*Invited lecture/Review*

# Glycosylation Research in Bovines-the Significance and Recent Updates

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**Abstract:**
Glycosylation is an enzymatic process of attaching carbohydrate chains, glycans, to biomolecules, thereby influencing their biological features. Understanding the glycosylation patterns and mechanisms in bovines (*Bos taurus*) has the potential to bring improvements in various fields, aspects such as reproduction, herd health management, and the quality and safety of milk and meat products. The article, starting with a glimpse into glycobiology, will continue with overviewing the previous 5-year achievements of glycosylation in bovines, collated during a recent PubMed search. Hereafter, more details about the four studies will follow as the selected examples and go along with the concluding remarks and general future research directions.

**Keywords:** data mining, glycosylation, bovines

Glycosylation represents the enzymatic formation of a glycoconjugate, which consists of carbohydrate chains, or glycans, covalently linked to different biomolecules such as proteins, lipids, or RNA (Reily et al., 2019; Trbojević Akmačić et al., 2022). Glycobiology is a comprehensive science focusing on glycans, with the research spanning their biological chemistry, biosynthesis, evolution, (patho)physiological roles, analysis, and biotechnology aspects. It initially appeared among natural sciences and rapidly attracted interest from many other fundamental, biomedical, and biotechnology disciplines (Varki et al., 2022).

The article will start with a brief explanation of glycobiology key postulates. It will continue with an insight into the reasons for researching glycosylation in bovines. After, four examples illustrating the relevance will follow. Concluding the article will provide a summary of the current findings' relevance and future research directions.

## 1. A glimpse into glycobiology

Mammals are estimated to possess trillion different branched glycan structures, formed through the combination of 17 monosaccharides with multiple glycosylation sites, numerous enzymes involved, and the potential for $\alpha$ or $\beta$ stereochemical conjugations (Reily et al., 2019). Various biomolecules serve as substrates for glycosylation, resulting in the formation of glycoproteins, proteoglycans, glycosphingolipids, glycosaminoglycans, or glycoRNA. In addition, glycobiology also grants interest for free oligosaccharides, adding further complexity to the field (Trbojević Akmačić et al., 2022).

Macro- and microheterogeneity are crucial concepts in understanding glycobiology. For example, in glycoproteins, multiple glycosylation sites on the polypeptide "backbone" constitute macroheterogeneity, while microheterogeneity indicates the presence of different glycans on the same glycosylation site. Finally, if only the N-glycan supplement distinct glycoproteins, they represent glycoforms (Varki et al., 2022).

In eukaryotes, N- and O-glycans are two major types of glycans. The N-glycans attach via the side chain of an asparagine (Asn) residue within the peptide sequence Asn-X- serine (Ser)/threonine (Thr), where X stands for any amino acid except proline. Their structural core consists of two acetylglucosamines (GlcNAc) conjoined with a mannose structure (Man), which holds two additional mannoses lined at positions 3 and 6 (Varki, Cummings et al., 2022). According to biosynthetic maturation, indicated by the structures extending the core, four glycan types exist: pauci-mannosidic, oligo-mannosidic, complex, and hybrid. In pauci-mannosidic extensions are lacking, while in the oligo-mannosidic, they form only of Man residues. The characteristic of the complex glycans is the presence of up to five antennae, whose structures start with the GlcNAc and continue with the repeating N-acetyllactosamine residues. Hybrid glycans have oligomannose extensions at core position 6, while up to two antennae appear at position 3. More precise biosynthesis phase annotation comes with additional derived traits like antennarity, sialylation, galactosylation, fucosylation (core/antennary), or bisection (de Haan et al., 2022). The attachment sites for O-glycans are Ser or Thr residues. The O-glycans (patho)biological contexts typically diverge into features related to O- N-Acetylgalactosamine (GalNAc) and O-GlcNAc glycans. For O-GalNAc glycans, four core structures are available, each with GalNAc bound on Ser or Thr residue, differing by the presence of Gal and GlcNAc. The structure of O-GlcNAc glycans is less complex because they contain only GlcNAc attached (Varki et al., 2022).

The endoplasmatic reticulum and Golgi complex are intracellular locations of the three-step N-glycosylation process, involving the generation of the lipid-linked oligosaccharide donors, co-translational glycan shifts onto glycosylation sites on polypeptide chain, and further processing of the glycans. Regulation of this comprehensive process relies on modifying the activity of enzymes involved, glycosyltransferases and glycosidases, at the transcriptional or posttranscriptional level (phosphorylation, glycosylation, availability of chaperones). Besides, the availability of their substrates also contributes to the regulation (Esmail et al., 2021).

At the molecular level, N-glycans play a substantial role in proper protein folding, assembling of multimeric proteins, and stabilizing their structures. In the physiological context,

N-glycosylation is involved in a network of comprehensive mechanisms such as cell adhesion, recognition of foreign pro- and eucaryotic cells, or receptor activation. N-glycosylation also attracted the attention of pathophysiology and clinical disciplines. Congenital disorder of glycosylation syndrome Type I, an autosomal recessive multi-system disorder, has a depleted N-glycosylation as the cause. Changes in cell membrane N-glycome are characteristic of malignancy, thus raising the potential for advanced oncological biomarkers discovery. Terminal sialylation and fucosylation usually change, while increased branching relates to growth, infiltration, and metastasis. N-glycome alterations are frequent in chronic diseases. The best example from that field is rheumatoid arthritis (RA), an autoimmune arthritis associated with changes to the galactosylation of Ig G, where the disease severity relates to these changes in antigen-specific IgGs. Research advancements prompt additional exploration into efforts on N-glycosylation as a potential therapeutic target (Esmail et al., 2021).

## 2. What is the importance of researching glycosylation in bovines?

Understanding the glycosylation patterns and mechanisms in bovines (*Bos taurus*) has the potential to bring improvements in various fields, aspects such as reproduction, herd health management, and the quality and safety of milk and meat products (Beletić et al., 2023a). For example, the results of a recent proteomic study in cows with the retained placenta (Beletić et al., 2023b) suggested as potential biomarkers lipopolysaccharide-binding protein and haptoglobin, both of which are glycoproteins (https://www.uniprot.org). A proteomic study of milk from dairy cows with subclinical mastitis (Beletić et al., 2022) indicated that a higher abundance of thrombospondin-1, a glycoprotein (https://www.uniprot.org), could differentiate between samples with *Staphylococcus* spp. and *Streptococcus* spp. as the causative agents.

Recently, we have performed a PubMed search for "glycosylation" and "*B. taurus*" using the following filters: full text available, the publication date of five years, and the preprints excluded. From the 244 initially retrieved results, the content analysis identified 88 as eligible, among which only one was a review article. These studies primarily focused on functional aspects and glycan profiles, with milk and tissues being the most common sample types. Among them, ten studies provided data on the total glycome of milk or tissue samples, while many others analyzed glycosylation of individual proteins, with fetuin being the most frequently studied (Beletić et al., 2023a). Apart from the associated (patho)biological relevance, which is still challenging, bovine fetuin is also worth mentioning in the analytical context as a frequent testing analyte during the development of glycoproteomics analytical approaches (Achim et al., 2023). Milk-related studies brought the data about total glycome, or individual proteins, like casein or IgG (Beletić et al., 2023a), which are of particular relevance in the assessment of milk quality and nutritive value (O'Riordan et al., 2014).

## 3. The recent updates-selected examples

In a recent paper, Dilimulati et al. (2023) presented intriguing findings about the involvement of N-glycosylation in the interaction between sperm and oocyte proteins in bovines. In mammalian species, a layer called zona pelucida (ZP) surrounds oocytes and has essential functions in oogenesis, fertilization, and preimplantation. The ZP structure has long interconnected fibrils harboring ZP glycoproteins (ZPGs), and ZP thickness, protein content, and N-/O-glycosylation represent a species specificity (Wassarman, 2008; Yonezawa, 2014). ZP in bovines contains three ZPG3 annotated bZP2, bZP3, and bZP4. In vitro results identified the N-terminal domain of bZP4 (ZP-N1) and the middle region of bZP4 as substantial for sperm binding but without evidence for N-glycosylation of ZP-N1 as necessary for this purpose (Dilimulati et al., 2022). Further research of the bZP4 middle region (including the hinge domain crucial for the bZP3-bZP4 complex formation, which is essential for sperm binding) showed species-specific sperm binding. Additional analyses associated the bZP4 middle region function with N-glycosylation at Asn-314 and marked N-glycosylation sites at Asn-314 (near the hinge region) and Asn-146 (within the hinge of the bZP3-bZP4 complex) as required for the unhampered sperm binding (Dilimulati et al., 2023). These findings significantly contribute to a better understanding of between-

species differences in ZP functions. Identification of sperm-associated factors responsible for sperm-ZP interactions and validation via in vivo bovine fertilization research represents the primary research efforts to achieve reliability in bovine reproduction practice (Dilimulati et al., 2022).

The review by Zlatina and Galuska (2021) comparatively assessed N-glycosylation traits of the bovine and human lactoferrin (Lf), primarily guided by the fact that the bovine variant often serves as a model for developing innovative therapeutic strategies. Lf is a whey protein present in most mammals. Human Lf (hLf) also occurs in other organs and fluids such as kidneys, lungs, liver, prostate, saliva, plasma, and immune cells, having numerous effects: antimicrobial defense, immunomodulation, antioxidant protection, or microbiome homeostasis (Kowalczyk et al., 2022). The primary structures of hLf and bovine Lf (bLf) have a 69% analogy. Five N-glycosylation sites exist on bLf (two more than hLf). Four sites have 100% occupancy, and the fifth varies between 15% in mature milk and 30% in colostrum. Consequently, two bLf variants appear, differing in glycosylation pattern, molecular mass, and colostrum/mature milk abundance. Structural analyses of bLf and hLf suggested the N-glycans' significance with potential effects on their functionality. Their removal prominently reduces the iron-binding properties, the primary Lf function, and the "weapon" against bacteria. The site-specific presence of N-glycans on bLf is a potential inhibitor of proteases. The large amount of high-mannose N-glycans, rare among extracellular proteins of mammalian species, also differentiates bLf from hLf and might induce a quick clearance of the gastrointestinal tract and bloodstream mediated by mannose-recognizing receptors on macrophages. Another feature of bLf glycome is the presence of N-Glycolylneuraminic acid, a non-human form of sialic acid, which, besides enhancing the clearance of dietary bLf, also links to antigenicity and potential adverse effects on the efficiency of the immune response. Notwithstanding the relevance of the described advances for deciphering the bLf effects in humans, prudence is necessary in their interpretation, originating from the comprehensive network of molecular (like amino acid sequence), physiological (breed, lactation time, or diet), and methodological features (Zlatina et al., 2021).

Fat, casein, and whey represent the three milk components (Haug et al., 2007). According to their amount, the whey proteins belong to highly ($\alpha$-lactalbumin, $\beta$-lactoglobulin, serum albumin, IgG, glycosylation-dependent cellular adhesion molecule 1 (GlyCAM-1), and Lf), moderate (IgA, IgM, lactoperoxidase, and osteopontin) and minor (lysozyme and folate binding protein) abundant group (O'Riordan et al., 2014; Tacoma et al., 2016). Whey proteins are ingredients in numerous food products, including infant formulas. Considering N-glycans' relevance for the glycoprotein functional properties, an assessment of the bovine whey total glycome profile would be significant for predicting the quality and safety of whey-containing food products. Valk-Weeber et al. (2020) analyzed the role of individual glycoproteins in determining the comprehensive N-linked glycoprofile of bovine whey. In accordance with the protein abundance, approximately 95% of *N*-glycans in the total glycome originated from Lf, IgG, and GlyCAM-1. As expected, Lf dominantly participated with oligomannose-type glycans. IgG was the source of fucosylated di-antennary glycans with N-Acetyllactosamine domains. GlyCAM-1 was the only whey glycoprotein carrying highly fucosylated and sialylated tri- and tetra-antennary glycans. Analyzing differences between early and late colostrum and mature milk revealed the complex interactions between the glycoprofile alterations (dominanly higher sialylation and fucosylation degree in early colostrum) and dynamics of glycoproteins concentrations.

Food fraud is a growing general issue in food safety and quality and is particularly common among animal products. Species identification is one of the key tasks in preventing adulteration problems with foods of animal origin (Smaoui et al., 2023). Tai et al. (2023) developed a glycopeptide-based analytical pipeline to identify six meat species: pork, beef, mutton, chicken, duck, and turkey. The first phase of their experiment was the sarcoplasmic proteome analyses using one-dimensional gel electrophoresis, which showed that enolase could differentiate livestock and poultry origin. Applying ultra-high-performance liquid chromatography with quadrupole time-of-flight mass spectrometry confirmed the species specificity of enolase glycoprofiles. The validation procedure, aiming to

identify livestock, poultry, and mixed meat, yielded promising results, which might be even superior to the currently available analytical platforms.

## 4. Conclusions

The pioneer insight provided in this article allowed for the conclusion on the availability of reliable data about glycosylation in the bovines and the incarnated challenges. As such, they are eligible as the starting point for further scientific efforts on their continuous appending, systematization, and multidisciplinary analyses.

**Conflicts of Interest:** The authors declare the following competing interests: G.L. is the founder and owner of Genos Ltd., a private research organization that specializes in high-throughput glycomic analysis and has several patents in this field. A.B., I.D.O., T.P., and J.K. are employees of Genos Ltd.

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