A comparative study of free oligosaccharides in the milk of domestic animals

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Abstract

The present study was conducted to obtain a comprehensive overview of oligosaccharides present in the milk of a variety of important domestic animals including cows, goats, sheep, pigs, horses and dromedary camels. Using an analytical workflow that included ultra-performance liquid chromatography–hydrophilic interaction liquid chromatography with fluorescence detection coupled to quadrupole time-of-flight MS, detailed oligosaccharide libraries were established. The partial or full characterisation of the neutral/fucosylated, phosphorylated and sialylated structures was facilitated by sequencing with linkage- and sugar-specific exoglycosidases. Relative peak quantification of the 2-aminobenzamide-labelled oligosaccharides provided additional information. Milk from domestic animals contained a much larger variety of complex oligosaccharides than was previously assumed, and thirteen of these structures have been identified previously in human milk. The direct comparison of the oligosaccharide mixtures reflects their role in the postnatal maturation of different types of gastrointestinal systems, which, in this way, are prepared for certain post-weaning diets. The potential value of animal milk for the commercial extraction of oligosaccharides to be used in human and animal health is highlighted.

Key words: Domestic animal milk: Oligosaccharides: Ultra-performance liquid chromatography–hydrophilic interaction liquid chromatography with fluorescence detection–MS: 2-Aminobenzamide: Relative quantification

Human milk and animal milk are rich sources of bioactive oligosaccharides, which are of great interest to the functional food industry. Many biological activities have been reported for certain milk oligosaccharides including prebiotic activity, anti-adhesion effects, anti-inflammatory properties, glycome-modifying activity, and a role in brain development and growth-related characteristics of intestinal cells[1–4].

Milk oligosaccharides are typically composed of three to ten monosaccharide units, including glucose (Glc), galactose (Gal) and *N*-acetyl-glucosamine (GlcNAc) as well as fucose and sialic acids. The core unit present at the reducing end of milk oligosaccharides is either lactose (Gal(β1–4)Glc) or *N*-acetyl-lactosamine (Gal(β1–4)GlcNAc)[5].

As the biological activity of milk oligosaccharides is dependent on their individual structural characteristics, a detailed knowledge of their composition is necessary. Human milk is a rich source of oligosaccharides (20 mg/l in colostrum; 12–13 mg/l in mature milk), and more than 200 structures have been identified so far[6,7]. Less is known about animal milk oligosaccharides as their concentration in milk is low. For example, the concentration of oligosaccharides in bovine colostrum is 1 g/l, whereas their concentration in mature milk is about 20-fold less[8,9]. Using a combination of advanced analytical techniques, structural libraries of up to forty and even more than fifty bovine milk oligosaccharides[10–12], twenty-nine porcine milk oligosaccharides[13], twenty caprine milk oligosaccharides[8] and twelve

oligosaccharides from camel milk have recently been established[14]. A few oligosaccharide structures have also been assigned for equine and ovine milk[15,16]. In contrast to human milk, the content of fucosylated oligosaccharides is rather low in the milk of domestic animal species. Most of the oligosaccharides in animal milk are sialylated, containing *N*-acetylneuraminic acid (Neu5Ac) and/or *N*-glycolylneuraminic acid (Neu5Gc)[5,17].

The different analytical methods used to study milk oligosaccharides make comparative analysis of oligosaccharide profiles difficult. Therefore, we carried out a study to obtain a comprehensive overview of oligosaccharides present in the milk of cows, goats, sheep, pigs, horses and dromedary camels. A combination of weak anion-exchange chromatography, ultra-performance liquid chromatography–hydrophilic interaction liquid chromatography with fluorescence detection (UPLC-HILIC-FLD) and complementary quadrupole time-of-flight MS as well as exoglycosidase sequencing allowed for the determination of the oligosaccharide sequences and linkages as well as their relative quantification. A direct comparison of the oligosaccharide mixtures could contribute to a better understanding of possible differences in their biological effects and highlight the potential value of animal milk for commercial oligosaccharide extraction.

Materials and methods

Standards and chemicals

Milk oligosaccharide standards (2′-fucosyllactose (2′-FL), 3-FL, 3′-sialyllactose (3′-SL), 6′-SL, 6′-sialylated *N*-acetyllactosamine (6′-SLN), lacto-*N*-neo-tetraose (LNnT), lacto-*N*-tetraose (LNT), lacto-*N*-neo-hexaose (LNnH) and lacto-*N*-hexaose (LNH)) were purchased from Carbosynth Limited. Gal(β1–4)Gal (β1–4)Glc, Gal(β1–3)Gal(β1–4)Glc and Gal(β1–6)Gal(β1–4) Glc were purchased from Dextra Laboratories. Chemicals and solvents were obtained from Sigma-Aldrich and were of LC or LC–MS grade, respectively.

Isolation of animal milk oligosaccharides

Bovine colostrum from Holstein-Friesian cows and porcine colostrum from Landrace pigs were obtained on-site at Teagasc Food Research Centre, Moorepark (Fermoy, Cork, Ireland). Equine colostrum from Draught foster mares was kindly provided by Coolmore Stud (Fethard, County Tipperary), while ovine colostrum from Scottish black-faced mountain sheep was kindly donated by Cashel Irish Farmhouse Cheese Makers (Fethard, County Tipperary). Mature milk from goats was kindly donated by Ardsallagh Goat Farm (Carrigtwohill, County Cork). Mature milk from dromedary camels was kindly provided by King Faisal University (Saudi Arabia). After collection, the milk samples ($n \geq 3$) from each animal species were pooled. The isolation of oligosaccharides from pooled animal milk and colostrum samples was carried out as described previously[11]. Briefly, the samples were defatted by centrifugation at 4°C (3850 *g*, 20 min, 4°C; Sorvall RC6 plus®). Caseins were precipitated at

pH 4·6. After neutralisation, large peptides were removed by ultrafiltration (5 kDa molecular-weight cut-off, Millipore® Helicon S10 Spiral Cartridge; Millipore). The permeates were freeze-dried and stored at −80°C until further processing. To remove lactose and residual peptides, the extracts were resolubilised in MilliQ® water and applied onto a Sephadex G-25 column (Pharmacia; 92 × 2·6 cm). Elution was carried out with deionised water (5 ml/min). Fractions were monitored for peptides according to the method of Bradford[18], and the lactose content was determined by high-performance anion-exchange chromatography[11]. Fractions low in peptide and lactose content were pooled and used for further characterisation.

Fluorescent labelling of animal milk oligosaccharides with 2-aminobenzamide

Animal milk oligosaccharides were fluorescently derivatised via reductive amination with 2-aminobenzamide (2-AB) and sodium cyanoborohydride in 30 % (v/v) acetic acid–dimethyl sulphoxide. A volume of 5 μl of labelling mixture was used per approximately 5 μg oligosaccharides and incubated at 65°C for 2 h. The samples were purified by ascending paper chromatography in acetonitrile (ACN)[19].

Fractionation of neutral and acidic oligosaccharides by weak anion-exchange chromatography

Weak anion-exchange chromatography was carried out on a Waters BioSuite™ DEAE anion-exchange column (7·5 × 75 mm; Waters), which was installed on a Waters 2795 Alliance system with a 2475 fluorescence detector (excitation wavelength (λ_{ex}) 330 nm; emission wavelength (λ_{em}) 420 nm). Solvent A was composed of 20 % acetonitrile and solvent B was composed of 0·1 M-ammonium acetate buffer, pH 7·0, in 20 % (v/v) acetonitrile. The elution conditions were 100 % solvent A (0–5 min), a linear gradient of 100–0 % solvent A (5–20 min), and column regeneration and cleaning in 100 % solvent B (20–22·5 min) and 100 % solvent A (23–30 min). The flow rate applied was 0·75 ml/min. The samples were stored at 5°C before injection, and the column temperature was kept at 25°C. The samples were injected in water, and a fetuin-*N*-glycan standard was used for calibration.

Digestion of oligosaccharides with exoglycosidases and alkaline phosphatase

Sugar- and linkage-specific exoglycosidases as well as an alkaline phosphatase were used to determine the fine structure of the milk oligosaccharides. Alkaline phosphatase derived from calf intestine (*EC* 3.1.3.1), 20 mU/ml, was purchased from Roche. The exoglycosidases used (all purchased from ProZyme) were as follows: *A. ureafaciens* α(2–3/6/8) sialidase (*EC* 3.2.1.18), 1–2 U/ml; *S. pneumoniae* α(2–3)-sialidase recombinant in *Escherichia coli* (*EC* 3.2.1.18), 1 U/ml; bovine kidney α(1–2/3/4) fucosidase (*EC* 3.2.1.51), 1 U/ml; almond meal α(1–3/4) fucosidase (*EC* 3.2.1.111), 3 mU/ml; β(1–3/4)-galactosidase from bovine testis (*EC* 3.2.1.23), 2 U/ml;

β(1–4)-galactosidase from *S. pneumoniae* (*EC* 3.2.1.23), 80 mU/ml; α(1–3/4)-galactosidase from coffee bean (*EC* 3.2.1.22), 0·5 U/ml; chicken liver α(1–3)-*N*-acetylgalactosaminidase (*EC* 3.2.1.49), 0·7 U/ml; jack bean β(1–2/3/4/6)-*N*-acetylhexosaminidase (*EC* 3.2.1.52), 10 mU/ml. All the enzymatic digestions were carried out using 2-AB-labelled milk oligosaccharides in 10 μl of solution containing enzymes at standard concentrations in the manufacturer's recommended buffers for 16 h at 37°C. After digestion, the enzymes were removed from the samples by filtration through a 10K microcentrifuge filter (Pall Life Sciences). The filtrate was dried in a Speed Vac and resuspended for analysis by UPLC-HILIC-FLD–(MS).

Oligosaccharide profiling by ultra-performance liquid chromatography–hydrophilic interaction liquid chromatography with fluorescence detection analysis

The 2-AB-labelled animal milk oligosaccharide mixtures were separated by ultra-performance liquid chromatography with fluorescence detection using a Waters BEH Glycan column (150 × 2·1 mm, 1·7 μm BEH (bridged ethylsiloxane/silica hybrid) particles), which was installed on a Waters H-Class instrument, consisting of a quaternary solvent manager, sample manager and fluorescence detector. Solvent A was composed of a 50 mM-ammonium formate buffer, pH 4·4, and solvent B was acetonitrile. The elution conditions were 12 % solvent A (0–1·5 min), 12–47 % solvent A (1·5–35 min), 70 % solvent A (35·5–36 min) and 12 % solvent A (36·5–40 min). The flow rate was 0·56 ml/min. The injection volume was 10 μl, and the samples were prepared in 80 % (v/v) acetonitrile. The samples were stored at 5°C before injection, and the separation temperature was 25°C. The fluorescence detection wavelengths were $\lambda_{ex} = 330$ nm and $\lambda_{em} = 420$ nm with a data collection rate of 20 Hz. A dextran hydrolysate ladder was used to convert retention times into glucose unit (GU) values. All the data were processed using the Waters Empower 3 chromatography workstation software.

Characterisation of oligosaccharides by ultra-performance liquid chromatography–hydrophilic interaction liquid chromatography with fluorescence detection–MS/MS analysis

UPLC-HILIC-FLD–MS and mass fragmentation (MS/MS) analysis of the 2-AB-labelled animal milk oligosaccharide mixtures was carried out on a Waters Acquity instrument with fluorescence detection connected to a Waters XEVO QTOF instrument. The Waters Acquity system consists of a binary solvent manager, sample manager and fluorescence detector and is equipped with a Waters BEH Glycan column (150 × 1 mm, 1·7 μm BEH particles). The solvents, elution conditions, fluorescence detector settings, injection volume, sample preparation method, sample storage conditions and column temperature were the same as those used for the UPLC-HILIC-FLD analysis. MS analysis was carried out in negative mode. The source capillary voltage was set to 2 kV and the source temperature was 100°C. The collision energy ramp for mass fragmentation was 30–42 V. The mass range for MS was set to *m/z* 400–2000 and that for MS/MS was set to *m/z* 100–2000. Data were processed using MassLynx software version 4.1 (Waters).

Sialic acid speciation

Sialic acids were released by mild acid hydrolysis (2 M-acetic acid, 80°C, 2 h). To a 5 μl aliquot of the released solution, 20 μl of DMB (1,2-diamino-4,5-methylenedioxybenzene) labelling solution (DMB in mercaptoethanol and sodium dithionite; Ludger) were added, and the mixture was incubated for 3 h at 50°C in the dark. The reaction was quenched by adding 475 μl of water. The labelled samples were diluted 1:10 and analysed using reversed-phase UPLC on a Waters XBridge BEH C18 (150 × 2·1 mm inner diameter, 3·5 μm column) with fluorescence detection ($\lambda_{ex} = 343$ nm; $\lambda_{em} = 448$ nm).

Results

Analysis of milk oligosaccharides by ultra-performance liquid chromatography–hydrophilic interaction liquid chromatography with fluorescence detection analysis

In the present study, oligosaccharides were extracted from pooled colostrum samples from cows, sheep, pigs and horses and pooled mature milk samples from goats and dromedary camels.

A global overview of the UPLC-HILIC-FLD profiles of the 2-AB fluorescently labelled milk oligosaccharides from the different animal species is shown in Fig. 1. Retention times were converted into GU values, enabling the independent comparison of different oligosaccharide profiles and the incorporation of the structures in our public access database (GlycoBase 3.2.2)[20]. Distinct and well-separated profiles were obtained for all the species. The profiles of all the milk oligosaccharides from domestic animals were dominated by one or few sialyllactose isomers (A6, A9, A11 and A15; their structural characterisation is described below and in Table 2). Lactose is the main carbohydrate component in domestic animal milk[5]. Before fluorescent labelling and analysis, the concentration of lactose in the samples was reduced by gel filtration. This procedure was essential to gain more signal intensity for the less-abundant milk oligosaccharides. As disaccharides other than lactose were also removed during sample preparation, they were not considered for characterisation and quantification.

Prefractionation and structural analysis of animal milk oligosaccharides

Detailed analysis of the complex oligosaccharide mixtures required subfractionation into neutral and acidic oligosaccharide pools. Therefore, the 2-AB-labelled oligosaccharide mixtures were subjected to preparative weak anion-exchange chromatography before the UPLC-HILIC-FLD analysis. Fluorescent labelling is stoichiometric and allows for relative quantification. The relative proportions of neutral and acidic oligosaccharides in the subfractions were determined by

peak quantification relative to the total baseline-integrated peak area set to 100 % (Tables 1 and 2). Subsequently, the relative proportions obtained for the subfractions were translated into total profiles to calculate the overall proportion of neutral and sialylated oligosaccharides (Table 4). The total oligosaccharide pools from the milk of all the domestic animals were composed of approximately 80–90 % acidic oligosaccharides (Table 4).

By means of exoglycosidase sequencing and subsequent analysis of the oligosaccharide profiles by the UPLC-HILIC-FLD–(MS/MS) analysis, twenty-nine neutral, forty-five sialylated and three phosphorylated structures were identified in animal milk (Tables 1–3). Detailed information on the specific mode of action of exoglycosidases on milk oligosaccharides has been reported in Mariño *et al.*[11]. Characteristic shifts in GU values upon exoglycosidase digestion allowed for detailed structural assignment of the animal milk oligosaccharides[11]. Of the oligosaccharide structures assigned for domestic animal milk, thirteen have been identified previously in human milk[6,7] and are highlighted in Tables 1 and 2.

Structural characterisation of neutral oligosaccharides from animal milk

Galactotrioses and galactotetraose. Galactotrioses (Gal(α1−3) Gal(β1−4)Glc, N6, Gal(β1−3)Gal(β1−4)Glc, N7, and

Fig. 1. (a) Ultra-performance liquid chromatography–hydrophilic interaction liquid chromatography with fluorescence detection profiles of 2-aminobenzamide-labelled free oligosaccharides from the milk of domestic animals. Major peaks are assigned according to Table 2 and their structures are given using (b) the Oxford symbol nomenclature[30]. *Lactose (removed during sample preparation). GU, glucose unit reference values; Glc, glucose; GlcNAc, *N*-acetyl-glucosamine; Gal, galactose; GalNAc, *N*-acetyl-galactosamine; Fuc, fucose; HexNAc, *N*-acetyl-hexosamine; Neu5Gc, *N*-glycolylneuraminic acid; Neu5Ac, *N*-acetylneuraminic acid.
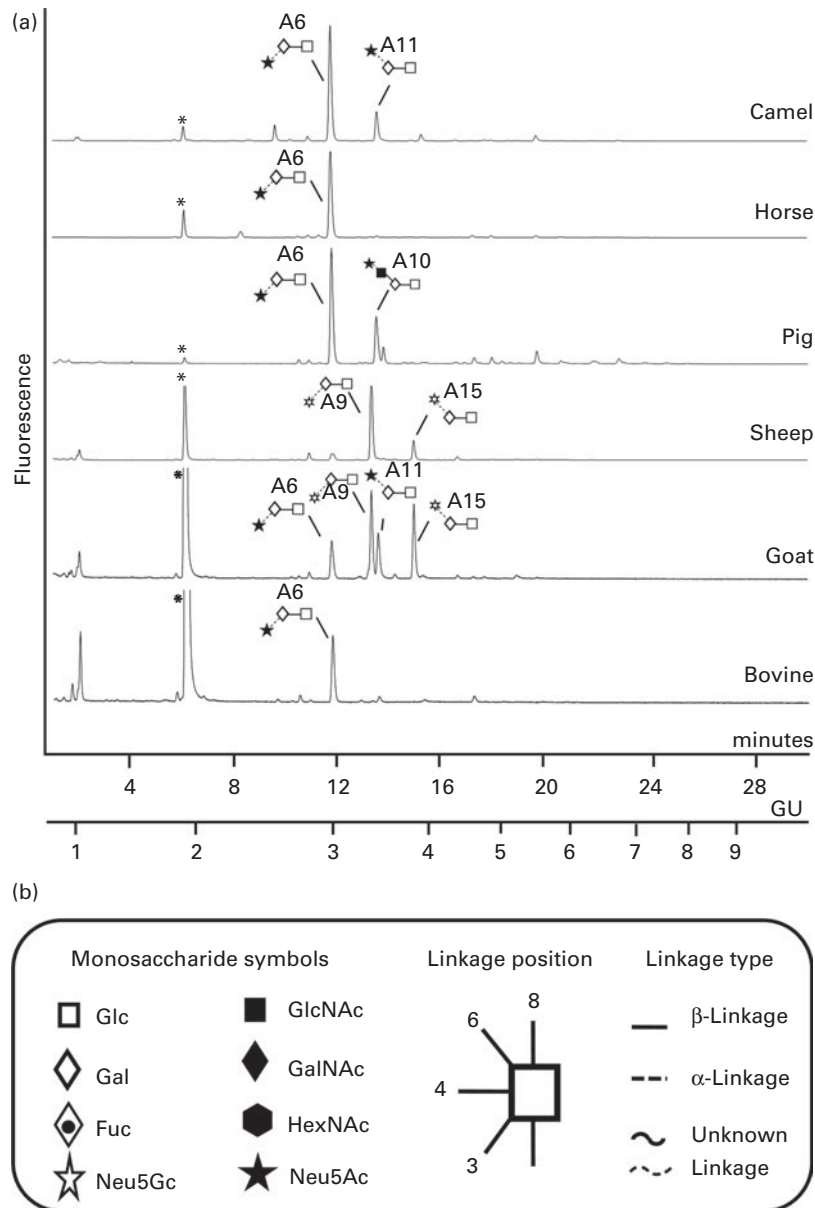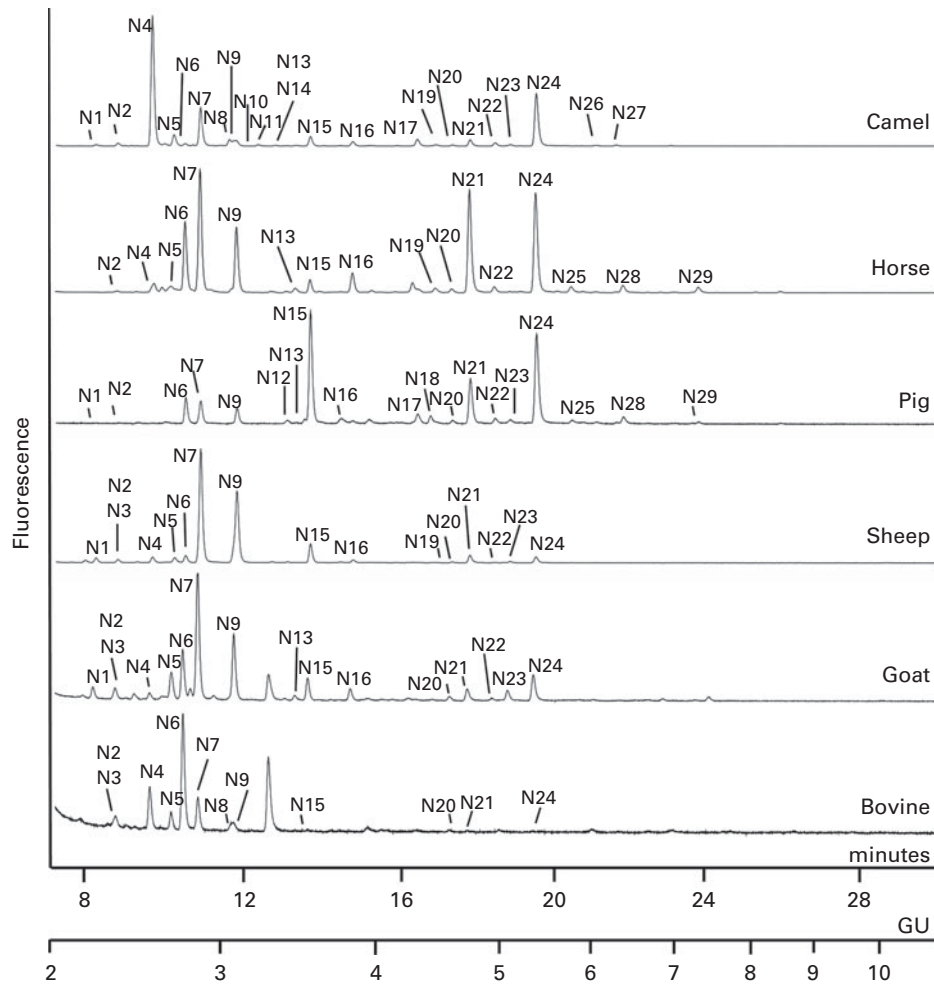
Fig. 2. Ultra-performance liquid chromatography–hydrophilic interaction liquid chromatography with fluorescence detection profiles of 2-aminobenzamide-labelled neutral oligosaccharide pools from the milk of domestic animals. Major peaks are assigned according to Table 1. GU, glucose unit reference values.

Gal(β1–6)Gal(β1–4)Glc, N9, with N7 and N9 being identified previously in human milk[7]) are the most abundant structures in the total neutral oligosaccharide pool. α-Galactotriose was selectively degraded by an α-specific galactosidase from coffee bean. Due to limitations in enzyme specificities, the (β1–3/6)-linked galactotrioses were identified by their respective standards. The (β1–4)-linked galactotriose (GU 2·74) was not detected in the milk of any of the animals. Trace amounts of an unknown galactotriose isomer (N10) and a galactotetrasaccharide (N13) were detected.

N-acetyl-hexosaminyl-lactoses. GalNAc(α1–3)Gal(β1–4)Glc (N4) and GlcNAc(β1–3)Gal(β1–4)Glc (N5) were identified with a β-N-acetylhexosaminidase-specific enzyme from jack bean and an α-N-acetylgalactosaminidase from chicken liver.

Lacto-N-neo-tetraose and iso-lacto-N-neo-tetraose. LNnT (Gal(β1–4)GlcNAc(β1–3)Gal(β1–4)Glc, N15), which is also present in human milk[7], and iso-LNnT (Gal(β1–4) GlcNAc(β1–6)Gal(β1–4)Glc, N16) were identified by their characteristic GU shifts following enzymatic digestion with a (β1–4)-specific galactosidase from *S. pneumoniae.*

Lacto-N-novo-pentaose and lacto-N-neo-hexaose. Gal(β1–4) GlcNAc(β1–6)(GlcNAc(β1–3))Gal(β1–4)Glc (N20), lacto-N-novo-pentaose I (LNnP I; Gal(β1–4)GlcNAc(β1–6)(Gal(β1–3)) Gal(β1–4)Glc, N21) and LNnH (Gal(β1–4)GlcNAc(β1–6) (Gal(β1–4)GlcNAc(β1–3))Gal(β1–4)Glc, N24), the latter of which is present in human milk[7], were degraded to their characteristic tetrasaccharide and/or trisaccharide cores by a (β1–4)-specific galactosidase from *S. pneumoniae* and a (β1–3/4)-specific galactosidase from bovine testis. Structural isomers (N18, N19 and N22) and an *N*-acetyl-lactosamine derivative of LNnH (N23) were also observed with some of the structures potentially being linear oligosaccharides, such as para-LNnH (Gal(β1–4)GlcNAc(β1–3)Gal(β1–4)GlcNAc (β1–3)Gal(β1–4)Glc) in human milk[7].

α-Galactosylated lacto-N-novo-pentaose and lacto-N-neo-hexaose. α-Galactosylated LNnP (N25) and mono-α-galacto-sylated and di-α-galactosylated LNnH (N28 and N29) were identified by digestion with the α-specific galactosidase from coffee bean. α-Galactosylated oligosaccharides are not present in human milk[7], but di-α-galactosylated LNnH has been observed previously in the milk from polar bears[21].

Fucosylated oligosaccharides. In contrast to human milk, merely eight neutral fucosylated oligosaccharides were identified in the total neutral oligosaccharide pool from animal milk, including fucosyllactoses (2′-FL (Fuc(α1−2)Gal(β1−4) Glc, N1) and 3-FL (Gal(β1−4)(Fuc(α1−3))Glc, N2)), fucosyl-*N*-acetyl-lactosamine (3-FLN (Gal(β1−4)(Fuc(α1−3))GlcNAc, N3)), fucosyl-galactosaminyllactose (GalNAc(α1−3)(Fuc(α1−2)) Gal(β1−4)Glc, N8), fucosylated galactotriose (N11), fucosylated LNnT (lacto-*N*-fucopentaose III; Gal(β1−4)(Fuc(α1−3)) GlcNAc(β1−3)Gal(β1−4)Glc, N17) and fucosylated LNnH (FLNnH; N26 and N27) isomers. Except N3, N8 and N11, these fucosylated structures have also been reported for human milk[7]. The fucosylated structures were identified with the aid of an (α1−3/4)-specific fucosidase from almond meal and an α(1−2/3/4)-specific fucosidase from bovine kidney.

Structural characterisation of acidic oligosaccharides from animal milk

Digestion of the samples with a sialidase from *A. ureafaciens* specific for (α2−3/6/8)-linkages and a recombinant sialidase from *S. pneumoniae* specific for (α2−3)-linkages provided linkage information on the underlying acidic structure.

Due to the differences in mass (*m/z*), MS analysis could be used to differentiate between Neu5Ac- and Neu5Gc-containing oligosaccharides (*m/z* Neu5Ac: 309·11; *m/z* Neu5Gc: 325·10). The MS charge state of multisialylated oligosaccharides indicates whether the Neu5Ac/Neu5Gc residues are attached to different sugar units in the oligosaccharide (doubly charged ions; $[M − 2H]^{2−}$) or are sequentially attached (singly charged ions; $[M − H]^{−}$).

The sialic acid composition of the animal milk oligosaccharides was determined by reversed-phase UPLC-UV after sample hydrolysis and DMB labelling. Major differences in the composition between animal species were observed. The acidic oligosaccharides from the milk of cows, pigs, horses and camels were decorated to ≥98% with Neu5Ac, while Neu5Gc contributed to 64 and 94% of the total sialic acid content in those from the milk of goats and sheep, respectively (Table 4). Neu5Ac is the sole sialic acid in human milk[7].

Sialylated lactose and N-acetyl-lactosamine. The total acidic oligosaccharide pool from animal milk was dominated by two pairs of sialylated lactose isomers, 3′-SL/6′-SL (Neu5Ac(α2−3) Gal(β1−4)Glc, A6/Neu5Ac(α2−6)Gal(β1−4)Glc, A11) and 3′GL/6′GL (Neu5Gc(α2−3)Gal(β1−4)Glc, A9/Neu5Gc(α2−6) Gal(β1−4)Glc, A15). In human milk, 3′-SL/6′-SL are also important components, but in contrast to animal milk, the
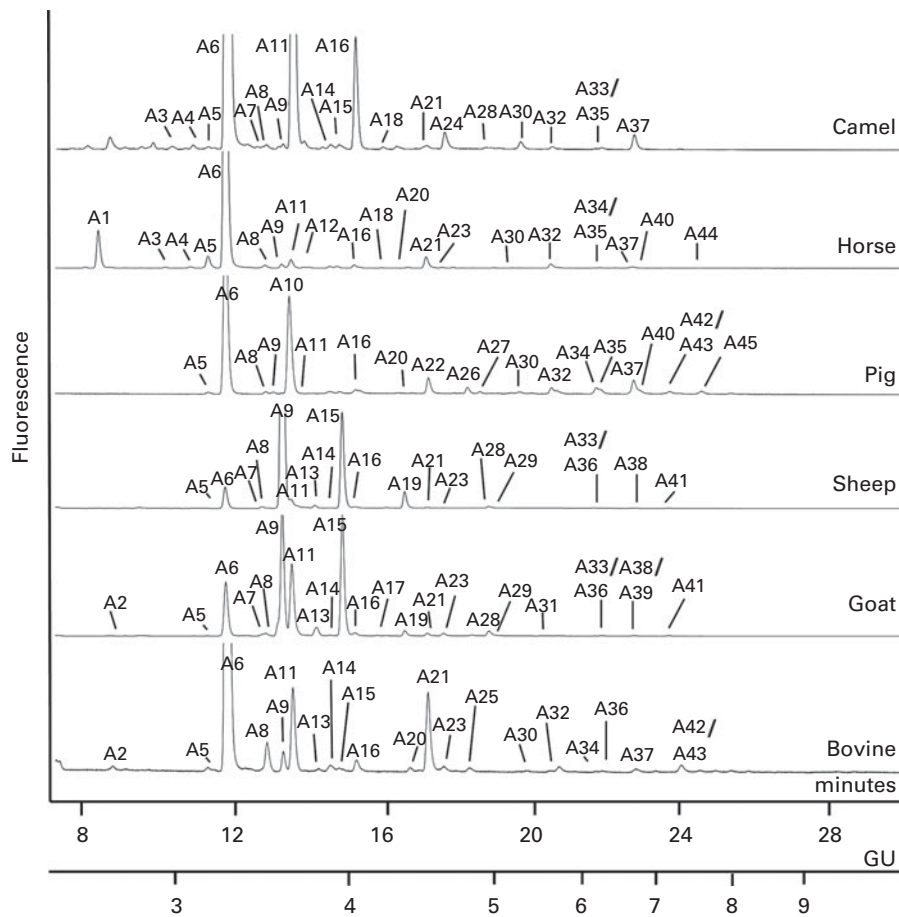

Fig. 3. Ultra-performance liquid chromatography−hydrophilic interaction liquid chromatography with fluorescence detection profiles of 2-aminobenzamide-labelled acidic oligosaccharide pools from the milk of domestic animals. Major peaks are assigned according to Table 2. GU, glucose unit reference values.

Table 1. Structures and their relative abundances in the neutral oligosaccharide pools from animal milk

No.	Name of the proposed structure [M − H]⁻ m/z observed	[M − H]⁻ m/z theoretical	Presence and relative peak abundance (FLD) in the neutral pool* Cow	Goat	Sheep	Pig	Horse	Camel	Symbol of the proposed structure†	GU*
N1‡	Fuc(α1−2)Gal(β1−4)Glc (2′-FL) 607·27	607·24	–	3⁽³¹⁾§	2	≤1⁽¹³⁾	–	≤1		2·39
N2‡	Gal(β1−4)(Fuc(α1−3))Glc (3-FL) 607·27	607·24	4‖⁽³²⁾	2‖	≤1‖	≤1	≤1	≤1		2·50
N3	Gal(β1−4)(Fuc(α1−3))GlcNAc (3-FLN) 648·32	648·26	4‖⁽³²⁾	2‖	≤1‖	–	–	–		2·50
N4	GalNAc(α1−3)Gal(β1−4)Glc 664·25	664·26	12⁽¹¹⁾	≤1	2	–	2	40		2·69
N5	GlcNAc(β1−3)Gal(β1−4)Glc 664·25	664·26	4⁽¹¹⁾	6⁽⁸⁾	2	–	2	4		2·81
N6	Gal(α1−3)Gal(β1−4)Glc 623·25	623·23	37⁽¹¹⁾	10⁽³¹⁾	2⁽³³⁾	6⁽¹³⁾	11	≤1		2·87
N7‡	Gal(β1−3)Gal(β1−4)Glc 623·25	623·23	11⁽¹¹⁾	27⁽⁸⁾	42⁽³³⁾	5⁽¹³⁾	20⁽¹⁶⁾	12⁽¹⁴⁾		2·95
N8	GalNAc(α1−3)(Fuc(α1−2))Gal(β1−4)Glc 810·34	810·31	2⁽¹¹⁾	–	–	–	–	2		3·14
N9‡	Gal(β1−6)Gal(β1−4)Glc 623·25	623·23	2⁽¹¹⁾	15⁽³¹⁾	31⁽³³⁾	4⁽¹³⁾	11⁽¹⁶⁾	≤1		3·15
N10	Gal-Gal-Glc 623·25	623·23	–	–	–	–	–	≤1		3·30
N11	Fuc + Gal-Gal(β1−4)Glc 769·31	769·29	–	–	–	–	–	≤1		3·40
N12	Gal(β1−3)(GlcNAc(β1−6))Gal(β1−4)Glc 826·36	826·31	–	–	–	≤1	–	–		3·46
N13	Gal-Gal-Gal(β1−4)Glc 785·30	785·28	–	≤1	–	≤1	≤1	≤1‖		3·53
N14	Gal(β1−4)GlcNAc-Gal(β1−4)GlcNAc 867·38	867·34	–	–	–	–	–	≤1‖		3·53

Table 1. *Continued*

No.	Name of the proposed structure [M − H]⁻ *m/z* observed	[M − H]⁻ *m/z* theoretical	Cow	Goat	Sheep	Pig	Horse	Camel	Symbol of the proposed structure†	GU*
N15‡	Gal(β1−4)GlcNAc(β1−3)Gal(β1−4)Glc (LNnT) 826·34	826·31	≤1[11]	5[8]	**7**	28[13]	≤1[15]	3		3·61
N16	Gal(β1−4)GlcNAc(β1−6)Gal(β1−4)Glc (iso-LNnT) 826·34	826·31	–	2[8]	≤1	≤1	3[15]	2		3·90
N17‡	Gal(β1−4)(Fuc(α1−3))GlcNAc(β1−3)Gal(β1−4)Glc (LNFP III) 972·40	972·37	–	–	–	3	–	2		4·39
N18	Gal + Gal(β1−4)GlcNAc-Gal(β1−4)Glc 988·40	988·36	–	–	–	2	–	–		4·49
N19	GlcNAc + Gal(β1−4)GlcNAc-Gal(β1−4)Glc 1029·45	1029·39	–	–	≤1	–	≤1	≤1		4·54
N20	Gal(β1−4)GlcNAc(β1−6)(GlcNAc(β1−3))Gal(β1−4)Glc 1029·45	1029·39	≤1[11]	≤1	≤1	≤1[13]	≤1	≤1		4·68
N21	Gal(β1−4)GlcNAc(β1−6)(Gal(β1−3))Gal(β1−4)Glc (LNnP I) 988·43	988·36	≤1[11]	2[8]	3	12[13]	18[16]	2[34]		4·83
N22	Gal(β1−4)GlcNAc + Gal(β1−4)GlcNAc-Gal(β1−4)Glc 1191·46	1191·44	–	≤1	≤1	2	≤1	≤1		5·07
N23	Gal(β1−4)GlcNAc + Gal-GlcNAc-Gal(β1−4)GlcNAc 1232·51	1232·47	–	2	≤1	≤1	–	≤1		5·21
N24‡	Gal(β1−4)GlcNAc(β1−6)(Gal(β1−4)GlcNAc(β1−3))Gal(β1−4)Glc (LNnH) 1191·46	1191·44	≤1[11]	6[8]	3	25[13]	19[15]	19[14]		5·47
N25	Gal(α1−3) + Gal(β1−4)GlcNAc(β1−6)(Gal(β1−3))Gal(β1−4)Glc 1150·42	1150·41	–	–	–	≤1	≤1	–		5·84
N26‡	Fuc + Gal(β1−4)GlcNAc(β1−6)(Gal(β1−4)GlcNAc(β1−3))Gal(β1−4)Glc (FLNH) 1337·52	1337·50	–	–	–	–	–	≤1		6·14
N27‡	Fuc + Gal(β1−4)GlcNAc(β1−6)(Gal(β1−4)GlcNAc(β1−3))Gal(β1−4)Glc (FLNH) 1337·52	1337·50	–	–	–	–	–	≤1		6·36

Table 1. *Continued*

No.	Name of the proposed structure		Presence and relative peak abundance (FLD) in the neutral pool*						Symbol of the proposed structure†	GU*
	[M − H]⁻ *m/z* observed	[M − H]⁻ *m/z* theoretical	Cow	Goat	Sheep	Pig	Horse	Camel		
N28	Gal(α1−3) + Gal(β1−4)GlcNAc(β1−6)(Gal(β1−4)GlcNAc(β1−3))Gal(β1−4)Glc									6·42
	1353·55	1353·49	–	–	–	2	≤1	–		
N29	2Gal(α1−3) + Gal(β1−4)GlcNAc(β1−6)(Gal(β1−4)GlcNAc(β1−3))Gal(β1−4)Glc									7·36
	1515·55	1515·55	–	–	–	≤1	≤1	–		

FLD, fluorescence detection; GU, glucose unit reference values; Fuc, fucose; Gal, galactose; Glc, glucose; FL, fucosyllactose; GlcNAc, *N*-acetyl-glucosamine; FLN, fucosyl-*N*-acetyl-lactosamine; GalNAc, *N*-acetylgalactosamine; LNnT, lacto-*N*-neo-tetraose; LNFP, lacto-N-fucopentaose; LNnP, lacto-*N*-novo-pentaose; LNnH, lacto-*N*-neo-hexaose; FLNH, fucosylated lacto-*N*-hexaose.

* Relative peak area and GU correspond to the average (av) observed for the respective species, which were each analysed in duplicate or triplicate (ΔGU(av) ≤ 0·03).
† Oxford symbol nomenclature[30] (Fig. 1(b)).
‡ Structures also reported for human milk[6,7].
§ Most recent reference was included for identified structures that were previously assigned for the respective milk samples.
|| Co-eluting structures; relative abundance accounts for both structures.

6′ isomer is more abundant than the 3′ isomer[7]. Animal milk additionally contained two pairs of SLN isomers (3′/6′-SLN (Neu5Ac(α2−3)Gal(β1−4)GlcNAc, A5/Neu5Ac(α2−6)Gal(β1−4)GlcNAc, A8) and 3′/6′GLN (Neu5Gc(α2−6)Gal(β1−4)GlcNAc, A7/Neu5Gc(α2−6)Gal(β1−4)GlcNAc, A13)) as well as five disialylated lactoses/*N*-acetyl-lactosamines (A20, A21 A23, A25, A28 and A31). The presence of two acetylated sialyllactose isomers (A1 and A2) was confirmed by mass fragmentation, and traces of a fucosyl-sialyllactose (Neu5Ac(α2−3)Gal(β1−4) (Fuc(α1−3))Glc, A12) were identified by enzymatic digestion.

Sialylated galactotriose and hexosaminyl-lactose. In animal milk, two pairs of sialylated galactotriose isomers (3″/6′-Neu 5Ac-galactotriose (A16 and A18) and 3″/6′-Neu5Gc-galactotriose (A17 and A19)) and two disialylgalactotrioses (3″-Neu5Ac-6′-Neu5Ac-galactotriose (A30) and 3″-Neu5Gc-6′-Neu5Gc-galactotriose (A33)) were identified. These isomers have not been detected in human milk[7] and had Gal(β1−3) Gal(β1−4)Glc backbone structures as revealed by sialidase digestion. Trace amounts of a galactotriose decorated with hexuronic acid were observed in camel milk (A24). As hexuronic acid is not a common milk oligosaccharide component, the oligosaccharide was assumed to be a degradation product or of different origin. In animal milk, two sialylated hexosaminyl-lactoses (3′/6′-Neu5Ac-hexosaminyl-lactose, A10 and A14) were also identified.

Sialylated lacto-N-neo-tetraose. In animal milk, 3′/6′-Neu5Ac-LNnT (A22/A26, the 6′-isomer has been identified previously in human milk[7]) was identified and traces of a Neu5Gc-LNnT isomer (A29) were also observed.

Sialylated lacto-N-novo-pentaose and lacto-N-neo-hexaose. In the present study, fourteen monosialylated and disialylated isomers and derivatives of LNnP and LNnH were observed in the milk of different animals (A27, A32 and A34−A45, with 6′-sialyl-lacto-*N*-neo-hexaose (SLNnH) (A37) also being identified previously in human milk[7]). The sialic acid residues are exclusively attached to the non-reducing terminal Gal units and located on different sugars in case of disialylation.

Structural characterisation of phosphorylated oligosaccharides in domestic animal milk

Phosphorylated oligosaccharides were identified with the help of a phosphatase sourced from calf intestine. To include oligosaccharides that are phosphorylated at the 1-O position of the reducing end sugar, the acidic oligosaccharide pools were incubated with calf-intestinal alkaline phosphatase before and after labelling the reducing end with fluorescent 2-AB. Glycans resulting from enzymatic digestion could be identified by their GU values in the UPLC-HILIC-FLD profiles. In contrast to human milk oligosaccharides, for which phosphorylation has not been observed[7], three phosphorylated structures were identified in animal milk in the present study (Table 3), namely *N*-acetyl-lactosamine-1-O-phosphate (P1), *N*-acetyl-lactosamine phosphate with phosphate group attached to a non-reducing position (P2) and lactose phosphate (P3).

Discussion

A structural library of neutral, fucosylated, sialylated and phosphorylated oligosaccharides from bovine, caprine, ovine, porcine, equine and dromedary camel milk was established in the present study. A total of forty-eight structures were identified in camel milk and forty-one structures were characterised in caprine milk. Furthermore, forty structures could be assigned for porcine and equine milk and thirty-eight and thirty-five structures were identified in ovine and bovine milk, respectively (Tables 1−4). Many of these oligosaccharides have not been reported previously, and many more could be present as it was not possible to assign all minor peaks, which accounted for less than 1% of the total oligosaccharide content. Indeed, supplementary structures have been reported previously[5,10,17]. This is particularly the case for bovine milk, which has been extensively studied and for which more than fifty structures have been identified, including interesting novel fucosylated structures present

Table 2. Structures and their relative abundances in the acidic oligosaccharide pools from animal milk

No.	Name of the proposed structure		Presence and relative peak abundance (FLD) in the acidic pool*						Symbol of the proposed structure†	GU*
	$[M-H]^-$ m/z observed	$[M-H]^-$ m/z theoretical	Cow	Goat	Sheep	Pig	Horse	Camel		
A1	Ac-O-Neu5Ac-Gal(β1−4)Glc									
	794·28	794·31	–	–	–	–	7	–		2·43
A2	Ac-O-Neu5Ac-Gal(β1−4)Glc									
	794·28	794·31	≤1[11]‡	≤1	–	–	–	–		2·47
A3	Neu5Ac-Hex-Hex									
	835·35	835·31	–	–	–	–	≤1	≤1		2·82
A4	Neu5Ac-Hex-Hex									
	835·35	835·31	–	–	–	–	≤1	≤1		2·96
A5	Neu5Ac(α2−3)Gal(β1−4)GlcNAc (3′-SLN)									
	793·33	793·30	≤1[12]	≤0·1[8]	≤0·1	≤1[13]	2	≤1		3·04
A6§	Neu5Ac(α2−3)Gal(β1−4)Glc (3′-SL)									
	752·29	752·27	75[11]	13[8]	4[35]	51[13]	81[36]	70[14]		3·15
A7	Neu5Gc(α2−3)Gal(β1−4)GlcNAc (3′-GLN)									
	809·34	809·29	–	≤1	≤1	–	–	≤0·1		3·37
A8	Neu5Ac(α2−6)Gal(β1−4)GlcNAc (6′-SLN)									
	793·33	793·30	3[11]	≤1[37]	≤0·1	≤1	≤1	≤1		3·40
A9	Neu5Gc(α2−3)Gal(β1−4)Glc (3′-GL)									
	768·30	768·27	≤1[11]	30[8]	70[35]	≤1	≤1	≤1		3·49
A10	Neu5Ac(α2−6)GlcNAc(β1−6)Gal(β1−4)Glc									
	955·34	955·35	–	–	–	24	–	–		3·55
A11§	Neu5Ac(α2−6)Gal(β1−4)Glc (6′-SL)									
	752·29	752·27	7[11]	18[8]	2	≤1[13]	2	19[14]		3·56
A12	Neu5Ac(α2−3)Gal(β1−4)(Fuc(α1−3))Glc									
	898·36	898·33	–	–	–	–	≤1	–		3·66
A13	Neu5Gc(α2−6)Gal(β1−4)GlcNAc (6′-GLN)									
	809·34	809·29	≤1[11]	2	≤1	–	–	–		3·72
A14	Neu5Ac(α2−3)GlcNAc(β1−3)Gal(β1−4)Glc									
	955·34	955·35	≤1[38]	≤1	≤0·1	–	–	≤1		3·84
A15	Neu5Gc(α2−6)Gal(β1−4)Glc (6′-GL)									
	768·30	768·27	≤1[11]	27[8]	17[35]	–	–	≤0·1		3·91

Table 2. *Continued*

No.	Name of the proposed structure [M − H]⁻ *m/z* observed	[M − H]⁻ *m/z* theoretical	Cow	Goat	Sheep	Pig	Horse	Camel	Symbol of the proposed structure†	GU*
					Presence and relative peak abundance (FLD) in the acidic pool*					
A16	Neu5Ac(α2−3)Gal(β1−3)Gal(β1−4)Glc									
	914·36	914·33	≤1[39]	≤1[40]	≤1	≤1	≤1	4[14]		4·00
A17	Neu5Gc(α2−3)Gal(β1−3)Gal(β1−4)Glc									
	930·38	930·32	–	≤1[8]	–	–	–	–		4·16
A18	Gal(β1−3)(Neu5Ac(α2−6))Gal(β1−4)Glc									
	914·36	914·33	–	–	–	–	≤0·1	≤0·1		4·23
A19	Gal(β1−3)(Neu5Gc(α2−6))Gal(β1−4)Glc									
	930·38	930·32	–	≤1[8]	3	–	–	–		4·40
A20	Neu5Ac(α2−8)Neu5Ac(α2−3)Gal(β1−4)GlcNAc (DSLN)									
	1084·45	1084·39	≤1[39]	–	–	≤1	≤1	–		4·44
A21	Neu5Ac(α2−8)Neu5Ac(α2−3)Gal(β1−4)Glc (DSL)									
	1043·41	1043·37	6[11]	≤1[8]	≤1	–	2	≤1		4·57
A22	Neu5Ac(α2−3)Gal(β1−4)GlcNAc(β1−3)Gal(β1−4)Glc (3′-SLNnT)									
	1117·45	1117·40	–	–	–	4[13]	–	–		4·62
A23	Neu5Gc(α2−8)Neu5Ac(α2−3)Gal(β1−4)Glc (GSL)									
	1059·43	1059·36	≤1[11]	≤1[8]	≤1	–	≤1	–		4·72
A24	HexAc-Gal-Gal(β1−4)Glc									
	799·27	799·26	–	–	–	–	–	≤1	**+ HexAc**	4·72
A25	Neu5Gc(α2−8)Neu5Gc(α2−3)Gal(β1−4)Glc (DGL)									
	1075·40	1075·36	≤1	–	–	–	–	–		4·97
A26§	Neu5Ac(α2−6)Gal(β1−4)GlcNAc(β1−3)Gal(β1−4)Glc (6′-SLNnT)									
	1117·45	1117·40	–	–	–	≤1[13]	–	–		4·98
A27	Neu5Ac(α2−3)Gal(β1−4)GlcNAc(β1−6)(GlcNAc(β1−3))Gal(β1−4)Glc									
	1320·40	1320·48	–	–	–	≤1	–	–		5·10
A28	Neu5Gc-Neu5Gc-Gal(β1−4)Glc (DGL)									
	1075·40	1075·36	–	2	≤1	–	–	≤0·1		5·14
A29	Neu5Gc + Gal(β1−4)GlcNAc(β1−3)Gal(β1−4)Glc (GLNnT)									
	1133·43	1133·40	–	≤0·1	≤0·1	–	–	–		5·37

Table 2. *Continued*

No.	Name of the proposed structure [M − H]⁻ *m/z* observed	[M − H]⁻ *m/z* theoretical	Presence and relative peak abundance (FLD) in the acidic pool*						Symbol of the proposed structure†	GU*
			Cow	Goat	Sheep	Pig	Horse	Camel		
A30	Neu5Ac(α2−3)Gal(β1−3)(Neu5Ac(α2−6))Gal(β1−4)Glc									
	602·21‖	602·21‖	≤0·1	–	–	≤1	≤0·1	≤1		5·48
A31	Neu5Gc-Neu5Gc-Gal(β1−4)Glc (DGL)									
	1075·40	1075·36	–	≤0·1	–	–	–	–		5·68
A32	Neu5Ac(α2−3) + Gal(β1−4)GlcNAc(β1−6)(Gal(β1−3))Gal(β1−4)Glc (3-SLNnP I)									
	1279·48	1279·46	≤1⁽¹¹⁾	–	–	≤1⁽¹³⁾	≤1	≤1		5·83
A33	Neu5Gc(α2−3)Gal(β1−3)(Neu5Gc(α2−6))Gal(β1−4)Glc									
	618·30‖	618·20‖	–	≤0·1⁽⁸⁾	≤0·1	–	–	≤0·1		6·34
A34	Neu5Ac(α2−6) + Gal(β1−4)GlcNAc(β1−6)(Gal(β1−3))Gal(β1−4)Glc (6-SLNnP I)									
	1279·48	1279·46	≤0·1⁽¹¹⁾	–	–	2⁽¹³⁾	≤0·1	–		6·38
A35	Neu5Ac(α2−3) + Gal(β1−4)GlcNAc(β1−6)(Gal(β1−4)GlcNAc(β1−3))Gal(β1−4)Glc (3-SLNnH)									
	1482·56	1482·54	≤1⁽¹¹⁾	–	–	≤1⁽¹³⁾	≤0·1	≤0·1		6·44
A36	Neu5Gc(α2−3) + Gal(β1−4)GlcNAc(β1−6)(Gal(β1−3))Gal(β1−4)Glc (3GLNnP I)									
	1295·59	1295·45	–	≤0·1	≤0·1	–	–	–		6·42
A37§	Neu5Ac(α2−6) + Gal(β 1−4)GlcNAc(β1−6)(Gal(β1−4)GlcNAc(β1−3))Gal(β1−4)Glc (6-SLNnH)									
	1482·56	1482·54	≤1⁽¹¹⁾	–	–	4⁽¹³⁾	≤1	≤1⁽¹⁴⁾		6·83
A38	Neu5Gc(α2−6) + Gal(β1−4)GlcNAc(β1−6)(Gal(β1−3))Gal(β1−4)Glc (6GLNnP I)									
	1295·59	1295·45	–	≤0·1	≤0·1	–	–	–		6·83
A39	Neu5Gc(α2−3) + Gal(β1−4)GlcNAc(β1−6)(Gal(β1−4)GlcNAc(β1−3))Gal(β1−4)Glc (3-GLNnH)									
	1498·53	1498·53	–	≤0·1	–	–	–	–		6·95
A40	2Neu5Ac + Gal(β1−4)GlcNAc(β1−6)(Gal(β1−3))Gal(β1−4)Glc (DSLNnP I)									
	784·78‖	784·77‖	–	–	–	≤1	≤0·1	–		6·96

The Symbol column contains glycan structure diagrams.

Table 2. *Continued*

No.	Name of the proposed structure		Presence and relative peak abundance (FLD) in the acidic pool*						Symbol of the proposed structure†	GU*
	[M − H]⁻ *m/z* observed	[M − H]⁻ *m/z* theoretical	Cow	Goat	Sheep	Pig	Horse	Camel		
A41	Neu5Gc(α2−6) + Gal(β1−4)GlcNAc(β1−6)(Gal(β1−4)GlcNAc(β1−3))Gal(β1−4)Glc (6GLNnH)									
	1498·53	1498·53	–	≤0·1	≤0·1	–	–	–		7·29
A42	2Neu5Ac + Gal(β1−4)GlcNAc(β1−6)(Gal(β1−3))Gal(β1−4)Glc (DSLNnP I)									
	784·78‖	784·77‖	≤1	–	–	≤1¶ [13]	–	–		7·41
A43	2Neu5Ac + Gal(β1−4)GlcNAc(β1−6)(Gal(β1−4)GlcNAc(β1−3))Gal(β1−4)Glc (DSLNnH)									
	886·32‖	886·31‖	≤1	–	–	≤1¶ [13]	–	–		7·41
A44	2Neu5Ac + Gal(β1−4)GlcNAc(β1−6)(Gal(β1−4)GlcNAc(β1−3))Gal(β1−4)Glc (DSLNnH)									
	886·32‖	886·31‖	–	–	–	–	≤0·1	–		7·76
A45	Neu5Ac + Gal(α1−3) + Gal(β1−4)GlcNAc(β1−6)(Gal(β1−4)GlcNAc(β1−3))Gal(β1−4)Glc									
	1644·59	1644·62	–	–	–	≤1	–	–		7·76

FLD, fluorescence detection; GU, glucose unit reference values; Neu5Ac, *N*-acetylneuraminic acid; Gal, galactose; Glc, glucose; GlcNAc, *N*-acetyl-glucosamine; SLN, sialylated *N*-acetyllactosamine; SL, sialyllactose; Neu5Gc, *N*-glycolylneuraminic acid; GLN, glycolyl-*N*-acetyllactosamine; GL, glycoyl-lactose; Fuc, fucose; DSLN, disialyl-*N*-acetyllactosamine; DSL, disialyl-lactose; SLNnT, sialyl-lacto-*N*-neo-tetraose; GSL, glycolyl-sialyl-lactose; HexAc, hexuronic acid; DGL, diglycolyl-lactose; GLNnT, glycolyl-lacto-*N*-neo-terarose; SLNnP, sialyl-lacto-*N*-neo-pentaose; SLNnH, sialyl-lacto-*N*-neo-hexaose; GLNnP, glycolyl-lacto-*N*-neo-pentaose; GLNnH, glycolyl-lacto-*N*-neo-hexaose; DSLNnP, disialyl-lacto-*N*-neo-pentaose; DSLNnH, sialyl-lacto-*N*-neo-hexaose.
* Relative peak area and GU correspond to the average (av) observed for the respective species, which were each analysed in duplicate or triplicate (ΔGU$_{(av)}$ ≤ 0·03).
† Oxford symbol nomenclature[30] (Fig. 1(b)).
‡ Most recent reference was included for identified structures that were previously assigned for the respective milk samples.
§ Structures also reported for human milk[6,7].
‖ *m/z* observed ([M − 2H]²⁻).
¶ Co-eluting structures; relative abundance accounts for both structures.

in minor proportions[10,11]. The qualitative and quantitative composition of oligosaccharides in animal milk is variable and complicates the comparison between different studies and species. On the one hand, variations might be due to the use of different analytical techniques and oligosaccharide extraction methods. On the other hand, the milk oligosaccharide composition is subject to genetic variation, and the number and concentration of glycans decrease rapidly over the course of lactation[22,23]. The most significant decrease in bovine colostrum oligosaccharides can be observed during the first 12−24 h after calving[9,22]. In particular, a shift towards

a higher percentage of neutral glycans is observed in time. Whereas sialylation is >80 % in bovine colostrums, it decreases to approximately 40 % in milk collected after 6 d[22]. Representative pools of colostrum milk (*n* ≥ 3) were used in the present study, except for camels and goats, for which pooled mature milk (*n* ≥ 3) was used. Indeed, twenty-three of the forty-five structures identified for camel milk belonged to the neutral oligosaccharide fraction, which had the highest number of neutral glycans observed for all the animal species in the present study and which might be due to the use of mature milk instead of colostrum. However, for mature

Table 3. Qualitative presence of phosphorylated oligosaccharides in the acidic oligosaccharide pools from animal milk

No.	Name of the proposed structure	Presence (X) in the acidic pool						Symbol of the proposed structure*
		Bovine	Goat	Sheep	Pig	Horse	Camel	
P1	Gal(β1−4)GlcNAc-O-P	–	–	X	–	X[36]†	X	
P2	P + Gal(β1−4)GlcNAc	X	–	–	–	–	–	
P3	P + Gal(β1−4)Glc	X[41]	X	X	–	X	X	

Gal, galactose; GlcNAc, *N*-acetyl-glucosamine; P, phosphate.
* Oxford symbol nomenclature[30] (Fig. 1(b)).
† Most recent reference was included for identified structures that were previously assigned for the respective milk samples.

Table 4. Overview of glycan structures in the milk of domestic animals identified in the present study (Number of structures and percentages)

	Bovine		Goat		Sheep		Pig		Horse		Camel	
	n	%	n	%	n	%	n	%	n	%	n	%
Total UPLC-HILIC-FLD oligosaccharide profile												
Peak area for neutral oligosaccharides		9		5		14		18		10		16
Peak area for acidic oligosaccharides		91		95		86		82		90		84
Total number of structures identified (neutral + acidic + phosphorylated)	35		40		37		39		40		45	
New structures identified for this species	5		19		31		20		32		38	
Structures in the neutral oligosaccharide pool	12		16		16		19		17		23	
Number and peak area of fucosylated structures in the neutral oligosaccharide pool	3	6	3	5	3	3	3	3	2*	<1	7	6
Structures in the acidic oligosaccharide pool	21		23		19		20		21		20	
Number and peak area of large structures† in the acidic oligosaccharide pool	6	1	4	<1	3	<1	9	10	6	1	3	1
Phosphorylated structures	2		1		2		–		2		2	
Structures also present in human milk	8		8		8		11		8		12	
Sialic acid composition (DMB speciation)												
Proportion of Neu5Ac/Neu5Gc		97/3		36/64		6/94		99·9/0·1		99/1		99·7/0·3

UPLC-HILIC-FLD, ultra-performance liquid chromatography−hydrophilic interaction liquid chromatography with fluorescence detection; DMB, 1,2-diamino-4,5-methylenedioxy-benzene; Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid.
* Whereof one structure in the acidic oligosaccharide pool.
† Large structures: ≧6 monosaccharide units.

caprine milk, the number and percentage of neutral oligo-saccharides were comparable with what was found for colostrum milk from other animal species.

Our comparative analysis revealed not only many similarities but also species-specific characteristics. Sialylated oligosaccharides accounted for approximately 80−90 % of the total oligosaccharide pools from the milk of all domestic animals. The neutral and acidic oligosaccharides from bovine, caprine and ovine milk were mainly of short chain length. The proportion of Neu5Gc-containing oligosaccharides in the acidic oligosaccharide fraction of caprine (64 %) and ovine (94 %) milk was highest, whereas only traces of Neu5Gc-containing oligosaccharides were observed in the milk from pigs and camels. Compared with the milk from other animal species, that from horses and camels contained a more balanced mixture of shorter and larger oligosaccharides. Interestingly, milk oligosaccharides from dromedary camels were exceptionally diverse and included seven fucosylated structures. The oligosaccharide mixture in porcine milk is unique. Among all the milk samples of the domestic animal species investigated, porcine milk contained the highest percentage of neutral oligosaccharides (approximately 20 %) and the most abundant variety of monosialylated and disialylated large oligosaccharides. Porcine milk oligosaccharides come closest to the human milk oligosaccharide mixture, which clearly demonstrates that milk oligosaccharides are tailor-made for the postnatal development of certain gastrointestinal systems and that these molecules aid in the preparation for certain post-weaning diets by acting as early dietary fibres. Humans and pigs are monogastric and therefore large bowel fermenters, whereas cows, goats and sheep are ruminants; camels have a three-chambered digestive system and horses have an extended caecal digestive system. Furthermore, humans and pigs are omnivores, whereas all other species are herbivores. Pigs are considered to be excellent models for human large bowel fibre metabolism, and the similarity between the human and porcine milk glycomes reinforces their usefulness in studying early-life nutritional programming and development of the gastrointestinal system[24,25].

The present study further highlights the distinct difference between the nature and number of oligosaccharides in human and domestic animal milk, in general, with merely thirteen common oligosaccharides (Tables 1−4). Different from domestic animal milk oligosaccharides, approximately 70 % of the human milk oligosaccharides are neutral and largely fucosylated. Human milk oligosaccharides contain neither Neu5Gc nor (α1−3)-linked galactose, and these non-human structural components might cause immunogenic reactions upon human consumption[26,27]. In human milk, oligosaccharides of the lacto-*N*-biose type I structure (Gal(β1−3)GlcNAc) predominate over those containing the *N*-acetyllactosamine type II structure (Gal(β1−4)GlcNAc). The milk of domestic animals contains exclusively type II oligosaccharides. In the milk of few other species, such as chimpanzees and elephants, type I oligosaccharides predominate over type II[28,29]. Type I oligosaccharides are specific for human milk and may serve as substrates for beneficial bifidobacteria in breast-fed infants[29].

Despite the obvious differences between animal and human milk oligosaccharides, the present study clearly demonstrates that milk from domestic animals contains a much larger variety of complex oligosaccharides than previously thought. Therefore, domestic animal milk is a highly attractive source of bioactive oligosaccharides, which once extracted can be potentially used in medical and functional foods. To justify their use, additional studies on their biological functions are required. The advanced knowledge on their structural composition will certainly help to gain new insights into the complex biochemical pathways underlying their potential health-promoting properties.

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