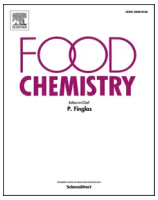
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Utilizing linkage-specific ethyl-esterification approach to perform in-depth analysis of sialylated *N*-glycans present on milk whey glycoproteins

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ABSTRACT

Glycosylation of milk whey proteins, specifically the presence of sialic acid-containing glycan residues, causes functional changes in these proteins. This study aimed to analyze the *N*-glycome of milk whey glycoproteins from various milk sources using a linkage-specific ethyl esterification approach with MALDI-MS (matrix-assisted laser desorption/ionization-mass spectrometry). The results showed that the *N*-glycan profiles of bovine and buffalo whey mostly overlapped. Acetylated *N*-glycans were only detected in donkey milk whey at a rate of 16.06%. a2,6-linked *N*-Acetylneuraminic acid (a2,6-linked NeuAc, E) was found to be the predominant sialylation type in human milk whey (65.16%). The amount of a2,6-linked NeuAc in bovine, buffalo, goat, and donkey whey glycoproteomes was 42.33%, 44.16%, 39.00%, and 34.86%, respectively. The relative abundances of a2,6-linked *N*-Glycolylneuraminic acid (a2,6-linked NeuGc, Ge) in bovine, buffalo, goat, and donkey whey were 7.52%, 5.41%, 28.24%, and 17.31%, respectively. Goat whey exhibited the highest amount of a2,3-linked *N*-Glycolylneuraminic acid (a2,3-linked NeuGc, Gl, 8.62%), while bovine and donkey whey contained only 2.14% and 1.11%, respectively.

1. Introduction

Protein glycosylation is a crucial post-translational modification that impacts the physical and chemical structure of proteins (Apweiler, Hermjakob, & Sharon, 1999). Glycosylation-induced alterations not only influence protein structures but also impact their functions, making glycosylated proteins integral to cellular processes (Blom, Sicheritz-Ponten, Gupta, Gammeltoft, & Brunak, 2004). This modification significantly contributes to the three-dimensional conformation of proteins and actively participates in mediating protein–protein interactions (Zhang, 2006). In addition, glycans act as specific ligands for cell recognition and cell–cell and cell-host interactions (Rabinovich, van Kooyk, & Cobb, 2012). Understanding the differentiation of glycans in these cellular processes is critical to revealing their functions (Kayili & Salih, 2022).

Milk is widely used as a source of protein in many food products. Various milk sources, such as bovine and goat, are commonly utilized as protein sources in infant formulas (Pekdemir et al., 2022). The components in milk have a wide range of essential functions, including the crucial role of free milk oligosaccharides (Duman et al., 2021). Free milk oligosaccharides have been found to directly contribute to the digestive system of newborns and serve as a carbon source for beneficial bacteria in the intestine (Wiciński, Sawicka, Gębalski, Kubiak, & Malinowski, 2020). Although many studies have been conducted on free milk oligosaccharides, the number of studies on complex oligosaccharides (i.e., glycans) that bind to milk proteins is limited. Milk proteins are a significant dietary source for newborns, consisting of three major proteins: caseins, whey proteins, and milk fat globule membrane proteins (Davoodi et al., 2016). Caseins consist of four isoforms: αs1, αs2, β, and κ. However, only κ- casein was glycosylated. Most whey proteins are glycosylated and consist mainly of globular proteins, such as β-lactoglobulin, a-lactalbumin, and serum albumin. Whey proteins also contain lactoferrin, lysozyme, immunoglobulins, and lactoperoxidase proteins (Andreas, Kampmann, & Mehring Le-Doare, 2015). Milk fat globule membrane proteins are coated with polar lipids and glycosylated proteins such as Mucin 1, xanthine oxidoreductase, and butyrophilin

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(Riccio, 2004). Milk proteome studies reveal that most human and mammalian milk proteins are heavily glycosylated, which modulates the biomodulatory properties of milk and its nutritional quality (Peterson, Cheah, Grinyer, & Packer, 2013).

The diverse array of *N*-glycome profiles of milk proteins can impact their biological properties and functions and may play a significant role in regulating milk composition and quality (Kaplan et al., 2022). Fucosylation was identified as a critical factor in the anti-pathogenic activities of human and bovine milk whey proteins based on investigations of their *N*-glycome (Wang, Wang, et al., 2017). In addition, it was observed that human and bovine milk *N*-glycome showed bifidogenic activity (Wang, Du, et al., 2017). Furthermore, a study highlighted that milk whey glycoproteins that contained sialic acid residues played a role in the immune system of newborns. Specifically, the sialic acid profiles of glycans and glycoproteins containing a2,6 and a2,3 binding changed during milk lactation (Lis-Kuberka, Berghausen-Mazur, & Orczyk-Pawiłowicz, 2017). In addition, lactoferrin can bind to and sequester iron, which can inhibit the growth of bacteria and viruses that require iron to proliferate (Duman et al., 2021). Human milk whey glycoproteins can also help reduce inflammation in the infant's body, which can be beneficial for preventing various diseases and conditions (Kruzel, Zimecki, & Actor, 2017). On the other hand, human milk whey *N*-glycome can serve as a prebiotic by promoting the growth of beneficial bacteria in the infant's gut (Ninonuevo et al., 2006). In particular, certain *N*-glycans have been shown to selectively promote the growth of Bifidobacterium, which is a beneficial bacterium that is commonly found in the infant's gut (S. Karav et al., 2016). Moreover, *N*-glycosylation of milk proteins may also impact milk quality, including factors such as milk yield, composition, and storage stability(Guan et al., 2022). Based on these results, it can be concluded that the *N*-glycome of milk whey proteins not only increases the complexity of their protein compositions but also contributes to their biological functions. Although considerable progress has been made in understanding the role of *N*-glycosylation in milk biology, further research is needed to fully elucidate the complex relationship between *N*-glycosylation and milk composition, structure, and function. Therefore, to gain a better understanding of these findings, robust analytical techniques should be employed for analyzing the glycan structures of milk glycoproteins.

Mass spectrometry has emerged as a valuable tool for the comprehensive analysis of *N*-glycan structures of proteins owing to its high sensitivity, speed, and accuracy. Recently, *N*-glycosylation analysis of milk whey proteins has been performed to identify *N*-glycosylation sites in milk whey proteins (Cao et al., 2017; Yang et al., 2017). Additionally, several *N*-glycomic studies have investigated the *N*-glycan structures of human, bovine, and goat whey glycome (Nwosu et al., 2012). However, these studies have limitations in terms of linkage-specific evaluation of sialic acid *N*-glycan structures. A novel approach for sialic acid analysis, ethyl-esterification, has been recently introduced in the glycomics field (Reiding et al., 2016a). This method involves ethylation of *N*-glycans containing a2,6-linked sialic acid, and lactonization of *N*-glycans containing a2,3-linked sialic acid. The resulting isomers can be separated based on the varying mass differences of +28 Da and −18 Da for a2,6 and a2,3N-glycan isomers, respectively.

This study aimed to perform a comprehensive analysis of the *N*-glycan profiles of milk whey *N*-glycomes, utilizing a linkage-specific ethyl-esterification approach through MALDI-MS. The detected *N*-glycans were verified by MALDI-TOF/TOF-MS/MS analysis. *N*-glycan traits were defined based on the distinctive structural features of *N*-glycans. The relative abundances of *N*-glycans and derived traits were assessed using the total area normalization approach. The study also included qualitative and quantitative comparisons of *N*-glycans and *N*-glycan traits among different species.

2. Materials and methods

2.1. Materials

Unless otherwise specified, all the reagents, solvents, and proteins were purchased from Sigma-Aldrich (St Louis, MO, USA) and used without further purification. PNGase F enzyme was obtained from Promega (Madison, WI, USA). The purified water used in all experiments was obtained using an Expe-Ultrapure Water System (Mirae St Co., Korea).

2.2. Sample collection

First, it should be emphasized that all samples used in the study were collected after approval from the Clinical Research Ethics Committee of Ataturk University, Medical Faculty (Decision Number: B.30.2. ATA.0.01.00/219). Mature milk samples belonging to animals (bovine, buffalo, goat, and donkey) were obtained from at least ten animal donors on local farms in Turkey. Human milk samples were obtained from Ataturk University Medical Hospital of the Department of Neonatology. The milk specimens were procured within the postpartum interval, specifically at around 1–2 months following parturition. Mature milk samples were mixed with an equal volume (1 mL) from each donor and used in the sample preparation steps.

2.3. Methods

2.3.1. Extraction of whey proteins from milk samples

Mature milk samples (0.5 mL) were collected from the milk pool of each species and diluted with water (0.5 mL). The samples were then centrifuged at 4000 rpm for 30 min. The samples were then centrifuged at 4000 rpm for 30 min. After removing the fat layer from the upper part, the milk samples were centrifuged again at 1500 rpm and +4 °C for 1 h. Under normal conditions, three layers (upper, middle, and lower) were expected to form in the milk samples. The upper layer contained fat, the middle layer contained whey proteins and free oligosaccharides, and the lower layer contained casein proteins. In this study, after the middle layer was carefully transferred to another microcentrifuge tube, a 90% cold ethanol solution (twice the volume of the intermediate layer phase) was added to precipitate whey proteins. Milk samples were incubated at −20 °C for 20 min. Following this, milk samples were centrifuged at 15,000 rpm to separate whey proteins. After removing the solution-containing phase from the upper part, the precipitated whey proteins were dried using a centrifugal concentrator and re-dissolved in 50 μL of a solution containing 2.0 % SDS before *N*-glycan release.

2.3.2. N-glycan release of whey glycoproteins

The denaturation of whey proteins, which were previously dissolved in 50 μL of 2.0 % SDS, was carried out by subjecting them to incubation at 90 °C for 10 min. Following this, 50 μL of 2.0 % Igepal-CA630 and 50 μL of 5X PBS were added to the resulting solution. In order to carry out enzymatic deglycosylation, 2 U of PNGase F enzyme was inserted into the samples, which were then subjected to incubation at 37 °C overnight. The experiments were conducted in triplicate.

2.3.3. Linkage-specific ethyl esterification of released N-glycans

A recently developed methodology (Reiding, Blank, Kuijper, Deelder, & Wuhrer, 2014) was adapted for the ethyl esterification of released *N*-glycans. Briefly, 2.0 μL of the released *N*-glycan solution was mixed with 10 μL of 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC) and 10 μL of hydroxy benzotriazole monohydrate (HOBt) solutions, both prepared in 0.5 M ethanol. The resulting mixture was incubated at 60 °C for 1 h, after which 20 μL of acetonitrile was added. The samples were then stored at −20 °C for 10 min before purification of the glycans.

2.3.4. Procainamide labeling

The labeling solution was prepared by dissolving procainamide tag (11 mg) and sodium cyanoborohydride (6 mg) in 100 μL DMSO: CH_3COOH (7:3, v/v). After preparing the two solutions, they were mixed in equal volumes. Twenty-five μL of this mixture was added to the samples and incubated at 65 °C for 2 h. The samples were then purified using a HILIC (hydrophilic interaction liquid chromatography)-based method.

2.3.5. Purification of N-glycans by Cotton-HILIC micropipettes

Purification of ethyl-esterified *N*-glycans was achieved using a procedure described previously (Selman, Hemayatkar, Deelder, & Wuhrer, 2011) with minor modifications. Cotton fibers (3 mm in length) were placed in a 100 μL pipette tip. The cotton-containing pipette tip was washed with 100 μL of deionized water and 100 μL of 85% acetonitrile. Subsequently, the prepared loading solution was passed through a pipette tip containing cotton at least 20 times. Following this step, to remove salt and other solvent residues, the pipette tip was washed three times with 85/14/1, v/v/v, acetonitrile/water/trifluoroacetic acid solution and three times with 85% acetonitrile. Elution was performed using 10 μL of water.

2.3.6. HPLC-HILIC-ESI-QTOF-MS/MS analysis

HPLC-HILIC-ESI-QTOF-MS/MS analyses were performed using an Agilent 1200 series HPLC equipped with a Bruker TIMS-TOF mass spectrometer. Waters Glycan BEH Amide 1.7 μm (2.1 mm ID × 15 cm L) HILIC column was used for the analytical separation of *N*-glycans. To obtain suitable loading conditions for purified procainamide-labeled *N*-glycans (25 μL), 75 μL of acetonitrile was added to the solution and 30 μL of it was injected for analysis. The flow rate was set at 0.2 mL/min, and the column temperature was set to 60 °C. Mobile phases A and B were 50 mmol/L ammonium formate (pH 4.3) and acetonitrile, respectively. Separation was performed using a linear gradient of 75–52% acetonitrile (v/v) over a 120-minute run. MS conditions were set to a capillary voltage of 4.5 kV, source temperature of 180 °C, nebulizer gas of 1.2 bar, drying gas of 7 L/min, and MS acquisition was set to be performed between 50 and 2800 *m/z* at a frequency of 1 Hz. MS/MS experiments were achieved by selecting the three most abundant precursor ions at a spectrum rate of 0.5 Hz to 2 Hz.

2.3.7. MALDI-MS analysis

Five microliters of the elution solution were dripped onto the MTP 384 Anchor Plate and allowed to dry at room temperature. Then, 1.0 μL of a 5.0 mg/mL sDHB matrix (w:w, 9:1, 2–5 dihydroxy benzoic acid:2-hydroxy-5-methoxy benzoic acid) solution in acetonitrile/1.0 mM NaOH at a ratio of 1/1 by volume was added to the dried samples. MALDI-MS analysis was performed using a Bruker rapifleXTM Tissuetyper instrument (Bremen, Germany). Analyses were carried out by collecting at least 10,000 laser pulses in reflectron mode with positive ionization. Prior to each analysis, mass calibration was performed using a peptide mixture. Mass spectra in the 1000–4000 mass range were obtained at an acceleration voltage of 25 kV. MS/MS spectra were acquired in the LIFT mode to perform structural verification of the detected *N*-glycans.

2.3.8. Data analysis

The relative abundances of *N*-glycans detected in whey glycome were calculated for each species based on the area of each mass peak of these *N*-glycans. MassyTools, a python software, was used to extract the areas detected *N*-glycan peaks from the spectra (Jansen et al., 2015). The spectra were converted to the appropriate format for the program (.xy), the *N*-glycan list, mass calibration information, and extraction criteria were entered into the software. These data are presented in Supporting Information Table S1. After the relative areas of each *N*-glycan were calculated using the software by considering the areas of their peaks, the data were visualized using the GraphPad Prism software. The Venn diagram depicting the comparison of *N*-glycan types was generated using a web-tool (Hulsen, 2022).

After converting the MS/MS data obtained from HPLC-HILIC-ESI-QTOF-MS/MS analyses into.xml file format using Bruker Data Analysis software, the data was imported to Protein Scape software. The GlycoQuest algorithm in Protein Scape software was used to determine the structures of procainamide-labeled glycans. MS and MS/MS tolerances were set to 0.05 Da and 0.01 Da, respectively. The Carbbank database was used as the glycan database. A filtration was applied to list glycans with a score and fragmentation coverage higher than 20.

3. Results

In this study, *N*-glycans released from milk glycoproteins were first profiled with MALDI-MS using the linkage-specific ethyl-esterification approach. This approach is frequently used to detect sialic acid linkage positions. The *N*-glycan spectra of each species were obtained. In contrast to the literature focusing only on *m/z* ratios of *N*-glycan components, fragmentation spectra of *N*-glycans were also obtained using MALDI-LIFT analysis. The graphical abstract shows an overview of the method used for profiling milk whey *N*-glycans.

The present study reports on the analysis of milk whey *N*-glycan profiles of various species, including human, bovine, buffalo, goat, and donkey, using the matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) technique. Prior to the analysis, the whey proteins were extracted and subjected to enzymatic treatment to release *N*-glycans, which were then treated with linkage-specific ethyl esterification. The relative abundances of the detected *N*-glycan structures and derived *N*-glycan traits were calculated using the total area normalization approach. Supporting Information Figs. 1-5 display the obtained MALDI-MS spectra for each species. The number of whey *N*-glycans identified in this study was 67, 75, 71, 99, and 66 for human, bovine, buffalo, goat, and donkey species, respectively. Subsequently, the fragmentation products of the *N*-glycans were transferred to the Glycoworkbench software and compared with the theoretical fragmentation products of the potential structure. The matched masses of the MS/MS peaks are presented in Supporting Information Tables S1–S5 for all the species. The Supporting Information Fig. S6 included MS/MS spectra of various species, which were obtained through MALDI-MS/MS analyses to confirm the presence of NeuAc and NeuGc sialylated glycan structures. The confirmation of isomers, particularly regarding the linkage location of NeuAc and NeuGc sialylation, was conducted by monitoring specific glycan fragments. For instance, the presence of a2,6-NeuAc structures (E) was indicated by a mass fragment of *m/z* 707.2 $[HNE + H]^+$, whereas the presence of a2,6-NeuGc structures (Ge) was indicated by a mass fragment of *m/z* 723.2 $[HNGe + H]^+$. In addition, the identification of sialylated structures was further confirmed by monitoring the mass differences from a given precursor ions, such as *m/z* 638.2 and 319.1, as previously reported (Reiding et al., 2016b). The presence of *O*-acetyl groups associated with sialic acids was confirmed through the observation of *m/z* 749.3 $[HNEAc + H]^+$ (Fig. S6E). Additionally, to obtain precise ion readings pertaining to specific *N*-glycan structures, procainamide-labeled *N*-glycans from milk samples were analyzed through an HPLC-HILIC-MS/MS system, in addition to the MALDI-MS/MS experiments. The core fucosylated *N*-glycan structures were determined by monitoring a specific fragment, *m/z* 587.3 $[proc + NF + H]^+$, which provided evidence of core-fucosylation. The confirmation of the presence of bisecting *N*-glycan structures was achieved by monitoring a specific fragment with an *m/z* of 1009.5 $[proc + H1N3 + H]^+$, which is considered diagnostic for this particular type of *N*-glycan structures (Kayili, 2020). It was revealed that the majority of fucosylated *N*-glycan structures carried fucose residues on their core, as indicated in Fig. S7 and Tables S1B-S5B.

When the peaks detected by MALDI-MS analysis were examined, those that were not suitable for the *N*-glycan core structure (H3N2) were determined only for *N*-glycans in human milk. The presence of free

human milk oligosaccharides (HMOs) in whey glycoproteins poses a challenge to their accurate analysis as the HMOs cannot be sufficiently separated from the proteins due to their varied branching from the core structure. Human milk has been found to contain the highest levels of these oligosaccharides among the various milk types. To remove free HMOs from whey glycoproteins, whey proteins were washed five times with a solution containing 90% ethanol. In addition, after the proteins were dissolved in 2% SDS, they were placed in a 30 kDa cut-off and washed twice with 2% SDS. Subsequently, the proteins were extracted from the cut-offs, and their analysis was performed using the specified method. However, complete removal of free HMOs was not achieved, suggesting a strong interaction between whey proteins and free HMOs. To precisely detect the free milk oligosaccharide peaks observed in MALDI-MS spectra in human milk, the remaining supernatant was dried and re-dissolved in 10 μL of water. Ethyl esterification was performed using 2 μL of the sample without the deglycosylation step. The obtained spectra are presented in Supporting Information Fig. S8. The potential interfering free HMO structures detected in the spectrum were identified by comparing the mass peaks with those of the whey *N*-glycome.

3.1. Comparison of N-glycan heterogeneity of milk whey glycoproteins

The numbers of detected *N*-glycans were 67, 75, 71, 99, and 66 for humans, bovines, buffalos, goats, and donkeys, respectively, as reported in Table S1-S5. A total of 152 different *N*-glycan types were identified across the five species, with 20, 4, 4, 31, and 18 glycan types unique to humans, bovines, buffalos, goats, and donkeys, respectively (Table S6-S7). Overlapping *N*-glycan types between species are presented in Supporting Information Table S8. Specifically, 43 *N*-glycans were common between human and bovine milk, 40 between human and buffalo milk, and 40 between human and goat milk. Furthermore, 40 *N*-glycan types were shared among human, cow, and buffalo milk. The whey *N*-glycome profiles of bovine and buffalo milk were found to be the most similar among the five species, as shown in the Venn diagram in Fig. 1. The glycan analysis using "deep learning" revealed that goat milk had a more diverse glycan profile compared to human milk and other types of milk, as presented in Table S6.

3.2. Comparison of milk N-glycan abundances of milk whey glycoproteins

The relative abundance of each *N*-glycan was then calculated using the total area normalization approach. The resulting relative abundances of all detected *N*-glycans for each milk type can be found in Supporting Information Tables S1-S5. The ten most abundant *N*-glycan structures for each milk type are illustrated in Fig. 2. Furthermore, *N*-glycans were categorized based on their various traits and structures, with formulas for the calculation of *N*-glycan traits provided in Supporting Information Table S9.

The relative abundances of the detected *N*-glycan traits are provided in Supporting Information Tables S10 and S11. Species-based variations between *N*-glycan traits are shown in Fig. 3. When the glycan groups were evaluated, the most abundant glycan group in the milk whey *N*-glycome profiles of all species was the complex type. In terms of high mannose content, the relative amounts of bovine and buffalo *N*-glycan profiles were found to be similar. The *N*-glycan profiles of human milk showed the highest fucosylation rates. Acetylated *N*-glycans were detected only in donkey milk (16.06%). The milk type richest in sialic acid-containing *N*-glycans was goat milk. Human milk was also rich in sialic acid-containing *N*-glycans (81.15%).

The comparison chart presented in Fig. 4 is based on sialic acid types, which were differentiated from each other in MALDI-MS analyses using the applied methodology. Notably, human milk lacks the genes responsible for synthesizing NeuGc in the human genome. Thus, the glycan type containing this profile could not be determined in human milk. (Tangvoranuntakul et al., 2003). Analysis revealed that a2,6-linked NeuAc glycan type (E) and lactonized glycans (a2,3-linked NeuAc, L*)* were found most abundantly in the human milk profile. Goat milk was found to have the richest profile in terms of esterified NeuGc (Ge), accounting for 8.24%. Moreover, goat milk was also identified as having the richest profile in terms of lactonized (a2,3-linked, Gl) *N*-glycolyl neuraminic acid, with a percentage of 8.62%.

In the present study, sialylation profiles were compared among species based on the total sialylation rate. The results are presented in Fig. 5, which displays a pie chart of the sialylation profiles for each milk sample. Overall, the most prevalent type of sialylation observed across all species was a2-6 NeuAc (E), with a sialylation rate of 92.8%. In bovine and buffalo milk, the a2-6 NeuAc (E) sialylation rates were 79.88% and 87.24%, respectively. In contrast, a lower a2-6 NeuAc (E) sialylation rate of 48.57% was found in goat milk. Notably, goat milk had the highest a2-6 NeuGc (Ge) sialylation rate at 35.17%. The second-highest sialylation rate was observed in donkey milk, with a rate of 31.81%. The a2-6 NeuGc (Ge) sialylation rates in bovine and buffalo milk samples were 14.19% and 10.69%, respectively. In general, a2,3 NeuAc (L) and NeuGc (Gl) sialylation rates were lower than those of a2,6 type sialylation.

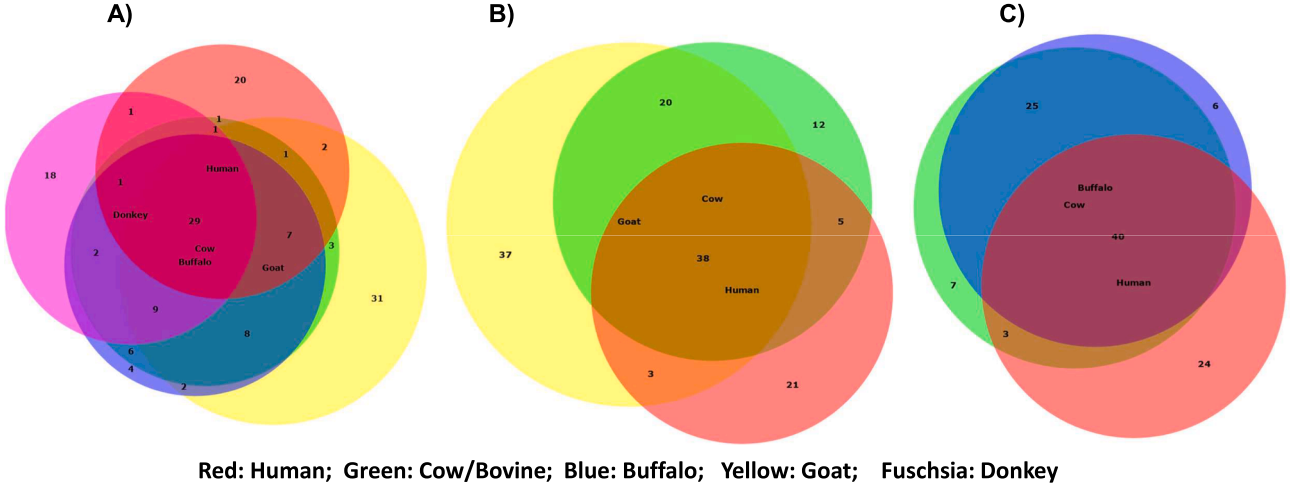


Fig. 1. Venn diagram of the *N*-glycans for the comparison of milk whey *N*-glycomes using deep learning approach. A) all species B) bovine/cow, human, and goat C) bovine/cow, human, and buffalo.

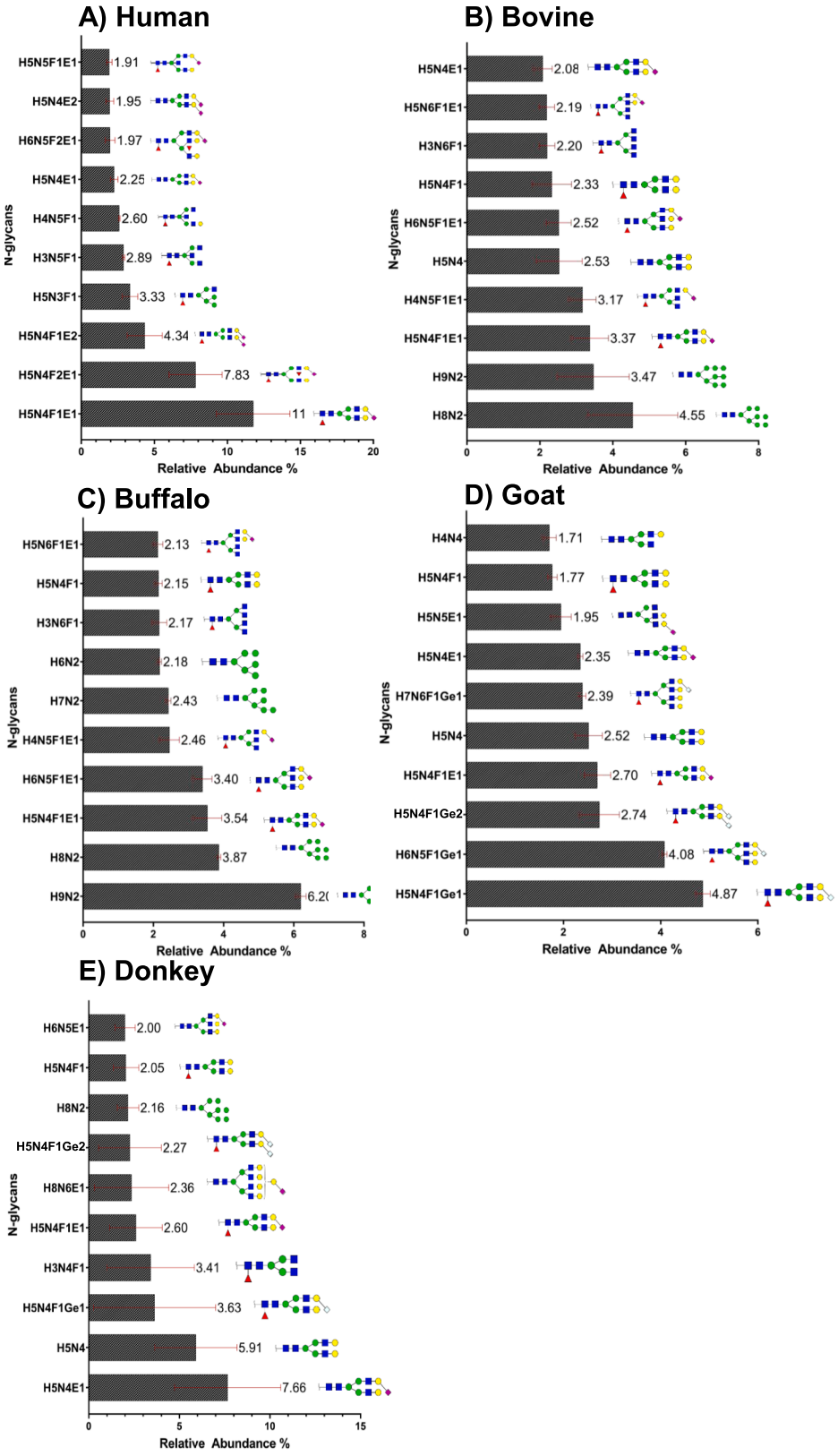


Fig. 2. The 10 most abundant *N*-glycan structures for A) human, B) bovine, C) buffalo, D) goat, E) donkey. Blue square: *N*-acetylglucose amine; yellow square: *N*-acetylgalactose amine; green circle: mannose; yellow circle: galactose; right slanted diamond (purple): α2,6-linked *N*-acetylneuraminic acid; left-slanted diamond (purple): a2,3-linked *N*-acetyl neuraminic acid; right slanted diamond (white): α2,6-linked *N*-glycolylneuraminic acid; left-slanted diamond (white): a2,3-linked *N*-glycolylneuraminic acid. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

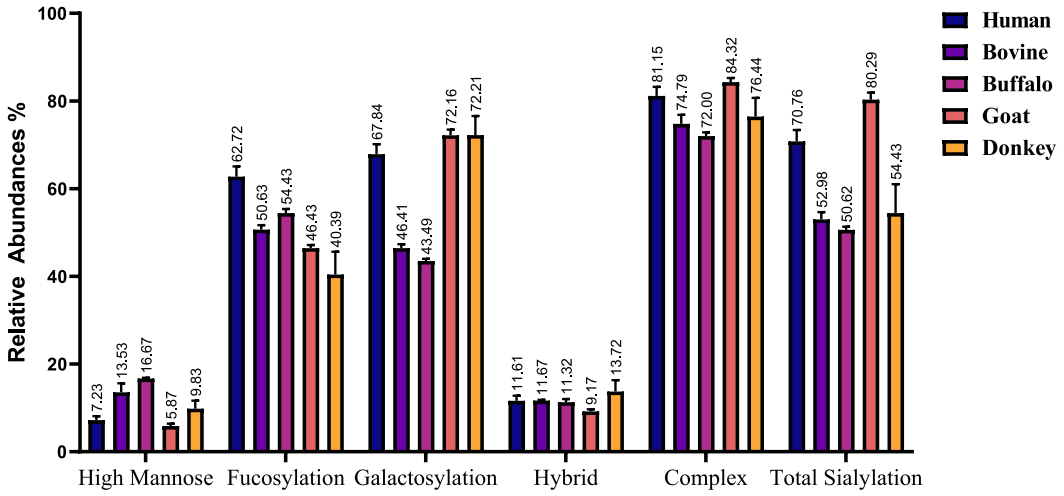


Fig. 3. Comparison of *N*-glycan trait relative abundances of milk whey glycoproteins.

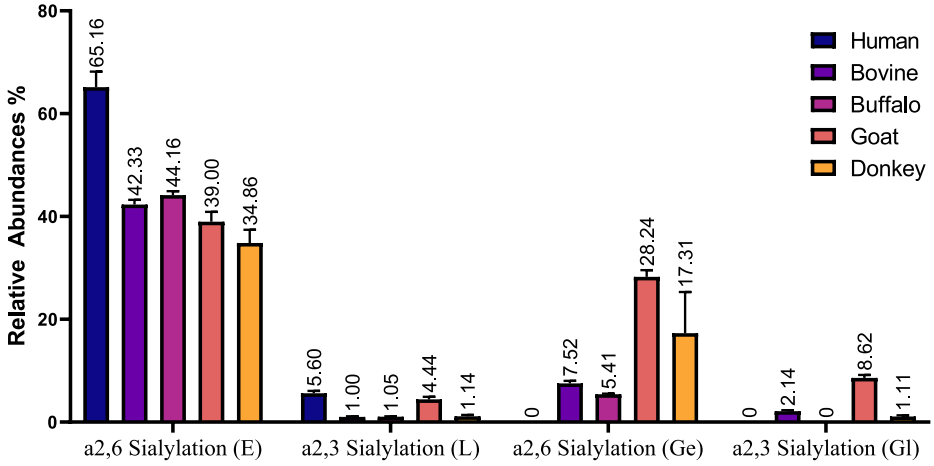


Fig. 4. Comparison of sialylated *N*-glycan trait relative abundances of milk whey glycoproteins.

4. Discussion

4.1. High-mannose content

The present study revealed that buffalo milk had the highest mannose content among the milk samples analyzed (16.67%), while bovine milk had a slightly lower mannose content (13.53%). A previous study compared the *N*-glycomes of human and bovine milk using high-performance microfluidic PGC chip liquid chromatography and reported that 6% and 10% of the *N*-glycomes were composed of high mannose for human and bovine milk, respectively (Nwosu et al., 2012). This finding is consistent with our results regarding high mannose distribution, where human milk and bovine milk *N*-glycomes were found to contain 7.23% and 13.53% high mannose, respectively. However, limited research has been conducted on the *N*-glycome profiles of goat milk. One study reported that high mannose *N*-glycans accounted for 17.9% of goat milk *N*-glycome profiles, whereas our analysis revealed only 5.87% high mannose *N*-glycans in the goat *N*-glycan pool (Lu et al., 2021).

4.2. Fucosylation

The results showed that human milk had the highest proportion of fucosylated *N*-glycans (62.72%) compared to the other species. Previous studies have reported varying degrees of fucosylation in different milk species. For instance, Nwosu et al. (2012b) reported fucosylation rates of 75% and 31% for human and bovine milk *N*-glycan pools, respectively. In our study, the fucosylation rates were 63% and 51% for human and bovine milk *N*-glycomes, respectively, which is consistent with the findings of Nwosu et al. (2012b). Another study indicated that high fucosylation and sialylation are common features of human milk *N*-glycome, whereas bovine milk has significantly less fucosylation and sialylation (Wang, Zhao, Tao, Li, Liu, & Dong, 2021). The same trend was observed in our study. Another study comparing human, bovine, and goat *N*-glycomes determined 52% fucosylation in bovine milk (Dong, Zhou, & Mechref, 2016). These results were consistent with our findings. However, they reported a relatively low number of *N*-glycans belonging to goat milk *N*-glycomes, indicating a difference in the profiles of *N*-glycan traits for goat milk. Another study reported a lower fucosylation rate of 18.55% in mature goat milk, while our study showed a much higher rate of 46.43% in goat milk *N*-glycome (Lu et al., 2021).

4.3. Sialylation

In this study, we observed that goat milk *N*-glycome exhibited the highest level of sialylation, with a total sialylation rate of 80.29%. Previous research has investigated alterations in goat milk *N*-glycome during different lactation stages, revealing that NeuGc and NeuAc-sialylated *N*-glycans constituted 5.3% and 3.7%, respectively (Lu et al., 2021). In contrast, we detected substantially higher total NeuAc-sialylated *N*-glycans (E and L) and total NeuGc *N*-glycans (Ge and Gl),

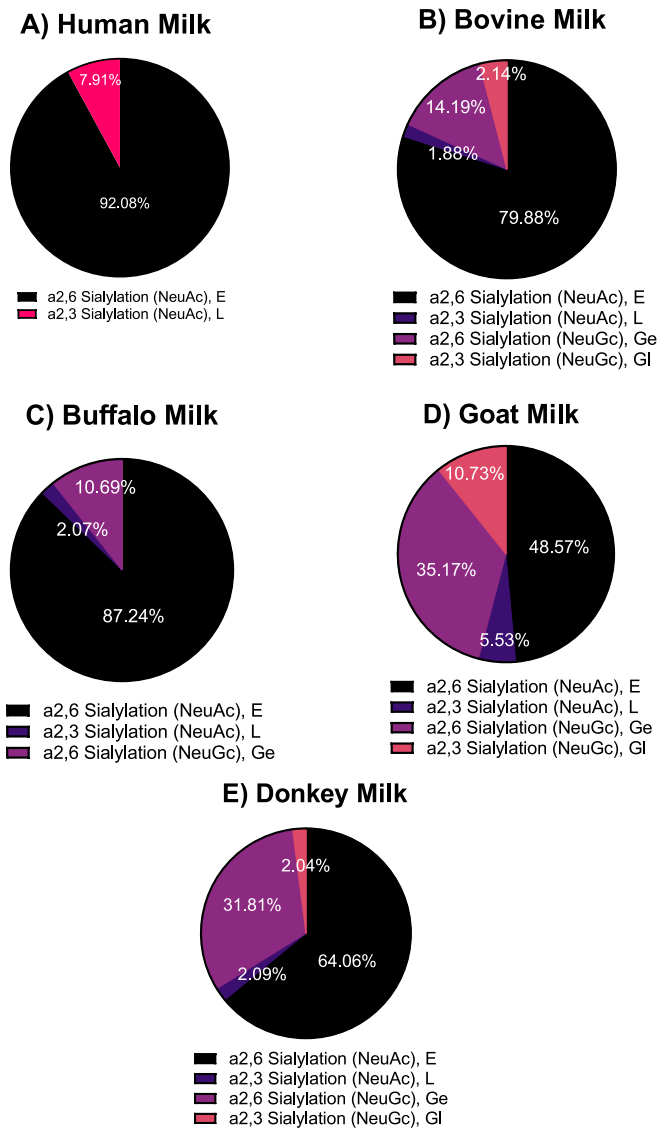


Fig. 5. Comparison of sialylated *N*-glycan trait ratios based on total sialylation distribution.

accounting for 43.44% and 36.86%, respectively. Regarding human and bovine milk *N*-glycome sialylation, one study detected 57% and 68% sialylation rates, respectively (Nwosu et al., 2012). We found that human and bovine milk *N*-glycome sialylation was 70.76% and 52.98%, respectively. Differences in the results may stem from variations in the number of *N*-glycan structures identified across the studies. For example, whereas Nwosu et al. (2012b) observed 38 *N*-glycan structures in human milk and 51 *N*-glycan compositions in the bovine milk *N*-glycan pool, we characterized 67 *N*-glycans belonging to human milk *N*-glycoproteins and 75 *N*-glycans belonging to bovine milk *N*-glycoproteins.

4.4. Assessing biological activities of milk N-glycans dependent on their structures

Several studies in the literature examining the biological role of milk *N*-glycans have been performed on total milk *N*-glycome profiles. For example, a recent research has demonstrated the prebiotic activity of milk *N*-glycans, indicating their ability to promote the growth of beneficial gut bacteria in neonates (Karav et al., 2019; Karav et al., 2016). They have determined that B. infantis was capable of consuming 73% of neutral and 92% of sialylated *N*-glycans, whereas B. lactis exhibited limited degradation, breaking down only 11% of neutral and less than 1% of sialylated *N*-glycans. According to the findings, goat milk may potentially have a positive impact on the gut microbiome due to its high content of sialylated *N*-glycans (80.29%). Conversely, previous research has indicated that NeuGc-type *N*-glycans present in red meat can lead to inflammation (Samraj et al., 2015). As goat milk *N*-glycans contain a relatively significant proportion of NeuGc type sialylation (36.86%, NeuGe, and NeuGl), it may not be a suitable source for milk formulas. However, further studies are necessary to comprehend this phenomenon. On the other hand, it has been determined that sialylated *N*-glycans can effectively inhibit the adhesion of pathogenic Escherichia to intestinal epithelial cells (Guan et al., 2022; Mu et al., 2019). In addition, it has been discovered that the sialic acid residues present on proteins have the potential to function as competitive substrates, thereby interfering with the binding of viruses to cell surface receptors (Yu et al., 2018). Research by Wang et al. (2017) identified fucosylation as a crucial element contributing to the anti-pathogenic properties of human and bovine milk whey proteins based on analysis of their *N*-glycome (Wang, Wang, et al., 2017). Moreover, a strong correlation has been observed between variations in the levels of fucosylated human milk oligosaccharides (HMOs) and milk *N*-glycans and the proliferation of Bifidobacterium spp. and Lactobacillus spp. in the gastrointestinal tract of neonates has been determined during the early and late lactation phases, respectively (Bai et al., 2018). According to a recent report, the sialylation and mannosylation of bovine lactoferrin were found to have an impact on the immunomodulatory properties of cells (Figueroa-Lozano et al., 2018). In conclusion, milk *N*-glycan types should be considered to prepare milk formulas based on these investigations.

4.5. Analytical method assessment

The utilization of the ethyl-esterification method for *N*-glycan profiling in milk is unprecedented in the scientific literature, offering an opportunity to characterize sialylated isomers. Additionally, there has been a paucity of investigations conducted on milk *N*-glycomes of buffalo and donkey whey glycoproteins, thus impeding a comparative analysis of their respective *N*-glycan profiles. Consequently, the present study serves to augment the existing knowledge base by providing novel insights into these two species' *N*-glycomes. The lack of identification of glycans with LacdiNAc in our study may be attributable to the specific analytical approach utilized, which may have limited our ability to detect such glycans. The analytical techniques used in our study did not allow for the identification of certain glycan types, such as those containing the LacdiNAc structure. Additionally, it is important to consider that the expression of these glycans in milk glycoproteins is known to be influenced by various factors, including lactation stage, breed, and individual variations in the glycosylation machinery. It is noteworthy that LacdiNAc structures are predominantly found in specific glycoproteins, such as lactoperoxidase, which may explain their absence in our study due to their relatively low abundance. Therefore, it is recommended that future studies utilize site-specific glycosylation analysis to better understand the glycosylation patterns of milk glycoproteins.

5. Conclusion

In the study, whey *N*-glycans obtained from various milk samples were analyzed using an advanced glycomic approach, and the *N*-glycan structures of the whey glycoproteins of each species were determined. The relative abundances of each detected *N*-glycan and *N*-glycan trait were calculated and compared between species. The analysis revealed that the *N*-glycan profiles of bovine and buffalo whey mostly overlap. The *N*-glycan profile of goat milk whey was determined to have the highest heterogeneity. The study also showed that human milk *N*-glycans had the highest fucosylation rate compared to other species. *N*-glycan structures containing total NeuGc were determined in all species

except human whey glycoproteins. Human milk whey *N*-glycans mostly contained a2,6-linked NeuAc (E) sialylation (65.16%). Moreover, goat milk was determined to have the highest amount of a2,6-linked NeuGc-containing *N*-glycans (Ge, 28.24%). These findings shed light on the structural diversity of *N*-glycans in milk whey glycoproteins and provide insight into the potential functional roles of these glycans in various species.

CRediT authorship contribution statement

Haci Mehmet Kayili: Conceptualization, Methodology, Investigation, Data curation, Writing – original draft. **Nazire Barlas:** Methodology, Investigation, Data curation. **Deniz Baran Demirhan:** Methodology, Investigation, Data curation. **Mehmet Emrah Yaman:** Software, Data curation, Writing – review & editing. **Mehmet Atakay:** Data curation, Writing – review & editing. **Ülkü Güler:** Data curation, Writing – review & editing. **Mustafa Kara:** Methodology, Writing – review & editing. **Kadir Serafettin Tekgunduz:** Methodology, Writing – review & editing. **Bekir Salih:** Methodology, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.136166.

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