Neu5Acα-sp3	α-Neu5Ac
Neu5Acα-sp9	α-

Spacer form of saccharide	Common name
	Neu5AcBn
Neu5Gcα-sp3	α-Neu5Gc
9-NAc-Neu5Acα-sp3	9-Nac-α-Neu5Ac
3-O-Su-GlcNAcβ-sp3	3-O-Su-β-GlcNAc
Fucα1-2Galβ-sp3	H_{di}
Fucα1-3GlcNAcβ-sp3	
Fucα1-4GlcNAcβ-sp3	Le
Galα1-2Galβ-sp3	
Galα1-3Galβ-sp3	B_{di}
Galα1-3GalNAcβ-sp3	$T_{\alpha\beta}$
Galα1-3GalNAcα-sp3	$T_{\alpha\alpha}$
Galα1-3GlcNAcβ-sp3	
Galα1-4GlcNAcβ-sp3	α-LacNAc
Galα1-6Glcβ-sp4	Melibiose
Galβ1-2Galβ-sp3	
Galβ1-3GlcNaAcβ-sp3	Le^c
Galβ1-3Galβ-sp3	
Galβ1-3GalNAcβ-sp3	$T_{\beta\beta}$
Galβ1-3GalNAcα-sp3	TF
Galβ1-4Glcβ-sp4	Lac
Galβ1-4Galβ-sp4	
Galβ1-4GlcNAcβ-sp3	LacNAc
Galβ1-6Galβ-sp4	
GalNAcα1-3GalNAcβ-sp3	Fs-2
GalNAcα1-3Galβ-sp3	A_{di}
GalNAcα1-3GalNAcα-sp3	Core 5
GalNAcβ1-3Galβ-sp3	
GalNAcβ1-4GlcNAcβ-sp3	LacdiNAc
Glcα1-4Glcβ-sp3	Maltose
Glcβ1-4Glcβ-sp4	Cellobiose

Spacer form of saccharide	Common name
Glcβ1-6Glcβ-sp4	Gentiobiose
GlcNAcβ1-3GalNAcα-sp3	Core 3
GlcNAcβ1-3Manβ-sp4	
GlcNAcβ1-4GlcNAcβ-Asn	Chitobiose-Asn
GlcNAcβ1-4GlcNAcβ-sp4	Chitobiose
GlcNAcβ1-6GalNAcα-sp3	Core 6
Manα1-2Manβ-sp4	
Manα1-3Manβ-sp4	
Manα1-4Manβ-sp4	
Manα1-6Manβ-sp4	
Manβ1-4GlcNAcβ-sp4	
Manα1-2Manα-sp4	
Galβ1-3(6-O-Su)GlcNAcβ-sp3	6-O-Su-Le^c
Galβ1-4(6-O-Su)Glcβ-sp2	6-O-Su-Lac
Galβ1-4(6-O-Su)GlcNAcβ-sp3	6-O-Su-LacNAc
GlcNAcβ1-4(6-O-Su)GlcNAcβ-sp2	6-O-Su-chitobiose
3-O-Su-Galβ1-3GalNAcα-sp3	3`-O-Su-TF
6-O-Su-Galβ1-3GalNAcα-sp3	6`-O-Su-TF
3-O-Su-Galβ1-4Glcβ-sp2	SM3
6-O-Su-Galβ1-4Glcβ-sp2	6`-O-Su-Lac
3-O-Su-Galβ1-3GlcNAcβ-sp3	3`-O-Su-Le^c
3-O-Su-Galβ1-4GlcNAcβ-sp3	3`-O-Su-LacNAc
4-O-Su-Galβ1-4GlcNAcβ-sp3	4`-O-Su-LacNAc
6-O-Su-Galβ1-3GlcNAcβ-sp3	6`-O-Su-

Spacer form of saccharide	Common name
	Le^c
6-O-Su-Galβ1-4GlcNAcβ-sp3	6`-O-Su-LacNAc
GlcAβ1-3GlcNAcβ-sp3	
GlcAβ1-3Galβ-sp3	
GlcAβ1-6Galβ-sp3	
GlcNAcβ1-4-[HOOC(CH_3)CH]-3-O-GlcNAcβ-sp4	GlcNAc-Mur
GlcNAcβ1--[HOOC(CH_3)CH]-3-O-GlcNAcβ-L-alanyl-D-i-glutaminyl-L-lysine	GMDP-Lys
Neu5Acα2-3Galβ-sp3	GM_4
Neu5Acα2-6Galβ-sp3	
Neu5Acα2-3GalNAcα-sp3	3-$SiaT_n$
Neu5Acα2-6GalNAcα-sp3	$SiaT_n$
Neu5Gcα2-6GalNAcα-sp3	NeuGc-T_n
3-O-Su-Galβ1-4(6-O-Su)Glcβ-sp2	3`,6-di-O-Su-Lac
3-O-Su-Galβ1-4(6-O-Su)GlcNAcβ-sp2	3`,6-di-O-Su-LacNAc
6-O-Su-Galβ1-4(6-O-Su)Glcβ-sp2	6,6`-di-O-Su-Lac
6-O-Su-Galβ1-3(6-O-Su)GlcNAcβ-sp2	6,6`-di-O-Su-Le^c
6-O-Su-Galβ1-4(6-O-Su)GlcNAcβ-sp2	6,6`-di-O-Su-LacNAc
3,4-O-Su_2-Galβ1-4GlcNAcβ-sp3	3`,4`-di-O-Su-LacNAc
3,6-O-Su_2-Galβ1-4GlcNAcβ-sp2	3`,6`-di-O-Su-LacNAc

Spacer form of saccharide	Common name
4,6-O-Su_2-Galβ1-4GlcNAcβ-sp2	4`,6`-di-O-Su-LacNAc
4,6-O-Su_2-Galβ1-4GlcNAcβ-sp3	4`,6`-di-O-Su-LacNAc
Neu5Acα2-8Neu5Acα2-sp3	$(Sia)_2$
3,6-O-Su_2-Galβ1-4(6-O-Su)GlcNAcβ-sp2	3`,6,6`-tri-O-Su-LacNAc
GalNAcβ1-4(6-O-Su)GlcNAcβ-sp3	6-O-Su-LacdiNAc
3-O-Su-GalNAcβ1-4GlcNAcβ-sp3	3`-O-Su-LacdiNAc
6-O-Su-GalNAcβ1-4GlcNAcβ-sp3	6`-O-Su-LacdiNAc
6-O-Su-GalNAcβ1-4-(3-O-Su)GlcNAcβ-sp3	6`-Su-3-O-Ac-LacdiNAc
3-O-Su-GalNAcβ1-4(3-O-Su)-GlcNAcβ-sp3	3,3`-O-Su_2-LacdiNAc
3,6-O-Su_2-GalNAcβ1-4GlcNAcβ-sp3	3`,6`-Su_2-LacdiNAc
4,6-O-Su_2-GalNAcβ1-4GlcNAcβ-sp3	4`,6`-Su_2-LacdiNAc
4,6-O-Su_2-GalNAcβ1-4-(3-O-Ac)GlcNAcβ-sp3	4`,6`-Su_2-3-O-Ac-LacdiNAc
4-O-Su-GalNAcβ1-4GlcNAcβ-sp3	4`-O-Su-LacdiNAc
3,4-O-Su_2-Galβ1-4GlcNAcβ-sp3	3`,4`-Su_2-LacdiNAc
6-O-Su-GalNAcβ1-4(6-O-Su)GlcNAcβ-sp3	6,6`-O-Su-LacdiNAc
Galβ1-4(6-O-Su)GlcNAcβ-sp2	6-O-Su-LacNAc
4-O-Su-GalNAcβ1-4GlcNAcβ-sp2	4`-O-Su-LacdiNAc

Spacer form of saccharide	Common name
Neu5Acα2-6GalNAcβ-sp3	
Neu5Gcα2-3Gal-sp3	NeuGcα3Gal
Fucα1-2Galβ1-3GlcNAcβ-sp3	Le^d H (type 1)
Fucα1-2Galβ1-4GlcNAcβ-sp3	H (type 2)
Fucα1-2Galβ1-3GalNAcα-sp3	H (type 3)
Fucα1-2Galβ1-4Glcβ-sp4	H (type 6
Galα1-3Galβ1-4Glcβ-sp2	
Galα1-3Galβ1-4GlcNAcβ-sp3	Galili (tri)
Galα1-4Galβ1-4Glcβ-sp3	Pk, Gb_3, $GbOse_3$
Galα1-4Galβ1-4GlcNAc-sp2	P_1
Galα1-3 Galβ-sp3	B_{tri}
Fucα1-2 Galβ1-2Galα1-4GlcNAcβ-sp4	
Galβ1-3Galβ1-4GlcNAcβ-sp4	
Galβ1-4GlcNAcβ1-3GalNAcα-sp3	
Galβ1-4GlcNAcβ1-6GalNAcα-sp3	
Fucα1-4 GlcNAcβ-sp3	Le^a
Galβ1-3 Galβ1-4 GlcNAcβ-sp3	Le^x
Fucα1-3 GalNAcα1-3 Galβ-sp3	A_{tri}
Fucα1-2 GalNAcβ1-4Galβ1-4Glcβ-sp3	GA_2, $GgOse_3$
$(Glcα1-4)_3β$-sp4	Maltotriose
$(Glcα1-6)_3β$-sp4	Isomaltotriose
GlcNAcβ1-2Galβ1-3GalNAcα-sp3	
GlcNAcβ1-3Galβ1-3GalNAcα-sp3	
GlcNAcβ1-3Galβ1-4Glcβ-sp2	

Spacer form of saccharide	Common name
GlcNAcβ1-3Galβ1-4GlcNAcβ-sp3	
GlcNAcβ1-4Galβ1-4GlcNAcβ-sp2	
GlcNAcβ1-4GlcNAcβ1-4GlcNAcβ-sp4	Chitotriose
GlcNAcβ1-6Galβ1-4GlcNAcβ-sp2	
GlcNAcβ1-6 GalNAcα-sp3	Core 2
Galβ1-3 GlcNAcβ1-6 GalNAcα-sp3	Core 4
GlcNAcβ1-3 Manα1-6 Manβ-sp4	Man_3
Manα1-3 Galβ1-3GalNAcβ1-3Gal-sp4	$T_{ββ}$-Gal
Galβ1-4Galβ1-4GlcNAc-sp3	
Fucα1-4 GlcNAcβ-sp3	Su-Le^a
3-O-Su-Galβ1-3 3-O-Su-Galβ1-4 GlcNAcβ-sp3	Su-Le^x
Fucα1-3 Neu5Acα2-6 GalNAcα-sp3	6-SiaTF
Galα1-3 Neu5Acα2-6 GalNAcα-sp3	
Galβ1-3 Neu5Acα2-3Galβ1-3GalNAcα-sp3	3`-Sia-TF
Neu5Acα2-3Galβ1-4Glcβ-sp3	3`SL
Neu5Acα2-3Galβ1-4Glcβ-sp4	3`SL
Neu5Acα2-6Galβ1-4Glcβ-sp2	6`SL
Neu5Acα2-3Galβ1-4GlcNAcβ-sp3	3`SLN
Neu5Acα2-3Galβ1-3GlcNAcβ-sp3	3`-SiaLe^c
Neu5Acα2-6Galβ1-4GlcNAcβ-sp3	6`SLN
Neu5Gcα2-3Galβ1-	3`SLN

Spacer form of saccharide	Common name
4GlcNAcβ-sp3	(Gc)
Neu5Gcα2-6Galβ1-4GlcNAcβ-sp3	6`SLN (Gc)
9-NAc-Neu5Acα2-6Galβ1-4GlcNAcβ-sp3	
Neu5Acα2-3Galβ1-4-(6-O-Su)GlcNAcβ-sp3	6-Su-3`SLN
Neu5Acα2-3Galβ1-3-(6-O-Su)GalNAcβ-sp3	6-Su-3`SiaTF
Neu5Acα2-6Galβ1-4-(6-O-Su)GlcNAcβ-sp3	6-Su-6`SLN
Neu5Acα2-3-(6-O-Su)Galβ1-4GlcNAcβ-sp3	6`-Su-3`SLN
$(Neu5Acα2-8)_3$-sp3	$(Sia)_3$
Neu5Acα2-6Galβ1-3GlcNAc-sp3	6`-SiaLe^c
Neu5Acα2-6Galβ1-3(6-O-Su)GlcNAc-sp3	6Su-6`-SiaLe^c
Neu5Gcα2-3Galβ1-3GlcNAcβ-sp3	3`SiaLe^c (Gc)
Galα1-3 Galβ1-3GlcNAcβ-sp3	B (type 1)
Fucα1-2 Galα1-3 Galβ1-4GlcNAcβ-sp3	B (type 2)
Fucα1-2 Galα1-3 Galβ1-3GalNAcα-sp3	B (type 3)
Fucα1-2 Galα1-3 Galβ1-3GalNAcβ-sp3	B (type 4)
Fucα1-2 Galα1-3Galβ1-4 GlcNAcβ-sp3	αGalLe^x
Fucα1-3 GalNAcα1-3 Galβ1-3GlcNAcβ-sp3	A (type 1)
Fucα1-2 GalNAcα1-3 Galβ1-4GlcNAcβ-sp3	A (type 2)
Fucα1-2 Fucα1-4 GlcNAcβ-sp3	Le^b

Spacer form of saccharide	Common name
Fucα1-2Galβ1-3 Fucα1-2Galβ1-4 GlcNAcβ-sp3	Le^y
Fucα1-3 Galα1-3Galβ1-4GlcNAcβ1-3Galβ-sp3	Galili (tetra)
Galα1-4GlcNAcβ1-3Galβ1-4GlcNAcβ-sp3	
Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ-sp4	LNT
Galβ1-3GlcNAcβ1-3Galβ1-3GlcNAcβ-sp2	
Galβ1-3GlcNAcα1-3Galβ1-4GlcNAcβ-sp3	
Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ-sp3	
Galβ1-3GlcNAcα1-6Galβ1-4GlcNAcβ-sp2	
Galβ1-3GlcNAcβ1-6Galβ1-4GlcNAcβ-sp2	
Galβ1-3GalNAcβ1-4Galβ1-4Glcβ-sp3	Asialo-GM_1
Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ-sp2	LNnT
Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ-sp3	i
Galβ1-4GlcNAcβ1-6Galβ1-4GlcNAcβ-sp2	
Galβ1-4GlcNAcβ1-6 GalNAcα-sp3	
Galβ1-3 GalNacβ1-3GalαGalβ1-4Glcβ-sp3	Gb_4, P
$(Glcα1-4)_4β$-sp4	Maltotetraose
$(Glcα1-6)_4β$-sp4	Isomaltotetraose
GalNAcα1-6 Galβ1-3GalNAcα-sp3	A (type 3)

Spacer form of saccharide	Common name
Fucα1-2 GlcNAcβ1-6 Galβ1-4GlcNAcβ-sp3	Tk
GlcNAcβ1-3 Galβ1-3GlcNAcβ1-3Galβ1-3GlcNAcβ-sp3	Le^c3Le^c
3-O-SuGalβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ-sp3	
4-O-SuGalβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ-sp3	
GalNAcβ1-4 Galβ1-4Glcβ-sp2	GM2
Neu5Acα2-3 Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ-sp3	
Neu5Acα2-3Galβ1-4 GlcNAcβ-sp3	SiaLe^x
Fucα1-3 Fucα1-4 GlcNAcβ-sp3	SiaLe^a
Neu5Acα2-3Galβ1-3 Neu5Acα2-3Galβ1-4 6-O-Su-GlcNAcβ-sp3	
Fucα1-3 Neu5Acα2-3(6-O-Su)Galβ1-4 GlcNAcβ-sp3	
Fucα1-3 Neu5Acα2-6 GalNAcα-sp3	Sia_2-TF
Neu5Acα2-3Galβ1-3 Neu5Acα2-8Neu5Acα2-3Galβ1-4Glcβ-sp4	GD_3
Penta-nona saccharides	
Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ-sp4	LNFP-I
Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ-sp2	H (type 1) penta

Spacer form of saccharide	Common name
Galα1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ-sp4	Galili (penta)
Galα1-3 Galβ1-4	
Fucα1-2 GlcNAcβ-sp3	Ble^y
Fucα1-3 Galβ1-4GlcNAcβ1-6 GalNAcα-sp3	
Galβ1-4GlcNAcβ1-3 GlcNAcβ1-6 Galβ1-4GlcNAc-sp2	
Galβ1-4GlcNAcβ1-3 Galβ1-4GlcNAcβ1-6 Galβ1-4GlcNAcβ-sp2	
GlcNAcβ1-3 $(Glcα1-6)_5β$-sp4	Isomalto-pentaose
$(GlcNAcβ1-4)_5β$-sp4	Chito-pentaose
Manα1-6 Manα1-6	Man5
Manα1-3 Manβ-sp4	
Manα1-3 Fucα1-4 GlcNAcβ1-3Galβ1-4Glcβ-sp4	Le^b-Lac
Fucα1-2Galβ1-3 Fucα1-2Galβ1-4 GlcNAcβ1-3Galβ1-4Glcβ-sp4	Le^y-Lac
Fucα1-3 $(Galβ1-4GlcNAcβ1-3)_3$-sp3	$(LN)_3$
Galβ1-4GlcNAcβ1-6 Galβ1-4GlcNAc-sp2	I
Galβ1-4GlcNAcβ1-3 Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ-sp4	Gb_5
$(Glcα1-6)_6β$-sp4	Maltohexaose

Spacer form of saccharide	Common name
$(GlcNAcβ1-4)_6β$-sp4	Chitohexaose
$(A-GN-M)_2$-3,6-M-GN-GNβ-sp4	9-OS
$(GN-M)_2$-3,6-M-GN-GNβ-sp4	7-OS
Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ-sp2	3`SLN-LacNAc
Neu5Acα2-3 Galβ1-4 GlcNAcβ1-3Galβ-sp3	SiaLe^x-3Gal
Fucα1-3 Neu5Acα2-6 GlcNAcβ1-3Galβ1-4Glcβ-sp4	LSTb
Galβ1-3 GalNAcβ1-4 Galβ1-4Glc-sp2	GD_2
Neu5Acα2-8Neu5Acα2-3 Neu5Acα2-8Neu5Acα2-8Neu5Acα2-3Galβ1-4Glc-sp2	GT_3
GalNAcβ1-4 Galβ1-4Glc-sp2	GT_2
$(Neu5Acα2-8)_2$Neu5Acα2-3 Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ-sp3	6`SLN-LacNAc
Neu5Acα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ-sp4	LSTa
Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ-sp4	LSTd
Le^x1-6'(Le^c1-3')Lac-sp4	MFLNH III
LacNAc1-6'(Le^d1-3')Lac-sp4	MFLNH I
Le^x1-6'(6'-SLN1-3')Lac-sp4	MSMFLNnH
Le^x1-6'(Le^d1-3')Lac-sp4	DFLNH (a)
Le^cLe^x1-6'(Le^c1-	MF(1

Spacer form of saccharide	Common name
3')Lac-sp4	-3)iLNO
Le^x1-6'(Le^b1-3')Lac-sp4	TFLNH
(GlcAβ1-4GlcNAcβ1-3)$_8$-NH_2-ol	hyaluronic acid
(Sia2-6A-GN-M)$_2$-3,6-M-GN-GNβ-sp4	11-OS, YDS
Galβ1-3GlcNAc	Lacto-*N*-BioseI
Galβ1-4GlcNAc	N-Acetyllactosamine
Galβ1-4Gal	β1-4galactosyl-galactose
Galβ1-6GlcNAc	β1-6galactosyl-*N*-Acetylglucosamine
Galβ1-3GalNAc	β1-3galactosyl-*N*-acetylgalactosamine
	Galb1-3GalNAcβ1-4Galβ1-4Glc
Galβ1-3GlcNAcβ1-3Galβ1-4Glc	Lacto-*N*-tetraose
Galβ1-4GlcNAcβ1-3Galβ1-4Glc	Lacto-*N*-neotetraose
Galβ1-4GlcNAcβ1-6(Galβ1-4GlcNAcβ1-3)Galβ1-4Glc	Lacto-*N*-neohexaose
Galβ1-4GlcNAcβ1-6(Galβ1-3GlcNAcβ1-3)Galβ1-4Glc	Lacto-*N*-hexaose
Galα1-4Galβ1-4Glc	Globotriose
GalNAcα1-O-Ser	Tn Antigen
Galβ1-	Galac

Spacer form of saccharide	Common name
3GalNAcα1-O-Ser	tosyl-Tn Antigen
Galα1-3Gal	α1-3 Galacto-biose
Galα1-3Galβ1-4GlcNAc	Linear B-2 Tri-saccharide
Galα1-3Galβ1-4Glc	Linear B-6 Tri-saccharide
Galα1-3Galβ1-4Galα1-3Gal	α1-3, β1-4, α1-3 Galacto-tetraose
Galβ1-6Gal	β1-6Galactobiose
GalNAcβ1-3Gal	Terminal disaccharide of globotriose
GalNAcβ1-4Gal	Receptor for pili of *P. aeruginosa*
Galα1-4Galβ1-4GlcNAc	P1 Antigen
GalNAcα1-3Galβ1-4Glc	α-D-*N*-acetylgalactosaminyl-1-3Gal-β1-4Glc
Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1-6(Galβ1-3GlcNAcβ1-3)Galβ1-4Glc	iso-Lacto-*N*-octaose
GlcNAcβ1-4GlcNAc	*N,N'*-Diacetylchitobiose
GlcNAcβ1-4GlcNAcβ1-4GlcNAc	*N,N', N''*-Triacetylchitotriose
GlcNAcβ1-4GlcNAcβ1-	*N,N', N'',N''*

Spacer form of saccharide	Common name
4GlcNAcβ1-4GlcNAc	'-Tetraacetylchitotetraose
GlcNAcβ1-4GlcNAcβ1-4GlcNAcβ1-4GlcNAcβ1-4GlcNAc	*N,N', N'',N''', N''''*-Hexaacetylchitohexaose
GlcNAcβ1-4MurNAc	Bacterial cell wall Muramyl Disaccharide
GlcNAcβ1-2Man	β1-2-*N*-Acetylglucosamine-mannose
GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)Man	Biantennary *N*-linked core pentasaccharide
Manα1-2Man	α1-2-Mannobiose
Manα1-3Man	α1-3-Mannobiose
Manα1-4Man	α1-4-Mannobiose
Manα1-6Man	α1-6-Mannobiose
Manα1-6(Manα1-3)Man	α1-3, a1-6-Mannobiose
Manα1-6(Manα1-3)Manα1-6(Manα1-3)Man	α1-3, α1-3, α1-6-Mannopentaose
Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc	Lacto-*N*-fucopentaose I
Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc	Lacto-*N*-fuco-

Spacer form of saccharide	Common name
	pentaose II
Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc	Lacto-*N*-fucopentaose III
Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc	Lacto-*N*-difuco-hexaose I
Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4(Fucα1-3)Glc	Lacto-*N*-difuco-hexaose II
Fucα1-2Gal	H-disaccharide
Fucα1-2Galβ1-4Glc	2'-Fucosyl-lactose
Galβ1-4(Fucα1-3)Glc	3'-Fucosyl-lactose
Galβ1-4(Fucα1-3)GlcNAc	Lewis^x
Galβ1-3(Fucα1-4)GlcNAc	Lewis^a
GalNAcα1-3(Fucα1-2)Gal	Blood Group A-tri-saccharide
Fucα1-2Galβ1-4(Fucα1-3)Glc	Lacto difuco-tetraose
Galβ1-3(Fucα1-2)Gal	Blood Group B-Tri-saccharide
Fucα1-2Galβ1-4(Fucα1-3)GlcNAc	Lewis^y
Fucα1-2Galβ1-3GlcNAc	Blood Group H Type II Tri-saccharide
Fucα1-2Galβ1-3(Fucα1-4)GlcNAc	Lewis^b tetra-saccharide
SO_3-3Galβ1-3(Fucα1-4)GlcNAc	Sulpho Lewis^a
SO_3-3Galβ1-	Sulph

Spacer form of saccharide	Common name
4(Fucα1-3)GlcNAc	o Lewis^{x}
Galβ1-3GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc	Monofucosyl-para-Lacto-*N*-hexaose IV
Galβ1-4(Fucα1-3)GlcNAcβ1-6(Galβ1-3GlcNAcβ1-3)Galβ1-4Glc	Mono-fucosyl-lacto-*N*-hexaose III
Galβ1-4(Fucα1-3)GlcNAcβ1-6(Fucα1-2Galβ1-3GlcNAcβ1-3)Galβ1-4Glc	Difucosyllacto-*N*-hexaose
Galβ1-4(Fucα1-3)GlcNAcβ1-6(Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ1-3)Galβ1-4Glc	Trifucosyl-lacto-*N*-hexaose
Neu5Acα2-3Galβ1-3(Fucα1-4)GlcNAc	Sialyl Lewis^{a}
Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAc	Sialyl Lewis^{x}
Neu5Acα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc	Sialyl lacto-*N*-tetraose a
Galβ1-4(Fucα1-3)GlcNAcβ1-6(Neu5Acα2-6Galβ1-4GlcNAcβ1-3)Galb1-4Glc	Monosialyl, monofucosyllacto-*N*-neohexaose
Neu5Acα2-3Galβ1-4GlcNAc	2,3'-Sialyl lactosamine
Neu5Acα2-6Galβ1-4GlcNAc	2,6'-Sialyl-lactosamine
	LS-Tetrasaccharide a
Galβ1-3(Neu5Acα2-6)GlcNAcβ1-3Galβ1-4Glc	LS-Tetrasaccharide b
Neu5Acα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glc	LS-Tetrasaccharide c

Spacer form of saccharide	Common name
Neu5Acα2-3Galβ1-3(Neu5Acα2-6)GlcNAcβ1-3Galβ1-4Glc	Disialyl-lacto-*N*-tetraose
Neu5Acα2-3Galβ1-4Glc	2,3'-Sialyl-lactose
Neu5Acα2-6Galβ1-4Glc	2,6'-Sialyl-lactose
(Neu5Acα2-8Neu5Ac)n (n<50)	Colominic acid
Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-6(Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAc-Asn	Bi-antennary 2,6-sialylated-*N*-glycan-Asn
$C_{24}H_{36}O_{25}S_2Na_2$ (Mixed anomers. Tetrasaccharide of regular κ -carrageenan)	Neocarratetraose-41, 3-di-*O*-sulphate (Na^+)
$C_{24}H_{37}O_{22}SNa$ (Mixed anomers. Derived from C1003 by removal of the non-reducing terminal 4-sulphate)	Neocarratetraose-41-*O*-sulphate (Na^+)
$C_{36}H_{52}O_{40}S_4Na_4$ (Mixed anomers. A hybrid sequence comprising carrageenan disaccharides in the order κ -i- κ, derived from the carrageenan from *Chondrus crispus*)	Neocarrahexaose-24,41, 3, 5-tetra-*O*-sulphate (Na^+)
$C_{36}H_{53}O_{37}S_3Na_3$ (Mixed anomers. Hexasaccharide of regular κ -carrageenan)	Neocarrahexaose-41, 3, 5-tri-*O*-sulphate (Na^+)
$C_{48}H_{70}O_{49}S_4Na_4$ (Mixed anomers. Octasaccharide of regular κ -carrageenan)	Neocarraoctaose-41, 3, 5, 7-tetra-

Spacer form of saccharide	Common name
	O-sulphate (Na^+)
$C_{60}H_{87}O_{61}S_5Na_5$ (Mixed anomers. Decasaccharide of regular κ -carrageenan)	Neocarradecaose-41, 3, 5, 7, 9-penta-*O*-sulphate (Na^+)
$C_{12}H_{15}NO_{19}S_3Na_4$ (Predominant disaccharide produced from heparin by heparinase I and II)	ΔUA-2S ® GlcNS-6S Na_4 (I-S)
$C_{12}H_{16}NO_{16}S_2Na_3$ (Produced from heparinase II digestion of heparin and heparin sulphate)	ΔUA ® GlucNS-6S Na_3 (II-S)
$C_{12}H_{16}NO_{16}S_2Na_3$ (Produced from heparin by digestion with heparinase I and II)	ΔUA ® 2S-GlcNS Na_3 (III-S)
$C_{14}H_{18}NO_{17}S_2Na_3$ (Minor component produced from heparin by heparinase II)	ΔUA ® 2S-GlcNAc-6S Na_3 (I-A)
$C_{14}H_{19}NO_{14}SNa_2$ (Product of the action of heparinases II and III on heparin and heparan sulphate)	ΔUA ® GlcNAc-6S Na_2 (II-A)
$C_{14}H_{19}NO_{14}SNa_2$ (Minor product of the action of heparinase II on heparin)	ΔUA ® 2S-GlcNAc Na_2 (III-A)
$C_{14}H_{20}NO_{11}Na$ (Produced from heparin sulphate by digestion With heparinase III)	ΔUA ® GlcNAc Na (IV-A)
$C_{14}H_{19}NO_{14}SNa_2$ (Produced from various chondroitin sulphates By the action of chondroitinases ABC, B and AC-1)	ΔUA ® GalNAc-4S Na_2 (Δ Di-4S)
C14H19NO14SNa2 (Produced	ΔUA ®

Spacer form of saccharide	Common name
from various chondroitin sulphates By the action of chondroitinases ABC, AC-1 and C)	GalNAc-6S Na2 (Δ Di-6S)
$C_{14}H_{18}NO_{17}S_2Na_3$ (Produced from various chondroitin sulphates By the action of chondroitinases ABC, B and AC-1)	ΔUA ® GalNAc-4S,6S Na_3 (Δ Di-disE)
$C_{14}H_{18}NO_{17}S_2Na_3$ (Produced from various chondroitin sulphates by action of chondroitinase ABC and/or B. Most typically from chondroitin sulphate B (dermatan sulphate)	ΔUA ® 2S-GalNAc-4S Na_2 (Δ Di-disB)
$C_{14}H_{18}NO_{17}S_2Na_3$ (Produced from various chondroitin sulphates by the action of chondroitinase ABC)	ΔUA ® 2S-GalNAc-6S Na_3 (Δ Di-disD)
$C_{14}H_{17}NO_{20}S_3Na_4$ (Produced as a minor component by the action of chondroitinase ABC on various chondroitin sulphates, particularly B)	ΔUA ® 2S-GalNAc-4S-6S Na_4 (Δ Di-tisS)
$C_{14}H_{19}NO_{14}SNa_2$ (Produced as a minor component from various chondroitin sulphates by the action of chondroitinase ABC)	ΔUA ® 2S-GalNAc-6S Na_2 (ΔDi-UA2S)
$C_{14}H_{20}NO_{11}Na$ (The only unsaturated disaccharide produced from hyaluronic acid by the action of chondroitinase ABC or AC-1)	ΔUA ® GlcNAc Na (Δ Di-HA)
(GlcAβ1-3GlcNAcβ1-4)n (n=4)	Hyaluronan fragments (4mer)
(GlcAβ1-3GlcNAcβ1-4)n (n=8)	Hyaluronan fragm

Spacer form of saccharide	Common name
	ents (8mer)
(GlcAβ1-3GlcNAcβ1-4)n (n=10)	Hyaluronan fragments (10mer)
(GlcAβ1-3GlcNAcβ1-4)n (n=12)	Hyaluronan fragments (12mer)
(GlcA/IdoAα/β1-4GlcNAcα1-4)n (n=200)	Heparin
(GlcA/IdoAβ1-3(±4/6S)GalNAcβ1-4)n (n<250)	Chondroitin sulfate
((±2S)GlcA/Ido	Dermatan

Spacer form of saccharide	Common name
Aα/b1-3(±4S)GalNAcβ1-4)n (n<250)	sulfate
(GlcA/IdoAβ1-3(±6S)GalNAcβ1-4)n (n<250)	Chondroitin 6-Sulfate
(GlcAβ1-3GlcNAcβ1-4)n (n=4)	HA-4 (10mM)
(GlcAβ1-3GlcNAcβ1-4)n (n=6)	HA-6 (10mM)
(GlcAβ1-3GlcNAcβ1-4)n (n=8)	HA-8 (9.7mM)
(GlcAβ1-3GlcNAcβ1-4)n (n=10)	HA-10 (7.83 mM)
(GlcAβ1-3GlcNAcβ1-4)n (n=12)	HA-12 (6.5mM)

Spacer form of saccharide	Common name
(GlcAβ1-3GlcNAcβ1-4)n (n=14)	HA-14 (5.6mM)
(GlcAβ1-3GlcNAcβ1-4)n (n=16)	HA-16 (4.9mM)
	HA-30,000da 2.5mg/ml
	HA-107,000da 2.5mg/ml
	HA-190,000da 2.5mg/ml
	HA-220,0

Spacer form of saccharide	Common name
	00da 2.5mg/ml
	HA-1,600,000da 2.5mg/ml
	Heparin Sulfate 5mg/ml
(Glcβ1-3Glcβ1-3)n	Beta-1-3Glucan

Table S2. Glycans sourced from 2) Glycoseparations Ltd (Moscow, Russia).

Spacer form of saccharide	Common name
Fucα-sp3	L-α-Fuc
Galα-sp3	α-Gal
Galβ-sp3	β-Gal
GalNAcα-sp0	TnSer
GalNAcα-sp3	T_n
GalNAcβ-sp3	β-GalNAc
Glcα-sp3	α-Glc
Glcβ-sp3	β-Glc
GlcNAcβ-sp3	β-GlcNAc
GlcN(Gc)β-sp4	β-GlcN(Gc)
$HOCH_2(HOCH)_4CH_2NH_2$	Amino-glucitol
Manα-sp3	α-Man
Manβ-sp4	β-Man
ManNAcβ-sp4	β-ManAc
Rhaα-sp3	L-α-Rha
GlcNAcβ-sp4	β-GlcNAc
3-O-Su-Galβ-sp3	3-O-Su-β-Gal
3-O-Su-GalNAcα-sp3	3-O-Su-β-GalNac
6-O-Su-GlcNAcβ-sp3	6-O-Su-β-GlcNAc
GlcAα-sp3	α-glucoronic acid
GlcAβ-sp3	β-glucoronic acid
6-H_2PO_3Glcβ-sp4	β-Glc6P
6-H_2PO_3Manα-sp3	α-Man6P
Neu5Acα-sp3	α-Neu5Ac
Neu5Acα-sp9	α-Neu5AcBn

Spacer form of saccharide	Common name
Neu5Gcα-sp3	α-Neu5Gc
9-NAc-Neu5Acα-sp3	9-Nac-α-Neu5Ac
3-O-Su-GlcNAcβ-sp3	3-O-Su-β-GlcNAc
Fucα1-2Galβ-sp3	H_{di}
Fucα1-3GlcNAcβ-sp3	
Fucα1-4GlcNAcβ-sp3	Le
Galα1-2Galβ-sp3	
Galα1-3Galβ-sp3	B_{di}
Galα1-3GalNAcβ-sp3	$T_{\alpha\beta}$
Galα1-3GalNAcα-sp3	$T_{\alpha\alpha}$
Galα1-3GlcNAcβ-sp3	
Galα1-4GlcNAcβ-sp3	α-LacNAc
Galα1-6Glcβ-sp4	Melibiose
Galβ1-2Galβ-sp3	
Galβ1-3GlcNaAcβ-sp3	Le^c
Galβ1-3Galβ-sp3	
Galβ1-3GalNAcβ-sp3	$T_{\beta\beta}$
Galβ1-3GalNAcα-sp3	TF
Galβ1-4Glcβ-sp4	Lac
Galβ1-4Galβ-sp4	
Galβ1-4GlcNAcβ-sp3	LacNAc
Galβ1-6Galβ-sp4	
GalNAcα1-3GalNAcβ-sp3	Fs-2
GalNAcα1-3Galβ-sp3	A_{di}
GalNAcα1-3GalNAcα-sp3	Core 5
GalNAcβ1-3Galβ-sp3	
GalNAcβ1-4GlcNAcβ-sp3	LacdiNAc
Glcα1-4Glcβ-sp3	maltose
Glcβ1-4Glcβ-sp4	Cellobiose
Glcβ1-6Glcβ-sp4	Gentiobiose

Spacer form of saccharide	Common name
GlcNAcβ1-3GalNAcα-sp3	Core 3
GlcNAcβ1-3Manβ-sp4	
GlcNAcβ1-4GlcNAcβ-Asn	Chitobiose-Asn
GlcNAcβ1-4GlcNAcβ-sp4	Chitobiose
GlcNAcβ1-6GalNAcα-sp3	Core 6
Manα1-2Manβ-sp4	
Manα1-3Manβ-sp4	
Manα1-4Manβ-sp4	
Manα1-6Manβ-sp4	
Manβ1-4GlcNAcβ-sp4	
Manα1-2Manα-sp4	
Galβ1-3(6-O-Su)GlcNAcβ-sp3	6-O-Su-Le^c
Galβ1-4(6-O-Su)Glcβ-sp2	6-O-Su-Lac
Galβ1-4(6-O-Su)GlcNAcβ-sp3	6-O-Su-LacNAc
GlcNAcβ1-4(6-O-Su)GlcNAcβ-sp2	6-O-Su-chitobiose
3-O-Su-Galβ1-3GalNAcα-sp3	3`-O-Su-TF
6-O-Su-Galβ1-3GalNAcα-sp3	6`-O-Su-TF
3-O-Su-Galβ1-4Glcβ-sp2	SM3
6-O-Su-Galβ1-4Glcβ-sp2	6`-O-Su-Lac
3-O-Su-Galβ1-3GlcNAcβ-sp3	3`-O-Su-Le^c
3-O-Su-Galβ1-4GlcNAcβ-sp3	3`-O-Su-LacNAc
4-O-Su-Galβ1-4GlcNAcβ-sp3	4`-O-Su-LacNAc
6-O-Su-Galβ1-3GlcNAcβ-sp3	6`-O-Su-Le^c
6-O-Su-Galβ1-4GlcNAcβ-sp3	6`-O-Su-LacNAc
GlcAβ1-3GlcNAcβ-sp3	

Spacer form of saccharide	Common name
GlcAβ1-3Galβ-sp3	
GlcAβ1-6Galβ-sp3	
GlcNAcβ1-4-[HOOC(CH_3)CH]-3-O-GlcNAcβ-sp4	GlcNAc-Mur
GlcNAcβ1--[HOOC(CH_3)CH]-3-O-GlcNAcβ-L-alanyl-D-i-glutaminyl-L-lysine	GMDP-Lys
Neu5Acα2-3Galβ-sp3	GM4
Neu5Acα2-6Galβ-sp3	
Neu5Acα2-3GalNAcα-sp3	3-$SiaT_n$
Neu5Acα2-6GalNAcα-sp3	$SiaT_n$
Neu5Gcα2-6GalNAcα-sp3	NeuGc-T_n
3-O-Su-Galβ1-4(6-O-Su)Glcβ-sp2	3`,6-di-O-Su-Lac
3-O-Su-Galβ1-4(6-O-Su)GlcNAcβ-sp2	3`,6-di-O-Su-LacNAc
6-O-Su-Galβ1-4(6-O-Su)Glcβ-sp2	6,6`-di-O-Su-Lac
6-O-Su-Galβ1-3(6-O-Su)GlcNAcβ-sp2	6,6`-di-O-Su-Le^c
6-O-Su-Galβ1-4(6-O-Su)GlcNAcβ-sp2	6,6`-di-O-Su-LacNAc
3,4-O-Su_2-Galβ1-4GlcNAcβ-sp3	3`,4`-di-O-Su-LacNAc
3,6-O-Su_2-Galβ1-4GlcNAcβ-sp2	3`,6`-di-O-Su-LacNAc
4,6-O-Su_2-Galβ1-4GlcNAcβ-sp2	4`,6`-di-O-Su-LacNAc
4,6-O-Su_2-Galβ1-4GlcNAcβ-sp3	4`,6`-di-O-Su-LacNAc
Neu5Acα2-8Neu5Acα2-sp3	$(Sia)_2$

Spacer form of saccharide	Common name
3,6-O-Su_2-Galβ1-4(6-O-Su)GlcNAcβ-sp2	3`,6,6`-tri-O-Su-LacNAc
GalNAcβ1-4(6-O-Su)GlcNAcβ-sp3	6-O-Su-LacdiNAc
3-O-Su-GalNAcβ1-4GlcNAcβ-sp3	3`-O-Su-LacdiNAc
6-O-Su-GalNAcβ1-4GlcNAcβ-sp3	6`-O-Su-LacdiNAc
6-O-Su-GalNAcβ1-4-(3-O-Su)GlcNAcβ-sp3	6`-Su-3-O-Ac-LacdiNAc
3-O-Su-GalNAcβ1-4(3-O-Su)-GlcNAcβ-sp3	3,3`-O-Su_2-LacdiNAc
3,6-O-Su_2-GalNAcβ1-4GlcNAcβ-sp3	3`,6`-Su_2-LacdiNAc
4,6-O-Su_2-GalNAcβ1-4GlcNAcβ-sp3	4`,6`-Su_2-LacdiNAc
4,6-O-Su_2-GalNAcβ1-4-(3-O-Ac)GlcNAcβ-sp3	4`,6`-Su_2-3-O-Ac-LacdiNAc
4-O-Su-GalNAcβ1-4GlcNAcβ-sp3	4`-O-Su-LacdiNAc
3,4-O-Su_2-Galβ1-4GlcNAcβ-sp3	3`,4`-Su_2-LacdiNAc
6-O-Su-GalNAcβ1-4(6-O-Su)GlcNAcβ-sp3	6,6`-O-Su-LacdiNAc
Galβ1-4(6-O-Su)GlcNAcβ-sp2	6-O-Su-LacNAc
4-O-Su-GalNAcβ1-4GlcNAcβ-sp2	4`-O-Su-LacdiNAc
Neu5Acα2-6GalNAcβ-sp3	
Neu5Gcα2-3Gal-sp3	NeuGcα3Gal
Fucα1-2Galβ1-3GlcNAcβ-sp3	Le^d, H (type 1)
Fucα1-2Galβ1-4GlcNAcβ-sp3	H (type 2)

Spacer form of saccharide	Common name
Fucα1-2Galβ1-3GalNAcα-sp3	H (type 3)
Fucα1-2Galβ1-4Glcβ-sp4	H (type 6
Galα1-3Galβ1-4Glcβ-sp2	
Galα1-3Galβ1-4GlcNAcβ-sp3	Galili (tri)
Galα1-4Galβ1-4Glcβ-sp3	P^k, Gb3, $GbOse_3$
Galα1-4Galβ1-4GlcNAc-sp2	P_1
Galα1-3	
Galβ-sp3	B_{tri}
Fucα1-2	
Galβ1-2Galα1-4GlcNAcβ-sp4	
Galβ1-3Galβ1-4GlcNAcβ-sp4	
Galβ1-4GlcNAcβ1-3GalNAcα-sp3	
Galβ1-4GlcNAcβ1-6GalNAcα-sp3	
Fucα1-4	
GlcNAcβ-sp3	Le^a
Galβ1-3	
Galβ1-4	
GlcNAcβ-sp3	Le^x
Fucα1-3	
GalNAcα1-3	
Galβ-sp3	A_{tri}
Fucα1-2	
GalNAcβ1-4Galβ1-4Glcβ-sp3	GA_2, $GgOse_3$
$(Glcα1-4)_3β$-sp4	Maltotriose
$(Glcα1-6)_3β$-sp4	Isomaltotriose
GlcNAcβ1-2Galβ1-3GalNAcα-sp3	
GlcNAcβ1-3Galβ1-3GalNAcα-sp3	
GlcNAcβ1-3Galβ1-4Glcβ-sp2	
GlcNAcβ1-3Galβ1-4GlcNAcβ-sp3	
GlcNAcβ1-4Galβ1-	

Spacer form of saccharide	Common name
4GlcNAcβ-sp2	
GlcNAcβ1-4GlcNAcβ1-4GlcNAcβ-sp4	Chitotriose
GlcNAcβ1-6Galβ1-4GlcNAcβ-sp2	
GlcNAcβ1-6	
GalNAcα-sp3	Core 2
Galβ1-3	
GlcNAcβ1-6	
GalNAcα-sp3	Core 4
GlcNAcβ1-3	
Manα1-6	
Manβ-sp4	Man_3
Manα1-3	
Galβ1-3GalNAcβ1-3Gal-sp4	$T_{ββ}$-Gal
Galβ1-4Galβ1-4GlcNAc-sp3	
Fucα1-4	
GlcNAcβ-sp3	Su-Le^a
3-O-Su-Galβ1-3	
3-O-Su-Galβ1-4	
GlcNAcβ-sp3	Su-Le^x
Fucα1-3	
Neu5Acα2-6	
GalNAcα-sp3	6-SiaTF
Galα1-3	
Neu5Acα2-6	
GalNAcα-sp3	
Galβ1-3	
Neu5Acα2-3Galβ1-3GalNAcα-sp3	3`-Sia-TF
Neu5Acα2-3Galβ1-4Glcβ-sp3	3`SL
Neu5Acα2-3Galβ1-4Glcβ-sp4	3`SL
Neu5Acα2-6Galβ1-4Glcβ-sp2	6`SL
Neu5Acα2-3Galβ1-4GlcNAcβ-sp3	3`SLN
Neu5Acα2-3Galβ1-	3`-SiaLe

Spacer form of saccharide	Common name
3GlcNAcβ-sp3	[c]
Neu5Acα2-6Galβ1-4GlcNAcβ-sp3	6`SLN
Neu5Gcα2-3Galβ1-4GlcNAcβ-sp3	3`SLN (Gc)
Neu5Gcα2-6Galβ1-4GlcNAcβ-sp3	6`SLN (Gc)
9-NAc-Neu5Acα2-6Galβ1-4GlcNAcβ-sp3	
Neu5Acα2-3Galβ1-4-(6-O-Su)GlcNAcβ-sp3	6-Su-3`SLN
Neu5Acα2-3Galβ1-3-(6-O-Su)GalNAcβ-sp3	6-Su-3`SiaTF
Neu5Acα2-6Galβ1-4-(6-O-Su)GlcNAcβ-sp3	6-Su-6`SLN
Neu5Acα2-3-(6-O-Su)Galβ1-4GlcNAcβ-sp3	6`-Su-3`SLN
$(Neu5Acα2-8)_3$-sp3	$(Sia)_3$
Neu5Acα2-6Galβ1-3GlcNAc-sp3	6`-SiaLe[c]
Neu5Acα2-6Galβ1-3(6-O-Su)GlcNAc-sp3	6Su-6`-SiaLe[c]
Neu5Gcα2-3Galβ1-3GlcNAcβ-sp3	3`SiaLe^c (Gc)
Galα1-3	
Galβ1-3GlcNAcβ-sp3	B (type 1)
Fucα1-2	
Galα1-3	
Galβ1-4GlcNAcβ-sp3	B (type 2)
Fucα1-2	
Galα1-3	
Galβ1-3GalNAcα-sp3	B (type 3)
Fucα1-2	
Galα1-3	
Galβ1-3GalNAcβ-sp3	B (type 4)
Fucα1-2	
Galα1-3Galβ1-4	
GlcNAcβ-sp3	αGalLe^x

Spacer form of saccharide	Common name
Fucα1-3	
GalNAcα1-3	
Galβ1-3GlcNAcβ-sp3	A (type 1)
Fucα1-2	
GalNAcα1-3	
Galβ1-4GlcNAcβ-sp3	A (type 2)
Fucα1-2	
Fucα1-4	
GlcNAcβ-sp3	Le^b
Fucα1-2Galβ1-3	
Fucα1-2Galβ1-4	
GlcNAcβ-sp3	Le^y
Fucα1-3	
Galα1-3Galβ1-4GlcNAcβ1-3Galβ-sp3	Galili (tetra)
Galα1-4GlcNAcβ1-3Galβ1-4GlcNAcβ-sp3	
Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ-sp4	LNT
Galβ1-3GlcNAcβ1-3Galβ1-3GlcNAcβ-sp2	
Galβ1-3GlcNAcα1-3Galβ1-4GlcNAcβ-sp3	
Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ-sp3	
Galβ1-3GlcNAcα1-6Galβ1-4GlcNAcβ-sp2	
Galβ1-3GlcNAcβ1-6Galβ1-4GlcNAcβ-sp2	
Galβ1-3GalNAcβ1-4Galβ1-4Glcβ-sp3	Asialo-GM_1
Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ-sp2	LNnT
Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ-sp3	i
Galβ1-4GlcNAcβ1-	

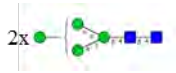
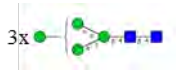
Spacer form of saccharide	Common name
6Galβ1-4GlcNAcβ-sp2	
Galβ1-4GlcNAcβ1-6	
GalNAcα-sp3	
Galβ1-3	
GalNacβ1-3GalαGalβ1-4Glcβ-sp3	Gb_4, P
$(Glcα1-4)_4β$-sp4	Malto-tetraose
$(Glcα1-6)_4β$-sp4	Isomalto-tetraose
GalNAcα1-6	
Galβ1-3GalNAcα-sp3	A (type 3)
Fucα1-2	
GlcNAcβ1-6	
Galβ1-4GlcNAcβ-sp3	Tk
GlcNAcβ1-3	
Galβ1-3GlcNAcβ1-3Galβ1-3GlcNAcβ-sp3	Le^c3Le^c
3-O-SuGalβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ-sp3	
4-O-SuGalβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ-sp3	
GalNAcβ1-4	
Galβ1-4Glcβ-sp2	GM_2
Neu5Acα2-3	
Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ-sp3	
Neu5Acα2-3Galβ1-4	
GlcNAcβ-sp3	SiaLe^x
Fucα1-3	
Fucα1-4	
GlcNAcβ-sp3	SiaLe^a
Neu5Acα2-3Galβ1-3	
Neu5Acα2-3Galβ1-4	
6-O-Su-GlcNAcβ-sp3	
Fucα1-3	

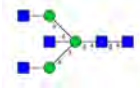
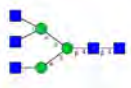
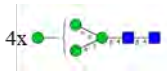
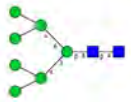
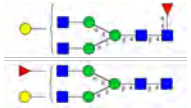
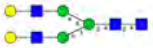
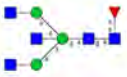
Spacer form of saccharide	Common name
Neu5Acα2-3(6-O-Su)Galβ1-4	
GlcNAcβ-sp3	
Fucα1-3	
Neu5Acα2-6	
GalNAcα-sp3	Sia_2-TF
Neu5Acα2-3Galβ1-3	
Neu5Acα2-8Neu5Acα2-3Galβ1-4Glcβ-sp4	GD_3
Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ-sp4	LNFP-I
Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ-sp2	H (type 1) penta
Galα1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ-sp4	Galili (penta)
Galα1-3	
Galβ1-4	
Fucα1-2 GlcNAcβ-sp3	Ble^y
Fucα1-3	
Galβ1-4GlcNAcβ1-6	
GalNAcα-sp3	
Galβ1-4GlcNAcβ1-3	
GlcNAcβ1-6	
Galβ1-4GlcNAc-sp2	
Galβ1-4GlcNAcβ1-3	
Galβ1-4GlcNAcβ1-6	
Galβ1-4GlcNAcβ-sp2	
GlcNAcβ1-3	
$(Glcα1-6)_5β$-sp4	Isomalto-pentaose
$(GlcNAcβ1-4)_5β$-sp4	Chito-pentaose
Manα1-6	
Manα1-6	Man5
Manα1-3 Manβ-sp4	
Manα1-3	
Fucα1-4	

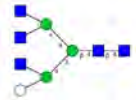
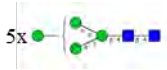
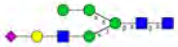
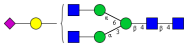
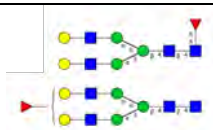
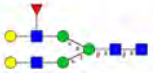
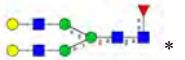
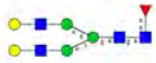
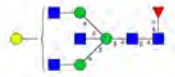
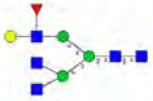
Spacer form of saccharide	Common name
GlcNAcβ1-3Galβ1-4Glcβ-sp4	Le^b-Lac
Fucα1-2Galβ1-3	
Fucα1-2Galβ1-4	
GlcNAcβ1-3Galβ1-4Glcβ-sp4	Le^y-Lac
Fucα1-3	
$(Galβ1-4GlcNAcβ1-3)_3$-sp3	$(LN)_3$
Galβ1-4GlcNAcβ1-6	
Galβ1-4GlcNAc-sp2	I
Galβ1-4GlcNAcβ1-3	
Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ-sp4	Gb_5
$(Glcα1-6)_6β$-sp4	Malto-hexaose
$(GlcNAcβ1-4)_6β$-sp4	Chito-hexaose
$(A-GN-M)_2$-3,6-M-GN-GNβ-sp4	9-OS
$(GN-M)_2$-3,6-M-GN-GNβ-sp4	7-OS
Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ-sp2	3`SLN-LacNAc
Neu5Acα2-3 Galβ1-4	
GlcNAcβ1-3Galβ-sp3	SiaLe^x-3Gal
Fucα1-3	
Neu5Acα2-6	
GlcNAcβ1-3Galβ1-4Glcβ-sp4	LSTb
Galβ1-3	
GalNAcβ1-4	
Galβ1-4Glc-sp2	GD_2
Neu5Acα2-8Neu5Acα2-3	
Neu5Acα2-8Neu5Acα2-8Neu5Acα2-3Galβ1-4Glc-sp2	GT_3
GalNAcβ1-4	
Galβ1-4Glc-sp2	GT_2
$(Neu5Acα2-8)_2$Neu5Acα2-3	

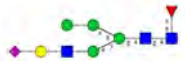
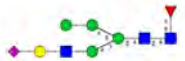
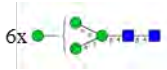
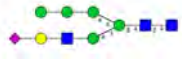
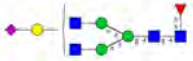
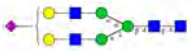
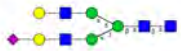
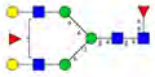
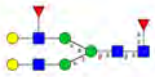
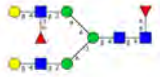
Spacer form of saccharide	**Common name**
Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ-sp3	6`SLN-LacNAc
Neu5Acα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ-sp4	LSTa
Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ-sp4	LSTd
Le^x1-6'(Le^c1-3')Lac-sp4	MFLNH III
LacNAc1-6'(Le^d1-3')Lac-sp4	MFLNH I
Le^x1-6'(6'-SLN1-3')Lac-sp4	MSMFLNnH
Le^x1-6'(Le^d1-3')Lac-sp4	DFLNH (a)
Le^cLe^x1-6'(Le^c1-3')Lac-sp4	MF(1-3)iLNO
Le^x1-6'(Le^b1-3')Lac-sp4	TFLNH
(GlcAβ1-4GlcNAcβ1-3)$_8$-NH_2-ol	hyaluronin c acid
(Sia2-6A-GN-M)$_2$-3,6-M-GN-GNβ-sp4	11-OS, YDS

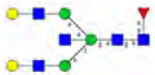
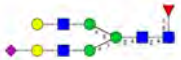
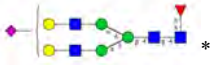
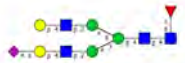
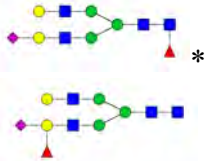
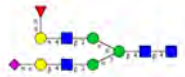
Table S3. A total of 65 *N*- and *O*-linked glycan structures on human milk proteins are described and grouped according to their increasing calculated experimental mass. This study identified 45 *N*-linked glycans (of which 28 have been previously reported) and 20 *O*-linked glycans (of which 16 have been previously reported). The theoretical masses of the possible *N*-and *O*-linked glycans were obtained from GlycoMod (http://web.expasy.org/glycomod/). Only the most abundant glycans were analysed in this study for their relative abundances and their proposed structures were compared with reported glycans on human milk glycoproteins in literature that corresponded to the same mass and monosaccharide composition. The 21 possible *N*- and *O*-linked glycan masses (N1, N7, N14, N20, N23, N27, N30, N31, N32, N35, N36, N37, N38, N39, N41, N44, N45, O9, O16, O19 and O20) detected at low abundances in this study have not been previously reported in the literature[1-11] used for comparison.

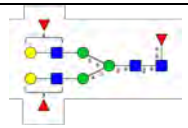
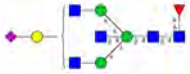
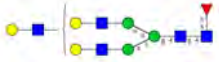
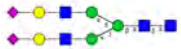
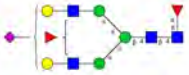
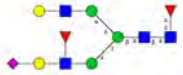
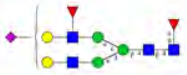
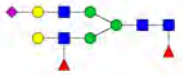
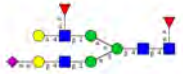
S/ No.	Calcu-lated experi-mental mass $[M-H]^{1-}$	Theore-tical mass $[M-H]^{1-}$	No. of isomers	Relative abund-ance (%)	Proposed glycans on total human milk proteins in this study	Glycans reported on human skim milk proteins in literature[1]	Glycans reported on human milk whey proteins in literature[2]	Glycans reported on specific human milk proteins in literature
N 1	1276.4	1276.5	ND	Trace		×	×	×
N 2	1235.3	1235.4	1	0.81		×	✓*	×
N 3	1397.4	1397.5	1	0.42		×		×
N 4	1479.4	1479.5	ND	Trace		×	✓*	×

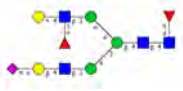
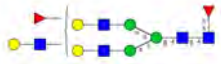
S/ No.	Calcu-lated experi-mental mass $[M-H]^{1-}$	Theore-tical mass $[M-H]^{1-}$	No. of isomers	Relative abund-ance (%)	Proposed glycans on total human milk proteins in this study	Glycans reported on human skim milk proteins in literature[1]	Glycans reported on human milk whey proteins in literature[2]	Glycans reported on specific human milk proteins in literature
N 5	1520.4	1520.6	1	2.41			×	×
N 6	1559.2	1559.5	1	0.67			✓*	×
N 7	1567.6	1567.5	1	0.28		×	×	×
N 8	1625.4	1625.6	ND	Trace	 Possible Le	×	✓*	×
N 9	1641.4	1641.6	1	0.29			 *	 BSSL[3] Human lactoferrin[5]
N 10	1666.6	1666.6	1	7.01			×	×

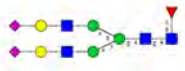
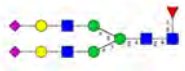
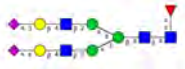
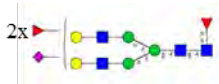
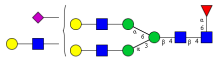
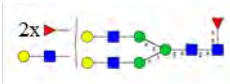
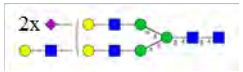
S/ No.	Calcu-lated experi-mental mass $[M-H]^{1-}$	Theore-tical mass $[M-H]^{1-}$	No. of isomers	Relative abund-ance (%)	Proposed glycans on total human milk proteins in this study	Glycans reported on human skim milk proteins in literature[1]	Glycans reported on human milk whey proteins in literature[2]	Glycans reported on specific human milk proteins in literature
N 11	1682.6	1682.6	1	1.16			×*	×
N 12	1721.4	1721.6	1	1.17		×	✓*	×
N 13	1729.6	1729.6	1	0.79		×		×
N 14	1770.6	1770.6	ND	Trace		×	×*	×*
N 15	1787.6	1787.6	3	2.03	 Possible Le	 Le		 BSSL[3] Human lactoferrin[4, 5]
N 16	1828.6	1828.7	1	5.31		 Le	×*	×

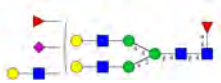
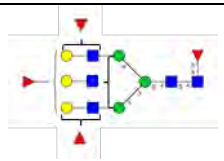
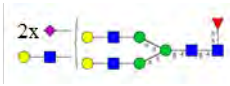
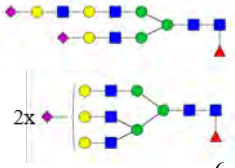
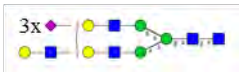
S/ No.	Calcu-lated experi-mental mass $[M-H]^{1-}$	Theore-tical mass $[M-H]^{1-}$	No. of isomers	Relative abund-ance (%)	Proposed glycans on total human milk proteins in this study	Glycans reported on human skim milk proteins in literature[1]	Glycans reported on human milk whey proteins in literature[2]	Glycans reported on specific human milk proteins in literature
N 17	1875.6	1875.7	1	0.63		×		×
N 18	1883.6	1883.6	1	1.31		×		×
N 19	1891.6	1891.7	1	0.87		×	✓*	×*
N 20	1916.6	1916.7	1	0.78		×	×*	×
N 21	1932.6	1932.7	1	11.21				 BSSL[3]
N 22	1933.6	1933.7	2	2.86	 Le	 Le	 Le	 Le Human lactoferrin[4, 5]

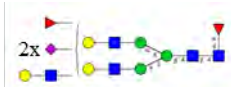
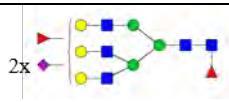
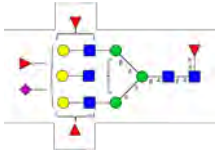
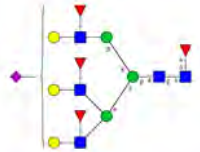
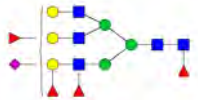
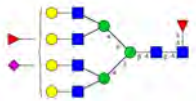
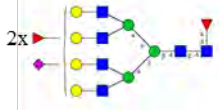
S/ No.	Calcu-lated experi-mental mass [M-H]$^{1-}$	Theore-tical mass [M-H]$^{1-}$	No. of isomers	Relative abund-ance (%)	Proposed glycans on total human milk proteins in this study	Glycans reported on human skim milk proteins in literature[1]	Glycans reported on human milk whey proteins in literature[2]	Glycans reported on specific human milk proteins in literature
N 23	1990.6	1990.7	1	0.15		×	×*	×
N 24	2078.6	2078.7	3	10.64				 BSSL[3] Human lactoferrin[4, 5] sIgA[7]  MFGM proteins[6]  sIgA[7]

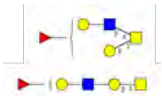
S/ No.	Calcu-lated experi-mental mass [M-H]$^{1-}$	Theore-tical mass [M-H]$^{1-}$	No. of isomers	Relative abund-ance (%)	Proposed glycans on total human milk proteins in this study	Glycans reported on human skim milk proteins in literature[1]	Glycans reported on human milk whey proteins in literature[2]	Glycans reported on specific human milk proteins in literature
N 25	2079.6	2079.8	2	0.16	 Possible Le	×	✓*	×
N 26	2119.6	2119.8	1	1.06		×	✓*	×*
N 27	2152.6	2152.8	ND	Trace		×	×*	×
N 28	2223.6	2223.8	1	25.82			 *	×
N 29	2224.6	2224.8	3	15.52	 Le/possible SLe	 SLe	 Le/possible SLe	 Le MFGM proteins[6] 

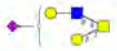
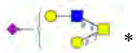
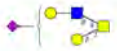
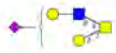
S/ No.	Calcu-lated experi-mental mass [M-H]$^{1-}$	Theore-tical mass [M-H]$^{1-}$	No. of isomers	Relative abund-ance (%)	Proposed glycans on total human milk proteins in this study	Glycans reported on human skim milk proteins in literature[1]	Glycans reported on human milk whey proteins in literature[2]	Glycans reported on specific human milk proteins in literature
								Le sIgA[7]  Le Human lactoferrin[5] sIgA[7]
N 30	2281.6	2281.8	1	0.36		×	×	×
N 31	2297.6	2297.8	ND	Trace		×	×	×*
N 32	2298.6	2298.8	ND	Trace	 Possible Le	×	×	×

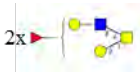
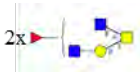
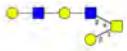
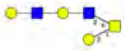
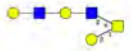
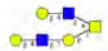
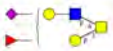
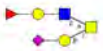
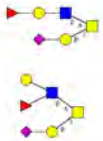
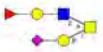
S/ No.	Calcu-lated experi-mental mass [M-H]$^{1-}$	Theore-tical mass [M-H]$^{1-}$	No. of isomers	Relative abund-ance (%)	Proposed glycans on total human milk proteins in this study	Glycans reported on human skim milk proteins in literature[1]	Glycans reported on human milk whey proteins in literature[2]	Glycans reported on specific human milk proteins in literature
N 33	2369.8	2369.8	1	4.47			×	 Human lactoferrin[5] MFGM proteins[6] sIgA[7]
N 34	2370.6	2370.9	2	0.78	 Possible Le/SLe	×	✓	×
N 35	2443.6	2443.9	2	0.41		×	×	×*
N 36	2444.6	2444.9	1	0.65	 Possible Le	×	×	×
N 37	2588.8	2588.9	ND	Trace		×	×	×

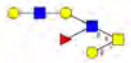
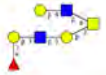
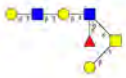
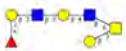
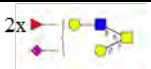
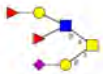
S/ No.	Calcu-lated experi-mental mass $[M-H]^{1-}$	Theore-tical mass $[M-H]^{1-}$	No. of isomers	Relative abund-ance (%)	Proposed glycans on total human milk proteins in this study	Glycans reported on human skim milk proteins in literature[1]	Glycans reported on human milk whey proteins in literature[2]	Glycans reported on specific human milk proteins in literature
N 38	2589.8	2589.9	ND	Trace	 Possible Le/SLe	×	×	×
N 39	2590.8	2591.0	ND	Trace	 Possible Le	×	×	×
N 40	2734.8	2735.0	ND	Trace		×	×	 MFGM proteins[6]
N 41	2879.8	2880.0	ND	Trace		×	×	×

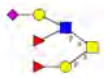
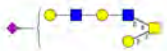
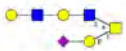
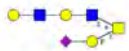
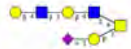
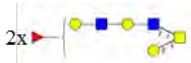
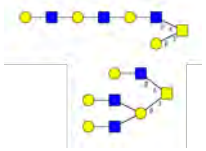
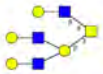
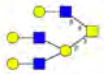
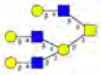
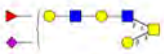
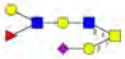
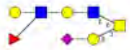
S/ No.	Calcu-lated experi-mental mass [M-H]$^{1-}$	Theore-tical mass [M-H]$^{1-}$	No. of isomers	Relative abund-ance (%)	Proposed glycans on total human milk proteins in this study	Glycans reported on human skim milk proteins in literature[1]	Glycans reported on human milk whey proteins in literature[2]	Glycans reported on specific human milk proteins in literature
N 42	2880.8	2881.0	ND	Trace	 Possible Le/SLe	×	×	 Possible Le/SLe MFGM proteins[6]
N 43	2881.8	2882.1	ND	Trace	 Possible Le/SLe	×	 SLe	 Le/SLe MFGM proteins[6]
N 44	2954.8	2955.1	ND	Trace	 Possible Le/SLe	×	×	×
N 45	3100.8	3101.1	ND	Trace	 Possible Le/SLe	×	×	×
O 1	587.1	587.2	1	1.69		×	×	 MFGM proteins[6]

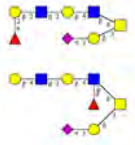
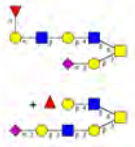
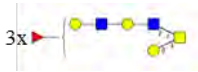
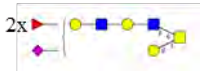
S/ No.	Calcu-lated experi-mental mass $[M-H]^{1-}$	Theore-tical mass $[M-H]^{1-}$	No. of isomers	Relative abund-ance (%)	Proposed glycans on total human milk proteins in this study	Glycans reported on human skim milk proteins in literature[1]	Glycans reported on human milk whey proteins in literature[2]	Glycans reported on specific human milk proteins in literature
O 2	675.3	675.2	2	2.42			× *	 * sIgA[9]  * MFGM proteins[6]
O 3	749.3	749.3	2	20.54				 MFGM proteins[6] Mature milk κ-casein[8] sIgA[9, 10]
O 4	878.3	878.3	3	2.46				 Skim milk mucins[11]
O 5	895.3	895.3	2	10.33	 Possible Le	×	 Le 	 Le MFGM proteins[6] 

S/ No.	Calcu-lated experi-mental mass [M-H]$^{1-}$	Theore-tical mass [M-H]$^{1-}$	No. of isomers	Relative abund-ance (%)	Proposed glycans on total human milk proteins in this study	Glycans reported on human skim milk proteins in literature[1]	Glycans reported on human milk whey proteins in literature[2]	Glycans reported on specific human milk proteins in literature
								MFGM proteins[6] sIgA[10]
O 6	952.3	952.3	1	0.30		×	×	 Mature milk κ-casein[8]
O 7	966.3	966.3	3	1.06		×*	×*	 * Skim milk mucins[11]
O 8	1040.3	1040.4	1	27.25		 *		 * MFGM proteins[6]  sIgA[10] Skim milk mucins[11]

S/ No.	Calcu-lated experi-mental mass [M-H]$^{1-}$	Theore-tical mass [M-H]$^{1-}$	No. of isomers	Relative abund-ance (%)	Proposed glycans on total human milk proteins in this study	Glycans reported on human skim milk proteins in literature[1]	Glycans reported on human milk whey proteins in literature[2]	Glycans reported on specific human milk proteins in literature
O 9	1041.3	1041.4	1	7.72	 Possible Le	×	×	×
O 10	1082.4	1082.4	1	0.12		×	×	 Mature milk κ-casein[8]
O 11	1114.3	1114.4	3	3.97				 MFGM proteins[6] sIgA[10]  sIgA[10]
O 12	1186.4	1186.4	4	9.66	 Possible Le/SLe		 Le	 MFGM proteins[6]

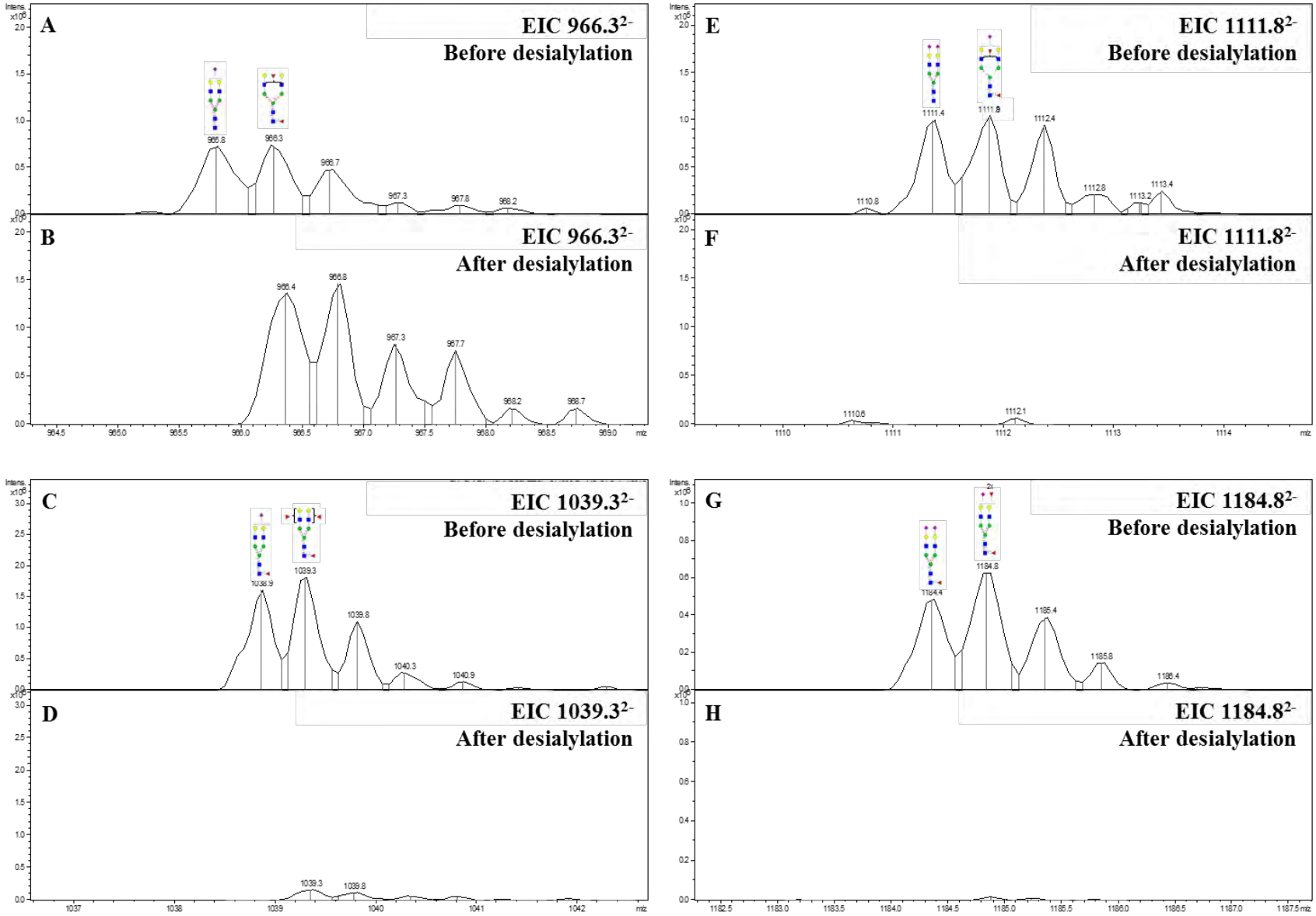
S/ No.	Calcu-lated experi-mental mass [M-H]$^{1-}$	Theore-tical mass [M-H]$^{1-}$	No. of isomers	Relative abund-ance (%)	Proposed glycans on total human milk proteins in this study	Glycans reported on human skim milk proteins in literature[1]	Glycans reported on human milk whey proteins in literature[2]	Glycans reported on specific human milk proteins in literature
								 Le MFGM proteins[6] sIgA[10] Skim milk mucins[11]
O 13	1260.4	1260.5	4	5.05	 Possible Le	×	 Le	 sIgA[10]  Le MFGM proteins[6] sIgA[10]  sIgA[10]
O 14	1332.5	1332.5	2	0.61	 Possible Le/SLe	×		

S/ No.	Calcu-lated experi-mental mass [M-H]$^{1-}$	Theore-tical mass [M-H]$^{1-}$	No. of isomers	Relative abund-ance (%)	Proposed glycans on total human milk proteins in this study	Glycans reported on human skim milk proteins in literature[1]	Glycans reported on human milk whey proteins in literature[2]	Glycans reported on specific human milk proteins in literature
							Le  SLe	 Le MFGM proteins[6]
O 15	1405.4	1405.5	2	3.72				 MFGM proteins[6] sIgA10 Skim milk mucins[11]
O 16	1406.5	1406.5	2	0.24	 Possible Le	×	×	×
O 17	1479.4	1479.5	3	1.42				 MFGM proteins[6] sIgA[10]
O 18	1551.4	1551.6	2	1.45	 Possible Le/SLe	 Le	 Le	 Le MFGM proteins[6]

S/ No.	Calcu-lated experi-mental mass [M-H]$^{1-}$	Theore-tical mass [M-H]$^{1-}$	No. of isomers	Relative abund-ance (%)	Proposed glycans on total human milk proteins in this study	Glycans reported on human skim milk proteins in literature[1]	Glycans reported on human milk whey proteins in literature[2]	Glycans reported on specific human milk proteins in literature
								 Le sIgA[10]  Possible Le/SLe Skim milk mucins[11]
O 19	1552.5	1552.6	ND	Trace	 Possible Le	×	×	×
O 20	1697.5	1697.6	ND	Trace	 Possible Le/SLe	×	×	×

Abbreviations: BSSL, bile salt-stimulated lipase; ✓, glycan structure is detected in human milk, but not shown; ×, glycan structure is not detected in

human milk; *, glycan structure detected in bovine milk[6]; HMW, high molecular weight; κ, kappa; MFGM, milk fat globule membrane; Le, Lewis-type structure; ND; not detected as trace levels; sIgA, secretory immunoglobulin A; S/No., structure number; SLe, sialyl Lewis-type structure. Data compiled from [1]Dallas *et al.* 2011; [2]Nwosu *et al.* 2012; [3]Mechref *et al.* 1999; [4]Matsumoto *et al.* 1982; [5]Samyn-Petit *et al.* 2001; [6]Wilson *et al.* 2008; [7]Pierce-Crétel *et al.* 1982; [8]Saito *et al.* 1988; [9]Pierce-Crétel *et al.* 1981; [10]Pierce-Crétel *et al.* 1989; [11]Hanisch *et al.* 1990.


A
EIC 966.3²⁻
Before desialylation
B
EIC 966.3²⁻
After desialylation
E
EIC 1111.8²⁻
Before desialylation
F
EIC 1111.8²⁻
After desialylation
C
EIC 1039.3²⁻
Before desialylation
D
EIC 1039.3²⁻
After desialylation
G
EIC 1184.8²⁻
Before desialylation
H
EIC 1184.8²⁻
After desialylation

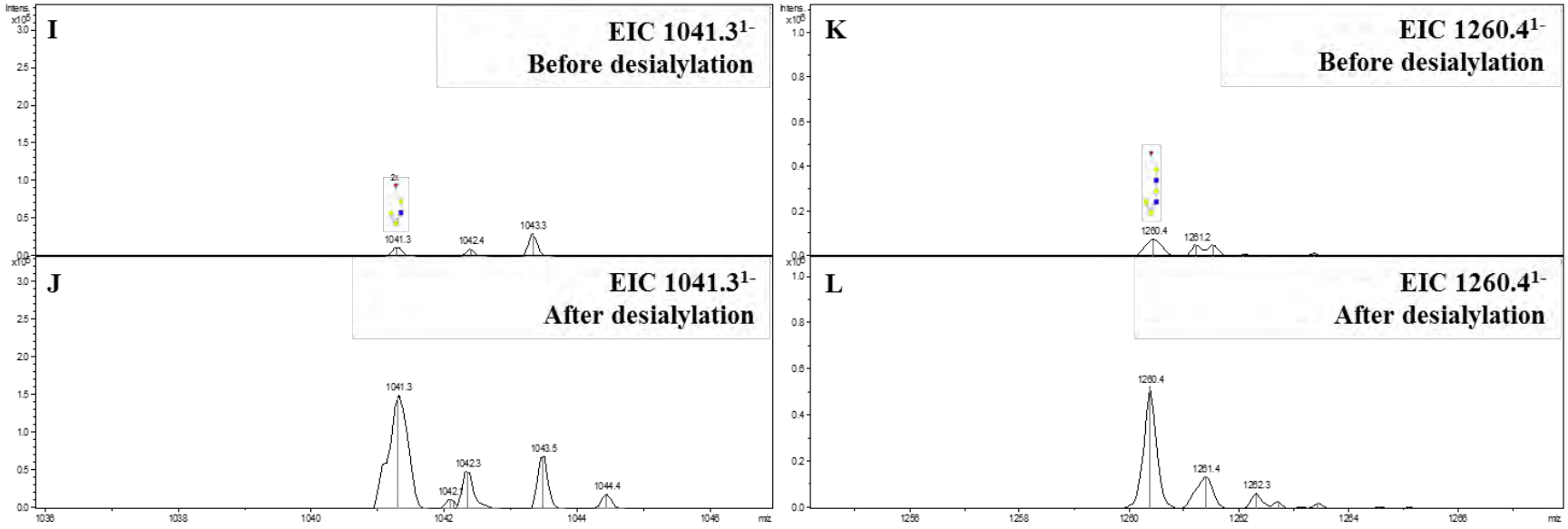
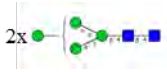
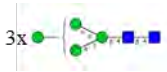
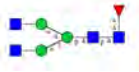
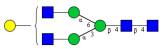
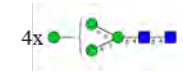
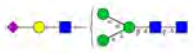
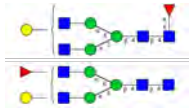
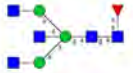
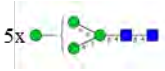
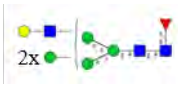
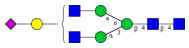
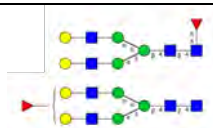
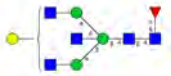


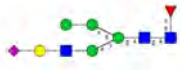
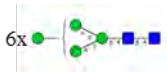
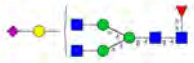
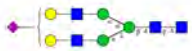
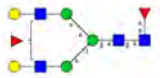
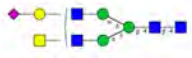
Fig. S1. *N*- and *O*-glycosylation changes of total human milk proteins with neuraminidase treatment analysed by LC-ESI-MS. The removal of terminal sialic acid residues to corresponding *N*- and *O*-linked glycan structures is shown between before and after 16 hours neuraminidase treatment. A - H show that the sialylated *N*-linked glycan structures were all significantly decreased after desialylation with α2-3,6,8 neuraminidase, confirming that these masses were *N*-linked glycans containing sialic acid and not di-fucose residues. However, the di-fucosylated *N*-linked glycan structures *m/z* [+ 0.5 Da] $^{2-}$, such as *m/z* [966.3]$^{2-}$ was increased. Similarly, for the *O*-linked glycans, I - L show two fucosylated core 2 structures were increased after desialylation *m/z* [1041.3] $^{2-}$ and 1260.4] $^{2-}$ indicating they were indeed di-fucosylated *O*-linked glycans and probably are the products of the same structure with an additional sialic acid, such as ~~as~~ *m/z* [1332.3]$^{1-}$ and *m/z* [1697.5]$^{1-}$, respectively (Figure 3.7). Abbreviation for EIC; extracted ion chromatogram.

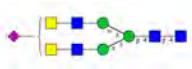
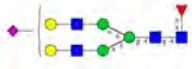
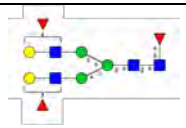
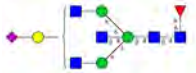
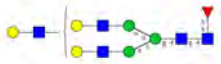
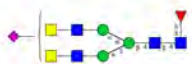
Table S4. Comparison of *N*-glycosylation profiles between bovine and human milk whey protein and surface proteins of HT-29 cells. All structures are grouped according to their increasing calculated experimental mass with the numbering corresponding to those structures (S/No) found in total milk (Supplementary Data, Table S3.). Eleven structures (A1 - A11) were not found in the total milk analysis described in Chapter 3. The compositions of the possible *N*-linked glycans were obtained from GlycoMod (http://www.expasy.ch/tools/glycomod/). Only the most abundant glycans were analysed in this study for their relative abundances. The proposed structures were compared with reported glycans on bovine and human milk whey glycoproteins and HT-29 cell membrane proteins from the literature (see footnote).

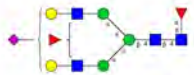
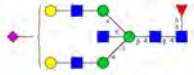
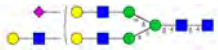
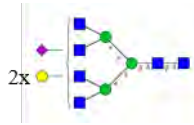
S/ No.	Calculated experimental mass $[M-H]^{1-}$	Theoretical mass $[M-H]^{1-}$	Proposed *N*-linked glycan structure from this study	Relative abundance on bovine milk whey proteins (%)	Relative abundance on human milk whey proteins (%)	Relative abundance on HT-29 cell membrane proteins (%)
N 2	1235.5	1235.4		Not detected[2]	Not detected[2]	Trace
N 3	1397.6	1397.5		1.62	0.91[1]	3.57
*A 1	1463.6	1463.5		1.08[2]	0.41	0.38[4]
N 4	1479.6	1479.5		2.39[2]	Trace[2]	Not detected
N 5	1520.6	1520.6		Not detected	1.61[1]	Not detected

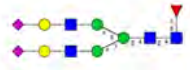
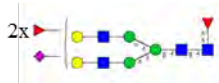
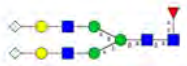
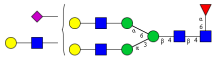
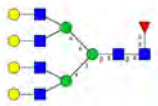
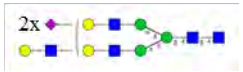
S/ No.	Calculated experimental mass $[M-H]^{1-}$	Theoretical mass $[M-H]^{1-}$	Proposed *N*-linked glycan structure from this study	Relative abundance on bovine milk whey proteins (%)	Relative abundance on human milk whey proteins (%)	Relative abundance on HT-29 cell membrane proteins (%)
N 6	1559.6	1559.5		1.40[2]	0.50[1,2]	8.21[4]
N 7	1567.6	1567.5		Not detected	0.38	0.54
N 8	1625.6	1625.6	 Possible Le	3.16[2]	1.90[2]	0.08
N 9	1641.8	1641.6		7.75[2]	1.06[1,2]	0.41
N 10	1666.8	1666.6		0.65	5.22[2]	0.34[4]
N 11	1682.8	1682.6		1.98[2]	0.54[1]	Not detected
N 12	1721.8	1721.6		6.28[2]	1.33[2]	20.51

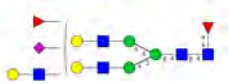
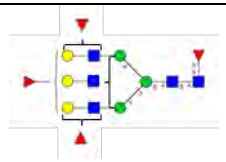
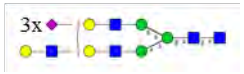
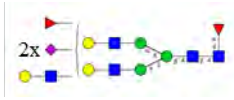
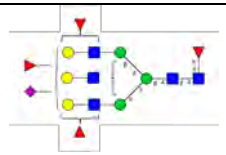
S/ No.	Calculated experimental mass $[M-H]^{1-}$	Theoretical mass $[M-H]^{1-}$	Proposed *N*-linked glycan structure from this study	Relative abundance on bovine milk whey proteins (%)	Relative abundance on human milk whey proteins (%)	Relative abundance on HT-29 cell membrane proteins (%)
*A 2	1723.8	1723.6		9.77[2]	Not detected	Not detected
N 13	1729.8	1729.6		1.98[2]	0.84[2]	1.98
*A 3	1746.6	1746.6		Not detected	Not detected	1.42
N 14	1770.8	1770.6		1.89[2,4]	2.76	Not detected
N 15	1787.8	1787.6	 Possible Le	7.98[2]	7.85[1,2]	2.32
N 16	1828.6	1828.7		2.97[2]	3.03[1]	Not detected[2]

S/ No.	Calculated experimental mass $[M-H]^{1-}$	Theoretical mass $[M-H]^{1-}$	Proposed *N*-linked glycan structure from this study	Relative abundance on bovine milk whey proteins (%)	Relative abundance on human milk whey proteins (%)	Relative abundance on HT-29 cell membrane proteins (%)
N 17	1875.8	1875.7		Not detected	0.49[2]	0.92
N 18	1883.8	1883.6		4.04[2]	2.12[2]	22.17
N 19	1891.8	1891.7		2.58[2,3]	0.88[2]	4.17
N 20	1916.8	1916.7		1.03[2]	4.97	Not detected
N 21	1932.6	1932.7		6.17[2,3]	7.71[1,2]	3.17
N 22	1933.8	1933.7	 Le	0.26[2]	0.97[1,2]	2.05
*A 4	1973.8	1973.7		6.54[2,3]	Not detected	Not detected

S/ No.	Calculated experimental mass $[M-H]^{1-}$	Theoretical mass $[M-H]^{1-}$	Proposed *N*-linked glycan structure from this study	Relative abundance on bovine milk whey proteins (%)	Relative abundance on human milk whey proteins (%)	Relative abundance on HT-29 cell membrane proteins (%)
*A 5	2015.0	2014.7		5.81[1]	Not detected	Not detected
*A 6	2045.8	2045.7		1.53	Not detected	3.12
N 24	2078.8	2078.7		3.04[2,3]	26.08[1,2,3]	5.64[4]
N 25	2080.0	2079.8	 Possible Le	Not detected[2]	Not detected[2]	3.35
N 26	2120.0	2119.8		8.39[2,3]	1.15[2]	0.17
N 27	2152.8	2152.8		Not detected[2]	Not detected	0.28
*A 7	2161.0	2160.8		6.00[2,3]	Not detected	Not detected

S/ No.	Calculated experimental mass $[M-H]^{1-}$	Theoretical mass $[M-H]^{1-}$	Proposed *N*-linked glycan structure from this study	Relative abundance on bovine milk whey proteins (%)	Relative abundance on human milk whey proteins (%)	Relative abundance on HT-29 cell membrane proteins (%)
N 28	2224.0	2223.8		0.93[1]	7.39[1,2]	1.81
N 29	2225.0	2224.8	 Le/possible SLe	Not detected	11.43[1,2,3]	8.30[4]
*A 8	2257.0	2256.8		0.14[2]	Not detected	Not detected
N 30	2282.0	2281.8		0.35	0.21	Not detected[4]
N 31	2298.0	2297.8		0.45[2,3]	Not detected	0.51
*A 9	2338.8	2338.8		0.39	Not detected	Not detected

S/ No.	Calculated experimental mass $[M-H]^{1-}$	Theoretical mass $[M-H]^{1-}$	Proposed *N*-linked glycan structure from this study	Relative abundance on bovine milk whey proteins (%)	Relative abundance on human milk whey proteins (%)	Relative abundance on HT-29 cell membrane proteins (%)
N 33	2370.0	2369.8		0.40	7.07[1,3]	2.14
N 34	2370.8	2370.9	 Possible Le/SLe	Not detected	Not detected[2]	0.79
*A 10	2402.0	2401.8		0.51[2]	Not detected	Not detected
N 35	2444.0	2443.9		0.54[3]	0.82	0.40
*A 11	2518.0	2517.9		Not detected	Not detected	0.22[4]
N 37	2588.8	2588.9		Not detected	Not detected	0.07

S/ No.	Calculated experimental mass $[M-H]^{1-}$	Theoretical mass $[M-H]^{1-}$	Proposed *N*-linked glycan structure from this study	Relative abundance on bovine milk whey proteins (%)	Relative abundance on human milk whey proteins (%)	Relative abundance on HT-29 cell membrane proteins (%)
N 38	2590.0	2589.9	 Possible Le/SLe	Not detected	0.37	0.50
N 39	2591.0	2591.0	 Possible Le	Not detected	Not detected	0.27
N 41	2879.8	2880.0		Not detected	Not detected	Trace
N 42	2881.0	2881.0	 Possible Le/SLe	Not detected	Not detected[3]	0.17
N 43	2882.0	2882.1	 Possible Le/SLe	Not detected	Not detected[2,3]	0.05

Abbreviations: [1]Glycan structures detected on *N*-linked glycoproteins of human milk whey reported by Dallas *et al.* 2011; [2]glycan structures detected on *N*-linked glycoproteins of bovine and/or human milk whey reported by Nwosu *et al.* 2012; [3]glycan structures detected on *N*-linked glycoproteins of

bovine or human milk fat globule membrane glycoproteins reported by Wilson *et al.* 2008; [4]glycan structures detected on *N*-linked glycoproteins of HT-29 cell reported by Vercoutter-Edouart *et al.* 2008. The *denotes new entry of a *N*-linked glycoprotein structure not seen in total human milk glycan analysis (Chapter 3).