The composition and dynamic nature of the N-linked glycoprofile of bovine milk serum and its individual proteins
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Chapter 9

Summary and discussion
Summary and Discussion

Introduction
Protein glycosylation is a modification of a protein with carbohydrate moieties, also known as glycans. These glycans can be synthesized from a number of monosaccharides, creating a diverse array of possible final structures. The glycan structure composition is known to vary between organisms (e.g. humans and cows), as well as in certain disease states and (to a limited extent) diet. The diversity of glycans also brings additional functionalities to the protein structure they are attached to, as the glycan moieties can interact with receptors and microorganisms. The glycans attached to proteins can also be a source of nutrition, both for the host and for the microorganisms in the gut. Many animal and plant products are rich sources of glycoproteins. In this work, we focus on the glycoproteins that are present in bovine milk. Bovine milk, next to being consumed directly, is processed into many dairy products, including infant formulas.

The functions of the glycan structures on whey glycoproteins have not been fully elucidated yet. In addition, it is unknown what factors (such as breed, genetics, stage of lactation) influence the final outcome of the glycans expressed on milk glycoproteins. Most of the N-linked glycosylated proteins of milk are present in the milk serum. This serum is the fluid that remains after removal of the caseins and is also commonly referred to as milk whey. The most common ways of producing whey are by coagulation of the casein proteins by enzymatic digestion with rennet (results in sweet whey), or by acidification (results in acid whey). Sweet whey contains more protein (~8 g/L), due to the presence of casein fragments, than acid whey (~5 g/L) where these fragments are not present. In this work, acid whey was used.

In order to answer the research questions, the glycans present on glycoproteins had to be visualized. For investigation of the functional properties of the glycans present on glycoproteins, proteins with different glycoprofiles, as well as their glycans were isolated in large quantities. Suitable methods for their isolation and characterization thus were developed, and applied to analysis of milk samples and proteins from different sources.

Variations occurring in N-linked glycosylation of Lactoferrin (Chapter 2)
Mammals produce an extra rich milk during the first days after parturition, also known as colostrum. Both human and bovine colostrum contain higher amounts of fat and protein, intended for the growth and development of the newborn. It is also very rich in bioactive glycosylated proteins, such as lactoferrin (LF) and immunoglobulins. Bovine LF is a protein with a known modulatory effect
on immunity and anti-pathogenic properties and is frequently added to human infant formulas. It has been demonstrated in both humans and cows that whey protein glycosylation, including LF, varies during the short colostrum phase. Early colostrum contains higher degrees of protein sialylation, as well as fucosylation. While a higher fucosylation and sialylation had been described for bovine LF, as well as differences occurring in its oligomannose profile, the exact changes in glycan structures were not known. In addition, the LF glycoprofiles after the brief colostrum period, and at the end of the lactation period, were not known. By applying a down-scaled protocol for the isolation of LF, in combination with optimized glycan labeling, sample cleanup and chromatography protocols, we were able to analyze a relatively large number of milk samples over two subsequent lactation periods of eight mixed-breed cows of an organically managed farm (Chapter 2). In addition, 10 commercial samples of LF powders were analyzed and their glycoprofiles compared. By combining mass spectrometry analysis with exoglycosidase assay protocols, we were able to perform a detailed structural analysis and identified the individual glycans present in the glycoprofile of LF. Using the unraveled glycoprofile, we were able to observe the changes occurring in the different LF samples, from colostrum, pre-dry period and mature milk, and in the commercial LF powders (Chapter 2). LF glycoprofiles of mature milk (1- and 3-months post-partum) and of commercial powders were highly similar in nature, despite differences in breed. This suggests that the glycosylation of LF is very tightly regulated and conserved. The highly stable glycoprofile of LF in mature milk between cows allowed for the calculation of a general distribution pattern of the oligomannoses of LF, where Man-9 was found to contribute 37.2% of the total glycans. This was used for quantifying LF and other proteins coeluting in the same chromatogram, using LF as an analytical standard (Chapter 7). In colostrum, rapid shifts were observed in the LF oligomannose profile over time. Smaller oligomannose structures (e.g. Man-5 and Man-6) were present in higher concentrations in early colostrum samples than in mature milk. In contrast, the largest oligomannose structure (Man-9) was present in lower concentrations in early colostrum samples. Within 72 hours, the LF glycoprofile developed into that observed in mature milk, where Man-9 is the dominant LF glycan. We also observed a higher degree of sialylation and fucosylation in early colostrum samples, decreasing over time. In this study, we also reported the presence of α(2,3) linked sialic acid in the earliest colostrum samples of some cows. Milk analyzed from the pre-dry period differed from mature milk in more resembling a late colostrum glycoprofile (Chapter 2). Changes in glycosylation thus may occur beyond the colostrum period, possibly to aid in the recovery of the mammary gland, or in anticipation of the newborn calf. Information about the effect of the glycoprofile on the function of the protein, or the effect of glycans in isolation, is currently lacking. In order to obtain such information, studies towards glycan functionality were performed (Chapters 4 and 5).
Obtaining pure $N$-linked glycans for functional studies (Chapter 3)

Glycans for evaluation of structure-function relationships should be highly pure to avoid that impurities influence the outcome of the subsequent studies. For functional analysis a few mg of pure glycans is required at the minimum. Considering that most glycoproteins contain $<10\%$ of glycan per weight, this required isolation of the glycans from a large-scale digest ($\sim500$ mg to 1 gram of protein, resulting in a digest of a few 100 mL). Our target protein LF contains approximately $7\%$ glycans per weight, of which the glycan portion is exclusively comprised of $N$-linked glycans. These $N$-linked glycans have a universal core of $\text{GlcNAc}_2\text{Man}_3$ and therefore can be released enzymatically by a single enzyme, most commonly peptide-$N$-glycosidase F (PNGase F). Glycan release by PNGase F works optimally with denatured and sodium dodecyl sulfate (SDS) linearized proteins. SDS inhibits PNGase F activity, and the detergent Nonidet P-40 was added to counteract the effects of SDS in the mixture. The amphiphilic nature of detergents like SDS and Nonidet P-40, makes them notoriously difficult to remove completely from complex samples. Removal of detergents from complex protein-carbohydrate mixtures, like the obtained PNGase F digests, was typically achieved by acetone precipitation. In this step, glycans and proteins are precipitated with acetone, and the detergent containing supernatant is removed. In this work we demonstrated that glycan precipitation using the typical 20:80 water:acetone ratio resulted in a selective loss of the smaller glycan structures in the mixture. For our target protein LF, with oligomannoses Man-5 to Man-9 as main glycan structures, this resulted in an observed loss of more than $>50\%$ of Man-5, while Man-9 was recovered completely. In precipitation experiments with Man-5 and Man-9 standards, we quantified that Man-5 recovery was typically $25\%$, while Man-9 recovery was much higher at $\sim80\%$. This effect was not limited to LF, but also was observed for other proteins with smaller glycans, such as Ribonuclease B, porcine Thyroglobulin and human LF. Conserving the complete glycoprofile is of utmost importance for a proper functional analysis. As an alternative, a method for detergent removal by a polystyrene resin was developed and applied. Following detergent removal, the glycans were further purified by a sequence of solid phase extraction steps. Using this optimized protocol, large-scale quantitative isolation of $N$-glycan structures was achieved from all mammalian glycoproteins tested. This protocol yielded fully purified glycans ($103\%$), with a final glycan recovery from the initial protein of $85\%$ while conserving the full range of glycans. In Chapter 2, the dynamic variation in LF glycosylation in colostrum was described, but little is known about the functional implications of this variation. The isolated glycans allowed investigation of the functional properties of LF glycans and the effect of glycoprofile alterations. (Chapters 4 and 5).
Summary and Discussion

The effect of LF and glycans on pattern recognition receptors of the immune system (Chapters 4 and 5)

In order to investigate the functional properties of the glycans of LF, and the effect of variations in the LF glycoprofile, assays were performed with pattern recognition receptors (PRRs) which are important immune signaling molecules on a variety of immune cells. For this purpose, pools of modified LF proteins were created. LF was treated with a mannosidase, trimming the oligomannose structures to create a profile containing shorter oligomannose structures than native LF and reducing the total mannose content by 25%. In addition, LF was treated with a sialidase, resulting in a protein with > 90% of the sialic acid removed. In addition to the modified proteins, glycans were removed and purified (Chapter 3) from all three pools (unmodified, mannosidase treated and sialidase treated) in order to study the effect of the glycans in isolation (Chapter 4 and 5). For the experiments, reporter cell lines were used that expressed Toll-like receptors (TLRs). The applied reporter cell lines were human acute monocytic leukemia-1 (THP1) and human embryonic kidney (HEK) cells. The THP1 cells express most TLRs. Stimulation of TLR in this cell line results in activation of the NF-κ B pathway. This activation of TLR dependent NF-κ B activation is MyD88 dependent. Therefore, a MyD88 truncated (necessary for TLR signaling) THP1 cell line was used to test whether the observed effects were TLR dependent. LF increased the release of NF-κ B significantly (0.0044 to 0.86, \( P < 0.01 \)) and was dependent of the Myd88 pathway, proving a TLR mediated response. LF with modified glycoprofiles had a different effect than the LF with the native glycoprofile. LF containing shorter oligomannoses had a two-fold larger effect than LF containing the native profile. De-sialylated LF had a significantly (\( P < 0.01 \)) reduced effect, demonstrating that the sialic acid of LF is important for receptor interaction. LF in colostrum contains a larger quantity of shorter oligomannoses (Man-5-6) and sialylated glycans. The results described in Chapter 4 suggest that these modifications both increase TLR signaling, thereby enhancing the immune response. Isolated glycans did not have an effect on the THP1 cells, indicating that the protein backbone is essential for the receptor activation. With the HEK cells expressing individual TLRs (TLR-2, TLR-3, TLR-4, TLR-5, TLR-7, TLR-8 and TLR-9), the TLRs responsible for the observed effects could be identified. TLR-3 is strongly (~10-fold) inhibited by LF, independent of the glycan composition. TLR-4 is activated 1.67-fold by LF with a native profile. LF with reduced mannose (a higher number of smaller oligomannoses) stimulates even more strongly (2.9-fold activation, \( P < 0.01 \)), while a de-sialylated profile has a lower, but still enhanced, stimulatory effect (1.4-fold activation, \( P < 0.001 \)) compared to the native profile. The isolated glycans did not have an effect on TLR-3 and TLR-4. This confirms the observation with THP1 cells that TLR-3 and TLR-4 activation is protein backbone dependent. However, from
the different responses obtained from LF with altered glycoprofiles, it can be extrapolated that the glycans can modify TLR response. In contrast to TLR-3 and TLR-4, LF protein did not have an effect on TLR-8, but instead the isolated glycans had a significant inhibitory effect. The observed inhibition (2.12-fold, \( P < 0.05 \)) was independent of any glycoprofile modifications. Since TLR-8 is a receptor implicated in autoimmune disease, the inhibitory effect of glycans can potentially be exploited therapeutically.

The inhibitory (antagonistic) effect of glycans on TLR-8 was investigated in more detail in Chapter 5. The effect of isolated glycans (native, mannosidase treated and sialidase treated) on TLR-8 activation was tested in two cell types, reporter HEK cells expressing individual TLRs and a primary cell, i.e. human monocyte-derived dendritic cells (MoDCs). The effect was compared with the immune suppressive compound chloroquine (CQN), which is a known TLR-8 antagonist. Two separate agonists, were used, R848 and ssRNA40 on which CQN is known to display different inhibitory effects. These differences are due to different mechanisms of TLR-8 inhibition of CQN, either by acidification of the endosome, or by direct interaction of CQN with the TLR ligands or TLR-8 configuration changes. Since in the HEK cell experiments, de-sialylated glycans inhibited TLR-8 similar to mannosidase treated and native glycans, the inhibitory effect could not be attributed to acidification of the endosome. Similarly, glycans cannot interact directly with ssRNA40 (like CQN does), therefore eliminating this mode of action for glycans. Therefore, it was concluded that glycans must interact directly with TLR-8 for its antagonistic effect. In the experiments with MoDCs, glycans were found to reduce the secretion of IL-6, but not IL-10 and TNF-\( \alpha \), which are reduced by CQN (in addition to IL-6). This effect was stronger when modified glycans (de-sialylated or reduced mannose) were used compared to the native glycans. The three pools of glycans did not differ in their inhibition of TLR-8 ssRNA40 induced activation, however modified glycan pools did induce a stronger inhibitory effect (compared to native glycans) on IL-6 secretion in MoDCs. This different effect observed in MoDCs is likely induced by other receptors than TLR-8. These results show that glycan composition can influence the immune response differently, even when isolated glycans are used instead of a glycoprotein complex. The anti-inflammatory characteristics of glycans are an interesting target for future studies, as glycosylated proteins are present in many food products. Adding glycans or glycoproteins with selected glycoprofiles to food products can therefore potentially increase the anti-inflammatory properties of the diet. While the whey protein LF is frequently added to infant formulas, complete whey and whey powders are also frequently processed into other food products, without isolation of their individual proteins. It is thus also important to consider the effects of (variations in) the overall whey glycoprofile.
Summary and Discussion

Analysis of the total whey glycoprofile (Chapters 6 and 7)

In order to study the overall N-linked glycoprofile of bovine whey and potential variations thereof, we developed a rapid analysis protocol. In this protocol, lactose that interferes with downstream glycan labeling, is removed by a protein precipitation step with ammonium acetate in a methanol solution (Chapters 6 and 7). The whey proteins were recovered completely and subsequently dissolved in digestion buffer (Chapter 7). After overnight digestion, a direct in-solution glycan labeling was applied, followed by a cellulose solid-phase extraction cleanup step (Chapters 6 and 7). The labeled glycans were then separated by applying a slowly sloping gradient in a hydrophilic interaction chromatography (HILIC) step and detected by fluorescent spectroscopy. A typical overall whey glycoprofile analysis obtained by this approach yields a complex HPLC chromatogram, which at first glance is difficult to interpret. Multiple (N-glycosylated) proteins are present in whey, and the overall whey glycoprofile thus represents the glycoprofiles of the mixture of proteins present. In many cases, the co-elution of multiple glycan structures creates combined peaks in the chromatogram, which were defined as peak clusters. Detailed information about the glycoprofiles of the individual protein is often lacking. The overall glycoprofile was analyzed by high-resolution mass spectrometry, which allowed for structural analysis of the many different glycan structures. By combining the mass spectrometry analysis with exoglycosidase assays, the structures in the complex chromatogram could be appointed to the different peaks present (Chapter 6). The overall whey glycoprofile was further deconstructed by analysis of the known major N-glycosylated proteins in the whey. Individual glycoprofiles of the proteins IgG, LF, Lactoperoxidase, α-Lactalbumin and Glycosylation-dependent cell adhesion molecule-1 (GlyCAM-1) were generated and their glycan structures analyzed. Signature glycan structures unique for the respective proteins were used as “fingerprints” to identify them in the overall whey glycoprofile. For example, LF mainly contributes oligomannose-type glycans to the overall whey glycoprofile, while IgG carries fucosylated di-antennary glycans with Gal(β1-4)GlcNAc (LacNAc) motifs. GlyCAM-1 is the only major whey glycoprotein contributing sialylated tri- and tetra-antennary structures (Chapter 6). While the individual glycan fingerprints allowed for identification of the protein of origin in the overall whey glycoprofile, it is also important to consider their relative concentration in the whey. By analysis of a mixture of the individual glycoproteins at typical native whey concentrations, the contribution of each protein to the overall whey glycoprofile could be estimated. This identified the IgG, LF and GlyCAM-1 proteins as the major N-glycosylated whey proteins, whereas the contributions of lactoperoxidase and α-lactalbumin were very minor (< 5% of total). Of the three major proteins, GlyCAM-1 contributes the vast majority of glycans to the overall whey glycoprofile (~75%, Chapter 8); the contribution of glycans from the other glycoproteins in the whey (LF, IgG) is significantly less (~25% Chapter 8).
Whey protein quantitation and the high concentration of GlyCAM-1 (Chapters 6 and 7)

The whey analysis method lends itself for in-depth analysis of a relatively large number (50-100) of milk samples in a short amount of time (1 week). We demonstrated a high repeatability of the method, with a coefficient of variation CV ≤ 5% of the generated chromatograms. The highly repeatable nature of the whey analysis method potentially allowed its application for qualitative glycoprofile screening and quantitation of its three major glycoproteins. By applying the glycoprofile analysis method to commercial whey protein powders, differences between whey protein concentrate (WPC), whey protein isolate (WPI) and demineralized whey were visualized. While LF and IgG were present in WPC and demineralized whey, their concentrations were much decreased in WPI, making GlyCAM-1 a relatively strong contributor to the overall glycoprofile in WPI (Chapter 7). For evaluation of protein concentration determination by glycoprofile analysis, 100 individual pooled tank milk samples were analyzed. After integration of the obtained glycoprofile chromatograms, 32 individual peak clusters were compared to ELISA determined immunoglobulin A, M and G, and LF concentrations (Chapter 7). Pearson correlations between the individual glycan clusters and the ELISA determined protein concentrations were calculated. Peak clusters with signature glycans indicative for LF and IgG correlated significantly with the ELISA determined protein concentrations, confirming a direct relationship between protein concentrations and the obtained glycoprofiles. For quantification of the glycoprotein concentration from the glycoprofile, a reference glycan was required with minimal interference from co-eluting structures. Analysis of the overall whey glycoprofile for the Man-9 signature glycan of LF allowed determination of the LF whey protein concentration in relation to a calibration curve of LF protein (Chapter 7). Following calculation of the molar quantity of the Man-9 glycan, the quantity of other peaks in the chromatogram could be determined. By quantifying signature glycans of IgG and GlyCAM-1, and considering their individual glycoprofiles and glycan occupancy, the whey concentrations of the IgG and GlyCAM-1 proteins also were estimated (Chapter 7). Finally, the determined concentrations of LF, IgG and GlyCAM-1 were compared with the ELISA determined (LF and IgG) and literature (GlyCAM-1) values. The IgG protein concentrations determined by glycoprofile analysis were in line with their ELISA determination, and with what is currently the acceptable range from literature, indicating that the glycoprofile analysis is a viable approach. The concentrations of LF determined from the glycoprofiles were higher than from ELISA analysis. This difference can be explained by considering that only native and not denatured LF is detected by ELISA, while the glycoprofile analysis does not discriminate between the two allows determination of total LF. Also, the
concentrations of GlyCAM-1 determined from glycoprofile analysis were much higher (> 1 g/L) than the values commonly reported in literature (0.3 g/L). The concentration of GlyCAM-1 in whey thus is likely higher than previously assumed, making GlyCAM-1 in fact one of the most abundant proteins in whey and in whey protein powders. The concentration and glycosylation of GlyCAM-1 was also followed over time in colostrum (Chapter 6). We observed relatively high concentrations of LF and IgG in the earliest collected colostrum sample, with a rapid decrease over the three-day colostrum period. The concentration of GlyCAM-1 was higher in colostrum samples, but not upregulated in a similarly strong fashion as LF and IgG; the GlyCAM-1 concentration decreased over time, reaching the lower levels typically observed in mature milk samples. We also observed changes in the glycosylation profile of GlyCAM-1, which in contrast to LF (Chapter 3), was not yet stable at 1 month. In some cows, changes in the GlyCAM-1 glycoprofile were also observed between 1-3 months post-partum milk. The regulation of the glycosylation of GlyCAM-1 thus is not as strict as for LF. This may allow modifications in the glycosylation of GlyCAM-1 by non-genetic effects, such as diet.

GlyCAM-1 glycosylation differs between cows, breeds and possibly diet (Chapter 8)

By applying the whey analysis method to two individual dietary intervention studies using Holstein-Friesian cows, the effect of diet as well as inter- and intra-cow variation on whey protein glycosylation was studied (Chapter 8). Two separate diet focused studies were evaluated for variations in the whey protein glycoprofile. In the first (Study A, 56 cows), rumen protected fat and protein was supplemented into the diet, while in the second (Study B, 6 cows) abomasal infusions of glucose, amino acids and fatty acids and combinations thereof, were performed. By combination of both studies, one of the main goals of the study described in this thesis, namely to investigate whether variations can occur in milk protein glycosylation, and if so, what underlies this variation. In order to quantify any observed changes in the glycoprofile, either due to changes in protein concentration or in glycosylation, the chromatograms were divided into 32 separate peak clusters, with structural assignment based on the information obtained in Chapter 6. The peak clusters were classified as originating primarily from GlyCAM-1, LF or IgG, and the percentage of GlyCAM-1 glycans against the total pool of N-glycans present in bovine milk serum was estimated. In addition, peak clusters containing fucosylated and/or sialylated structures were identified, and used to calculate the percentage of sialylated and/or fucosylated glycans in the total glycan pool. The majority of the fucosylated and sialylated glycans originated from GlyCAM-1. We observed that the degree of sialylation (59.7%), fucosylation (58.4%) and the proportion of GlyCAM-1 glycans (78%) remained relatively
constant during both Study A (dietary protein and fat) and Study B (metabolic substrate infusions). The highest variation was observed in the degree of fucosylation. With the exception of milk yield and days in milk affecting the total whey protein concentration and thus the total peak area of the obtained glycoprofiles, no significant effect of parity, dry matter intake, milk yield or days in milk could be observed for the level of sialylation and fucosylation. Evaluation of the glycoprofile chromatograms of Study A (dietary protein and fat) showed relatively large variations between the profiles of individual cows (inter-cow variation). Chromatograms could be classified based on their degree of fucosylation, ranging from (44-73%). Dietary supplementation of protein and fat did not induce changes in the degree of fucosylation, either as observed from the chromatograms, or by statistical evaluation. However, the results of Study A indicated that there is a large inter-cow variation in the degree of fucosylation of GlyCAM-1. In the samples of Study B (infusion of metabolic substrates) we observed a larger variation occurring over the experimental period, with the degree of fucosylation changing up to ± 18% over time. Individual peaks also varied significantly between samples from one cow. Since the observed variations in individual peaks, as well as in fucosylation levels, were much larger than observed in Study A (protein and fat supplementation, ≤ 4%), the nutrient levels in the small intestine appear to have effects on the observed milk-serum glycoprofile. In Study B, the additional metabolic substrates were added in isolation (e.g. added glucose, amino acids or fatty acids), as opposed to complete dietary fat and protein (Study A). It is possible that the supply of individual metabolic substrates leads to an imbalance inside the mammary cells, thereby affecting the processes underlying the fucosylation of the glycans of GlyCAM-1. We were however unable to significantly attribute the observed changes to any of the specific infusions. The lack of initial grouping by degree of fucosylation (inter-cow effect), the relatively short infusion periods (~1 week) or the low number of cows (n=6) may have hindered the statistical evaluation. However, the results from the infusion study demonstrated that particularly GlyCAM-1 glycosylation may change, most clearly in fucosylation. The observed variation in GlyCAM-1 fucosylation may affect receptor interaction, as observed for the glycoprofiles of lactoferrin (Chapter 4). Core fucosylation is often present on proteins that interact with the immune system via PRRs; GlyCAM-1 therefore also may have an immune modulatory function, which has yet to be discovered. The results obtained in this chapter suggest that inter-cow glycoprofile variation is larger than the changes induced by infusion. GlyCAM-1 fucosylation appears to be relatively flexible, offering possibilities for its manipulation by infusions/diet. Further studies should include variations in complex carbohydrates in the diet, and infusions with different monosaccharides that are directly linked to protein
glycosylation, e.g. fucose, mannose, galactose or N-acetyl-glucosamine. The functional implications of GlyCAM-1 fucosylation and its potential for variation remain to be explored.
**Concluding remarks and future research**

In this work, tools are presented for the rapid and efficient structural analysis of the glycans of milk glycoproteins and their function. The method for producing isolated N-glycans in a high purity allowed for functional analysis studies that were previously not possible. The in-depth analysis of LF glycosylation demonstrated its flexibility in colostrum and its rigidness in mature milk, independent of breed or cow. The oligomannose length, as well as degree of sialylation, affected LF interactions with PRRs. Lactoferrin isolated from colostrum potentially is a valuable addition to infant formulas. In addition, the presented methods allowed in-depth structural analysis of LF in relatively many milk or whey powder samples in a limited amount of time. Enzymatically modified LF glycans and their functional properties were investigated in Chapters 4 and 5. Functional studies with naturally occurring LF with different glycoprofiles remain to be done. This should be relatively straightforward, since LF can be isolated from (early) colostrum in order to obtain LF with increased sialylation, fucosylation and a higher abundance of shorter oligosaccharides. Comparisons can then be made between colostrum LF glycans and mature milk LF glycans.

The described methods can be used for an in-depth analysis of whey, providing information not only on the glycans present, but also about the proteins present and their concentrations (Chapters 6 and 7). This is achieved by using a small aliquot of whey, or protein powder, without isolation of the individual proteins, generating an overall glycoprofile that provides a large amount of detailed information in a single run. A multitude of possible applications can be devised, e.g. analysis of milk samples to study genetic or dietary effects on glycosylation or whey protein composition, or analysis of the effects of processing conditions on whey powder composition. The generated data could also be used to support future functional studies with whey or whey powders, as the relative amounts of glycans with a certain epitope are easily calculated from the chromatogram, allowing analysis of the effects of variations in glycan composition on functional properties.

The results described in this work give a more detailed insight into whey proteins and their glycosylation. Many questions remain unanswered. For example, the bovine immunoglobulin IgA and IgM glycoprofiles remain to be studied. Their contribution to the whey glycoprofile may be modest, as described in this work, but their functional properties remain highly interesting to study.

One of the major findings in this work is that GlyCAM-1 is such a dominant whey protein (> 1 g/L, compared to ~5 g/L total acid whey protein). The major contribution of GlyCAM-1 was surprising, as the generally reported whey concentration is 0.3 g/L, similar to LF and IgG. Current literature towards
GlyCAM-1 discusses its concentration, as well as its location in the milk (free or milk fat globule membrane associated). Information on its function in the milk or its functional properties is lacking. GlyCAM-1 also has an interesting glycoprofile, with a relatively large amount of sialylated and fucosylated glycan structures. Especially the fucosylation of GlyCAM-1 significantly differs between cows, as well as within-cow. Despite this information, there is little known about its exact function. In view of its high concentration in whey, it should be possible to generate a significant quantity of this protein for future functional studies. GlyCAM-1 variants with different levels of fucosylation in fact can be isolated, and used to study the effects of this altered fucosylation on, for example, the immune system. In Chapter 5 we show that glycans of LF inhibit TLR-8 and potentially also other PRRs to decrease cytokine production; it will be interesting to evaluate the functional properties of isolated glycans from GlyCAM-1 as well. GlyCAM-1 remains intact upon heating, whereas other proteins (LF+IgG) denature, therefore it is relatively simple to extract and produce GlyCAM-1. Isolation of glycans from heated whey by the method described in Chapter 3, will provide pure glycans from GlyCAM-1 for future functional studies. GlyCAM-1 and its isolated glycans may have therapeutic potential for autoimmune disorders.
Main findings of this thesis:

Chapter 1
- The literature research showed that there is a need for rapid and efficient methods for isolation and characterization (structural and functional) of bovine milk glycoproteins.
- It was unknown whether any kind of variation occurred in the glycosylation profile of the main milk serum glycoproteins over the course of a full lactation period.
- In case variations do occur, the inducing factors (lactation stage, diet, etc.) remained to be studied.

Chapter 2
- The glycoprofile of lactoferrin in colostrum contained more sialic acid and fucose and a higher amount of shorter oligomannoses than in mature milk.
- Glycosylation of lactoferrin is highly stable between cows (outside of the colostrum and pre-dry period) and in commercial lactoferrin powders. This indicates a strict regulation of the glycosylation of this protein.

Chapter 3
- A protocol was developed for the isolation of N-linked glycans of glycoproteins, conserving the complete glycoprofile.
- Precipitation of N-glycans in 80% acetone is not suitable for glycoproteins that contain a glycoprofile with short length glycans, as shorted glycans are not fully precipitated. Instead, a detergent binding resin should be used for detergent removal.

Chapter 4
- The pattern of glycans present in a glycoprofile modulates Toll-like receptor signaling, as demonstrated by significantly altered activation or inhibition by lactoferrin with modified glycoprofiles (desialylated or reduced mannose).
- Isolated lactoferrin glycans have an antagonistic effect, but only on TLR-8.

Chapter 5
- Isolated lactoferrin glycans affect TLR-8 signaling by a direct interaction with the receptor.
- Isolated lactoferrin glycans varying in profile composition did not induce different effects on TLR-8 in reporter cell lines, but a different response was seen in the dendritic cell line experiments. This may indicate that there are other (currently unknown) pattern recognition receptors that interact with these isolated glycans.
Chapter 6
- The three major N-linked glycosylated proteins in bovine milk serum were identified as immunoglobulin G, lactoferrin and GlyCAM-1. Each of these proteins contained a unique pattern of glycans by which they can be recognized in the overall whey glycoprofile.
- GlyCAM-1 glycans provide the highest contribution to the overall N-linked glycoprofile. Its profile somewhat varied in mature milk, compared to the stable profile of lactoferrin in the same samples. Regulation of GlyCAM-1 glycoprofile thus appears less strict than that of lactoferrin.

Chapter 7
- The developed isolation and analysis methods of bovine whey proteins is rapid and highly repeatable and lend themself for multiple applications, including whey protein concentration estimations by glycoprofile analysis, and whey powder analysis.
- GlyCAM-1 appears to have a much higher concentration in bovine whey than previously assumed. Of the three major glycoproteins, GlyCAM-1 is the dominant protein in whey powders.
- Different processing steps used in whey powder production also affected their (glycosylated) protein composition.

Chapter 8
- A large variation in the degree of fucosylation of GlyCAM-1 between cows was observed. Further research towards the (genetic) source of this variation is needed.
- Diet does not have a large (significant) effect on fucosylation of GlyCAM-1, but fluctuations within-cow were observed during abomasal infusions with different metabolites. This demonstrated that fucosylation of GlyCAM-1 is flexible to at least some extent.
- Current information on the GlyCAM-1 protein is rather limited. Its high concentrations in milk and whey powders, combined with its large contribution to the N-linked glycoprofile of milk, stimulates further research of this protein.