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# Non-targeted metabolomics by $^{1}$H nuclear magnetic resonance spectroscopy; an interspecies comparison of milk between dromedary, giraffe and white rhinoceros with observations on blesbok

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ABSTRACT

Various researchers have shown that milk metabolomes differ between domestic species regarding metabolite composition as well as metabolic pathways of milk synthesis. We contribute to this knowledge by presenting data of wild mammals. Inter-species differences were noted; 3-fucosyllactose was only detected in the milk of dromedary, and *N*-phenylacetylglycine and UDP-*N*-acetylglucosamine only in giraffe. Hippurate and 2-oxaloacetate were absent from milk of dromedary; creatinine and glutamate from giraffe; nicotinamide from white rhinoceros; and dimethylamine, creatine, valine, acetone, fumarate and 2-oxaloacetate from blesbok. With MetaboAnalyst 6.0, 15 statistically important metabolites of dromedary, giraffe and white rhinoceros were identified for KEGG metabolic pathway enrichment. Twelve metabolic pathways were identified as important with a specialization within species. In the giraffe mammary cells, the amino sugar and nucleotide sugar metabolism was observed as active and the alanine, aspartate and glutamate metabolism as inactive. The glycerophospholipid metabolism seemed highly active in the white rhinoceros and the galactose metabolism active in both the dromedary and white rhinoceros. Milk was available from only two blesbok, but data is presented as observation.

## 1. Introduction

The human breast milk metabolome had been studied in some detail (Dessì et al., 2018; Nolan et al., 2021; M. Li et al., 2022). Less is known of the milk metabolome of other mammals and most report on domesticated species such as cattle (*Bos taurus*) (Scano et al., 2014; Tenori et al., 2018; Settachaimongkon et al., 2021), goats (*Capra hircus*) (Scano et al., 2014), sheep (*Ovis aries*) (Cabrera et al., 2023), Bactrian camels (*Camelus bactrianus*) (R. Li et al., 2022) and donkeys (*Equus asinus*) (Li et al., 2020; Garhwal et al., 2023). The information on milk metabolomics finds application in dairy herd authenticity (Tenori et al., 2018), animal nutrition (Xu et al., 2020) and food quality and traceability (Rocchetti and O'Callaghan, 2021). Little information is currently available for other species such as the giant panda (Zhang et al., 2015), African elephant (Osthoff et al., 2023), Asian elephant (Galante et al., 2024) and white rhinoceros (Osthoff and Nieuwoudt, 2024).

It is difficult to compare milk metabolomes because the aims of studies often differ, with some focusing on specific metabolites while others opt for a non-targeted approach. The extraction methods may also differ between studies. Some reports highlight hydrophobic metabolites, for which analysis by gas chromatography (GC) is typically used (Scano et al., 2014; Settachaimongkon et al., 2021), while others focus on hydrophilic metabolites, where liquid chromatography (LC) is preferred (Dessì et al., 2018; Yang et al., 2024). Ever more sensitive techniques are employed for metabolite detection in both these chromatographic analyses such as mass spectrometry (MS), electrospray ionization (ESI)–MS/MS, triple quadrupole/linear ion trap (QTRAP) and quadrupole time-of-flight mass spectrometry (QTOF-MS). In recent years $^{1}$H-nuclear magnetic resonance spectroscopy ($^{1}$H NMR) has become popular as an analytical method for the characterization, detection and quantification of metabolites (Nolan et al., 2021; Garhwal et al., 2023). NMR was shown to have several unique characteristics such as being highly reproducible, allows identification of unknown metabolites and makes it possible to quantify absolute concentrations of

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all detected metabolites, even without internal standards. It is furthermore a non-destructive method that is suitable for the analysis of biological fluids such as serum and milk. These characteristics may outweigh its relatively low sensitivity and resolution (Gowda and Raftery, 2023).

A comparative metabolomic analysis of yak and human milk showed more than a 70 % variance. Yak milk contained mainly essential- and branched-chain amino acids, while human breast milk contained mainly non-essential amino acids or derivates (Li et al., 2023). In a comparative study of three ruminant species, cow, goat and sheep, major differences in milk metabolome were observed, with 414 of 587 (71 %) polar metabolite features and 210 of 233 (87 %) lipid features significantly different between species (Cabrera et al., 2023). *N*-acetylglucosamine, *N*-acetylgalactosamine (Rysova et al., 2020), talose and malic acid were reported as specific to cow milk, and valine and glycine specific to goat milk (Scano et al., 2014). A comparative study of four species, representative of three taxa, showed that metabolites as well as metabolic pathways may differ (Wu et al., 2021).

In the current study we investigated four different species; the dromedary (*Camelus dromedarius*), giraffe (*Giraffa camelopardalis*), white rhinoceros (*Ceratotherium simum*) and blesbok (*Damaliscus philipsii*). They are four different species that represent three different taxa, which are distinguished by their gastro-intestinal systems. The Tylopoda (camels) have a three-chamber stomach, the Ruminantia (giraffe, blesbok and other Pecora) have a four-chamber stomach, while the Perissodactyla (rhinoceroses, horses and tapirs) are single stomach hindgut fermenters (O'Donnell et al., 2017). In the milk, this difference is noted in the fatty acid composition of the milk fat. Milk of the ruminant families (Cervidae, Giraffidae and Bovidae), are characterized by the presence of up to 15 % short chain fatty acids (4:0 and 6:0). In the Camelidae these fatty acids occur at approximately 1 % and are absent in the Perissodactyla (Osthoff et al., 2021; Osthoff, 2022). The bacteria in the ruminant stomach produce high amounts of butyric and hexanoic acids, which are absorbed by the blood, transferred to the milk gland cells and directly incorporated in their fat. The intestine of the Camelidae have little of these microorganisms and non-ruminants have none (Bhatt et al., 2013; Boukerrou et al., 2023). The proximate composition of milk also differs between these species and taxa. The milk of camels contains approximately 4.5 % lactose, 4 % protein and 4–5 % fat (Farah, 1993), of the giraffes approximately 4.5 % lactose, 5 % protein and 7.5 % fat (Osthoff et al., 2017), while milk of rhinoceroses has a high lactose (>6 %), low protein (<1.6 %) and low fat (<0.2 %) content (Osthoff et al., 2021).

In a recent study the metabolome of colostrum and early lactation milk of the white rhinoceros was reported and compared to that of other species, specifically with data of the donkey, that is also a member of the Perissodactyla (Osthoff and Nieuwoudt, 2024). A conclusive parallel could not be drawn between different reports, firstly because different analytical methods were employed and secondly because the postpartum sampling intervals differed.

As mentioned, the milk composition and milk fat composition may differ between taxa but may be conserved between taxa (Osthoff et al., 2017; Osthoff, 2022). The aim of the current study was to determine whether the milk metabolome and perhaps preferred metabolic pathways also differ between taxa so that the difference in milk composition could be explained. The current work describes the metabolomic profile of the milk of four species, representing three taxa, to determine the extent of the differences. Furthermore, $^{1}$H NMR was used as analytical method of all to avoid the mentioned problems that prevent comparison of the metabolomes.

## 2. Materials and methods

The research reported here complies to the guidelines of the American Society of Mammalogy (Sikes, 2016) and the Animals Research Ethics of the University of the Free State (UFS-AED2016/0106 and UFS-AED2019/0052), Permission to do research in terms of section 20 of the Animal diseases act, 1984 (Act no. 35 of 1984) of South Africa (permit reference 12/11/1/4), Threatened or Protected Species Regulations (TOPS) (standing permit no. S07969, and registration certificate no. 29414) and South African National Parks Material Transfer Agreement 003/24. The collection of samples was opportunistic. Parity and sex of offspring of most of the sampled animals was unknown.

Dromedary milk was obtained from Koppieskraal Kameelplaas, Askham, Northern Cape province, South Africa. The animals are kept for commercial milk production and roamed on natural vegetation of Gordonia Duneveld (SVkd1) (Mucina and Rutherford, 2006) supplemented with lucern and teff. Five animals were sampled and 9 milk samples of 100 ml obtained. The stage of lactation of the animals was between 2 and 5 months.

The giraffes under study were from two different sites. The first was the Rooipoort Game reserve, Kimberley, Northern Cape, South Africa. Giraffes were sedated and fitted with radio transmitters during early summer of 2017, while transmitter removal was a year later (2018). Four of these were lactating females of which twelve milk samples of 50 ml were obtained. The animals were free roaming and browsed on natural vegetation of Kimberley Thornveld (SVk4) and Vaalbos Rocky Shrubland (SVk5) (Mucina and Rutherford, 2006). This reserve experienced extreme drought conditions during the two years when milk was collected. The second site was Sandveld Nature Reserve, Hoopstad, Free State Province, South Africa, where two animals were culled for managerial reasons and eight milk samples of 50 ml were obtained five minutes postmortem. They roamed on Kimberley Thornveld (SVk4) (Mucina and Rutherford, 2006). The stage of lactation of the giraffes ranged between three and five months.

Milk of two blesbok was obtained from a commercial game ranch 30 km west of Bloemfontein, Free State Province during an annual harvest cull. The animals roamed on vegetation of Dry Cymbopogon-Themeda Veld (Mucina and Rutherford, 2006). The animals were approximately five months in lactation. Milk (50 ml) was drawn not later than 15 min after death. Since only two samples were obtained from this species; the data was therefore reported as observation and was not included in the statistical metabolomic comparison.

Milk (50 ml) was obtained from seventeen white rhinoceroses from two semi-extensive wildlife ranches in the Northern Cape province of South Africa. The animals were in good health and roamed on natural vegetation, which was supplemented with lucerne during the dry times of 2017–2019. The natural vegetation fell in three savannah biomes; Schmidtsdrif Thornveld (SVk 6), Kuruman Mountain Bushveld (SVk 10), and the Olifantshoek Plains Thornveld (SVk 13) (Mucina and Rutherford, 2006). The rhinoceroses were tranquilized for routine veterinarian inspections and pregnancy tests with 6 mg etorphine, 2500 IU hyaluronidase and 40 mg azaperone with a Parmer dart. Reversal was by administration of diprenorphine. Eleven animals between 0.5 and 18 months lactation were sampled during May 2018, and four between 11 and 13 months lactation during May 2019. The same milk samples were previously used to determine the milk composition over lactation (Osthoff et al., 2021). The interindividual variation of all the milk components were found to be high and showed no trend of change over lactation. Hence all samples between 0.5 and 18 months lactation were included in the current study.

No milk letting agents were administered and milk was drawn from all animals by palpation of the teats while sustained pressure was exerted on the udder. Teats were milked out and milk from each teat was collected separately. Dromedaries were milked twice daily and produced between 7 and 9 L per milking. Giraffes and white rhinoceroses produced 5–40 ml milk per teat. Milk was kept on ice (1–2 h while in the field) until freezing facilities were available. For analysis, milk was thawed and mixed by swirling in a water bath set to 39 °C.

NMR analysis was carried out according to Erasmus et al. (2019), modified for extraction from milk. Milk samples were filtered with Amicon Ultra – 2 mL centrifugal units with 10 kDa membrane filters

(Merck, Darmstadt, Germany). Each centrifugal unit was pre-rinsed twice with 2 ml double distilled water at 4500 x g for 15 min in a Hettich EBA12 centrifuge with 1115 swing-bucket rotor (Hettich AG, Bäch, Switzerland). This step removes any trace amounts of glycerol from the membrane filter that may interfere with NMR signals. One ml of milk was placed in an Eppendorf tube, centrifuged at 12000 x*g* for five minutes and 600 µl serum was then filtered by centrifugation at 4500 x*g* for 30 min in a swing-bucket centrifuge using the above-mentioned membrane filters. To 540 µl of filtered serum, 60 µl NMR buffer solution (1,5 M potassium phosphate solution in deuterium oxide with internal standard trimethylsily1–2,2,3,3-tetradeuteropropionic acid {TSP}, pH 7.4) was added. The sample was mixed by vortex to ensure complete homogenicity and transferred to a 5 mm NMR tube for analysis.

The prepared samples were subjected to NMR spectroscopy on a Bruker Avance III HD NMR spectrometer, equipped with a triple resonance inverse (TXI) 1H {15 N, 13C} probe head and x, y, z gradient coils, at 500 MHz. $^{1}$H spectra were acquired as 128 transients in 32 K data points. The spectral width was 6002 Hz and an acquisition time of 2.72 s and receiver gain was set to 64. The sample temperature was maintained at 300 K and the $H_2O$ resonance was pre-saturated by single-frequency irradiation during a relaxation delay of 4 s with a 90° excitation pulse of 8 µs. Shimming of the sample was performed automatically on the deuterium signal. Fourier transformation and phase and baseline correction were done automatically. Software used for NMR processing was Bruker Topspin (V3.5). NMR spectral analysis, peak annotation and quantification were done using Bruker AMIX (V3.9.14).

Phase and baseline distortions of transformed spectra were corrected by using Topspin 3.2 (Bruker BioSpin, Billerica, Massachusetts, USA) and were automatically calibrated to the alanine signal at 1.48 ppm. Identification of signals was undertaken with the use of Chenomx (Edmonton, AB, Canada) or available assignments in the literature. The peaks of identified metabolites were fitted by a combination of a local baseline and Voigt functions according to the multiplicity of the NMR signal. The missing values were imputed using the k-nearest neighbor (kNN) algorithm (Benjamini and Hochberg, 1995) (with k = 5). These missing values were imputed with the lowest value of ethanol detected. Each metabolite was scaled to its mean value. Data were mean-centered and unit-variance scaled before multivariate analysis. Principal component analysis (PCA) and KODAMA (Addinsoft, 2020) were used to visualize the metabolomic data.

Statistical analysis was done using MetaboAnalyst 6.0 (Pang et al., 2024), a web server for metabolomics data analysis and interpretation, for the unsupervised principal component analysis (PCA), orthogonal partial least squares discriminant analysis (OPLS-DA), permutation tests of OPLS-DA and hierarchal cluster analysis. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (2024) was used to identify biosynthesis pathways.

## 3. Results

### 3.1. Metabolite profile

A total of 69 metabolites were detected in the milk of the three species (Table 1); 44 in the dromedary, 58 in the giraffe and 42 in the white rhinoceros samples (Fig. 1). Of these, 23 could not be annotated while 8 were only partially identified as UDP-saccharides and 5 as unassigned oligosaccharides (Table 1). Of the 46 annotated and partially identified metabolites, 34 were in dromedary milk, 42 in giraffe and 36 in rhinoceros. Of the annotated metabolites, 28 were shared between all three species, three between dromedary and giraffe, one between dromedary and white rhinoceros and six between giraffe and rhinoceros, while six metabolites were unique in the milk of dromedary and giraffe and only one in rhinoceros. The major component of the metabolome consisted of carbohydrates, of which only lactose, 3-fucosyllactose and 3′-siallyllactose were annotated, while the rest were only identifiable as saccharides, unassigned oligosaccharides and UDP-saccharides. Furthermore, there were amines, amino acids with derivatives, lipid derivatives, two nucleic acid derivatives, organic acids, and acetone. A PCA scatter plot (Fig. 2) of all the milk metabolites of the three species illustrated that the first two principal components (PC1 and PC2) accounted for 47.9 % and 29.2 % of the total variation, respectively. The samples were clustered within three groups according to species, indicating good repeatability of the samples. A heatmap (Fig. 3) was used to visualize the differences in metabolite abundance in the milk of the three species. The PCA plot and heat map showed that the giraffe milk samples formed two groups.

### 3.2. Significantly different metabolites

One-way ANOVA revealed 42 metabolites were significantly different between the 3 species, and a filtering step of significance based on an FDR correction (FDR <0.05) along with a Random Forest Model in Mean Decrease Accuracy >0.01 as well as by PERVANOVA with F-value >636.3, R-squared >0.96732 and *P*-value <0.001 yielded 26 metabolites which were subsequently identified. These are marked by an asterisk in Table 1. The dendrogram on the left side of the heat map (Fig. 3) shows clusters of milk metabolites that differ between the three mammals. There are three main clusters in which the significantly different metabolites fall, and each cluster is sub-divided into smaller clusters.

Saccharides are represented in each of these clusters and the top cluster mainly differentiates the milk metabolites of white rhinoceros from the other two species. Significantly different saccharides in the top cluster were unassigned oligosaccharide 2.07 ppm and UDP-saccharides at signals 5.36 ppm, 5.40 ppm, 5.46 ppm and 5.48 ppm. Lactose, glycerophosphocholine and acetate were detected in significantly high amounts in the white rhinoceros milk and in lower amounts in the milk of the other two species, while glutamate and creatinine were not detected in giraffe milk, and hippurate was absent in the dromedary milk.

The metabolites in the middle cluster of the heat map mainly distinguished dromedary milk from that of the other two species. The first important saccharides to be noted is 3-fucosyllactose, which was observed in the milk of only two of the five dromedaries, and 3′-siallyllactose, which occurred in amounts above 260 µM in dromedary milk, and in small quantities in the milk of a few giraffes and white rhinoceroses. Other saccharides found in the highest concentrations in dromedary milk were unassigned oligosaccharides 2.04 ppm, 2.06 ppm and 2.08 ppm.

Differentiating milk metabolites of giraffe milk were found in the bottom cluster of the heat map. UDP-*N*-acetylglucosamine and UDP-saccharides 5.61 ppm, 5.65 ppm and uridine were exclusive to giraffe milk, while UDP-saccharide 5.66 ppm was observed in the giraffe and white rhinoceros milk. Creatine, phosphocreatine, fumarate and pyruvate were found in significantly higher amounts in the milk of giraffe and dromedary, compared to the white rhinoceros milk, while nicotinamide did not occur in the latter.

It should be noted that, amongst the unassigned signals (Table 1), signals 7.94 ppm and 7.95 ppm occurred in amounts higher than 100 a. u. in dromedary milk, but were absent in giraffe and white rhinoceros milk, while 8.19 ppm occurred only in white rhinoceros milk, and 8.15 ppm was absent from giraffe milk.

Compared with the significantly different metabolites of the above three species, the milk of the blesbok contained no or only low amounts of amines, creatine, phosphocreatine, glutamate and *N*-phenylacetylglycine and high amounts of creatinine (Table 1). Of saccharides, lactose, 3′-sialyllactose, UDP-saccharide at 5,40 ppm, and unassigned oligosaccharides at signals 2.05 ppm, 2.07 ppm and 2.08 ppm were detected. The content of glycerophosphocholine, uridine, acetate, hippurate and pyruvate were in the same order of magnitude than that of the other three species, while fumarate was absent, and six unassigned signals were also detected.

**Table 1**
Metabolites detected in the milk of dromedary, giraffe and white rhinoceroses. Quantities of attenuated metabolites in μM and unassigned ones in arbitrary units. Metabolites with FDR < 0.05 and mean decrease accuracy > 0.01 are marked *.

| Group/metabolite | Dromedary | | Giraffe | | White rhinoceros | | Blesbok |
|---|---|---|---|---|---|---|---|
| | Range | Average ± STD | Range | Average ± STD | Range | Average ± STD | Range |
| Amines | | | | | | | |
| Dimethylamine | 37.7–407.0 | 175.9 ± 137.7 | 4.6–66.0 | 20.7 ± 13.8 | 23.8–67.2 | 45.6 ± 12.9 | 0 |
| *Nicotinamide | 7.9–31.6 | 21.0 ± 7.1 | 8.6–35.0 | 17.6 ± 9.4 | 0 | 0 | 0–20.4 |
| Amino acids & derivatives | | | | | | | |
| Acetylcarnitine | 63.4–189.2 | 109.3 ± 42.5 | 105.9–368.5 | 216.8 ± 105.4 | 16.7–170.6 | 47.0 ± 47.5 | 107.3–162.6 |
| Alanine | 18.8–114.7 | 65.8 ± 36.8 | 21.0–88.7 | 51.4 ± 19.5 | 22.6–208.7 | 92.1 ± 55.8 | 384.8–557.1 |
| *Creatine | 247.5–804.9 | 518.6 ± 187.3 | 1589.4–3469.8 | 2396.4 ± 681.1 | 46.7–982.2 | 323.9 ± 251.2 | 0 |
| *Phosphocreatine | 766.5–1640.7 | 1053.6 ± 306.9 | 945.2–3245.6 | 1716.0 ± 772.1 | 211.6–683.1 | 460.6 ± 128.3 | 0–1.0 |
| *Creatinine | 73.9–329.9 | 174.8 ± 91.5 | 0 | 0 | 50.1–1018.7 | 588.3 ± 320.9 | 995.1–1230.3 |
| Isoleucine | 8.4–45.1 | 27.7 ± 15.2 | 3.6–7.7 | 5.1 ± 1.2 | 0–88.0 | 9.6 ± 20.0 | 8.1–16.1 |
| *Glutamate | 58.6–120.1 | 85.4 ± 22.0 | 0 | 0 | 72.4–249.1 | 127.1 ± 51.6 | 0–203.8 |
| Tyrosine | 4.7–25.8 | 10.8 ± 6.8 | 0 | 2.6 ± 2.4 | 0–58.3 | 7.6 ± 13.3 | 15.0–27.5 |
| Valine | 32.8–83.1 | 61.6 ± 21.1 | 12.7–30.2 | 20.5 ± 5.0 | 4.6–120.7 | 25.3 ± 25.9 | 0 |
| **N*-phenylacetylglycine | 0 | 0 | 27.7–67.0 | 45.5 ± 14.1 | 0 | 0 | 0 |
| Carbohydrates & derivatives | | | | | | | |
| *UDP-N-Acetylglucosamine | 0 | 0 | 22.2–82.1 | 47.9 ± 25.0 | 0 | 0 | 0 |
| *UDP-saccharide at 5.66 ppm | 0 | 0 | 70.4–231.8 | 145.2 ± 61.2 | 0–61.4 | 11.7 ± 16.0 | 0 |
| *UDP-saccharide at 5.65 ppm | 0 | 0 | 79.1–597.6 | 284.1 ± 193.8 | 0 | 0 | 0 |
| *UDP-saccharide at 5.61 ppm | 0 | 0 | 129.2–419.7 | 252.7 ± 103.1 | 0 | 0 | 0 |
| UDP-saccharide at 5.50 ppm | 19.1–66.6 | 46.7 ± 19.5 | 548.0–1561.1 | 885.9 ± 309.8 | 50.1–1645.1 | 891.3 ± 332.1 | 0 |
| *UDP-saccharide at 5.46 ppm | 0 | 0 | 87.5–260.0 | 173.0 ± 50.7 | 20.9–299.8 | 186.8 ± 67.3 | 0 |
| UDP-saccharide at 5.48 ppm | 0 | 0 | 0 | 0 | 0–298.8 | 104.5 ± 64.4 | 0 |
| *UDP-saccharide at 5.40 ppm | 256.6–367.4 | 294.3 ± 34.8 | 488.2–952.7 | 683.6 ± 176.6 | 507.2–1157.0 | 905.9 ± 144.9 | 95.9–224.4 |
| *UDP-saccharide at 5.36 ppm | 0 | 0 | 178.2–497.7 | 306.0 ± 102.8 | 739.7–2851.0 | 2067.4 ± 448.3 | 0 |
| 3-Fucosyllactose | 0–512.6 | 139.7 ± 213.5 | 0 | 0 | 0 | 0 | 0 |
| *Lactose | 113,711.7–168,719.9 | 137,699.5 ± 17,911.4 | 103,991.0–139,135.1 | 123,762.8 ± 11,344.4 | 150,605.4–269,970.4 | 217,723.9 ± 27,906.8 | 85,702.5–93,162.9 |
| *3′-Sialyllactose | 266.5–675.4 | 405.3 ± 175.6 | 0–52.5 | 14.8 ± 21.3 | 0–43.9 | 14.0 ± 17.2 | 65.7–95.5 |
| *Unassigned oligosaccharide signal at 2.08 ppm | 333.6–616.6 | 431.3 ± 96.4 | 0–257.2 | 55.1 ± 91.8 | 0 | 0 | 142.2–205.4 |
| *Unassigned oligosaccharide signal at 2.07 ppm | 214.8–480.7 | 309.7 ± 93.5 | 1314.4–2975.1 | 2080.1 ± 566.6 | 1971.7–11,440.0 | 8063.2 ± 2253.6 | 225.1–352.0 |
| *Unassigned oligosaccharide signal at 2.06 ppm | 83.0–302.2 | 158.1 ± 67.4 | 0–1178.7 | 222.5 ± 325.6 | 0 | 0 | 0 |
| *Unassigned oligosaccharide signal at 2.05 ppm | 314.1–520.0 | 412.8 ± 81.4 | 90.1–219.9 | 121.0 ± 28.9 | 1179.3–3790.8 | 2033.1 ± 717.2 | 954.7–1032.8 |
| *Unassigned oligosaccharide signal at *0.04 ppm | 2295.5–4896.0 | 3220.0 ± 1075.2 | 335.0–526.0 | 413.3 ± 53.3 | 567.1–1463.1 | 1045.1 ± 322.2 | 0 |
| Carbonyls | | | | | | | |
| Acetone | 23.2–49.2 | 37.3 ± 10.6 | 2.78–12.34 | 16.8 ± 2.8 | 5.6–34.4 | 11.8 ± 7.1 | 0 |
| Lipid derivatives | | | | | | | |
| Choline | 36.1–229.0 | 104.6 ± 78.5 | 15.9–33.0 | 22.3 ± 4.4 | 6.4–234.1 | 74.5 ± 67.7 | 90.8–117.4 |
| *Glycerophosphocholine | 669.2–1121.7 | 896.9 ± 175.6 | 416.2–701.4 | 542.6 ± 79.2 | 1184.4–2326.2 | 1502.7 ± 269.0 | 704.5–810.9 |
| Phosphocholine | 622.9–854.3 | 781.6 ± 74.4 | 638.8–1145.9 | 835.6 ± 177.7 | 125.6–780.1 | 354.8 ± 183.4 | 156.2–269.7 |
| Nucleic acid derivatives | | | | | | | |
| Adenosine related | 0 | 0 | 0–5.6 | 1.6 ± 2.3 | 0 | 0 | 0 |
| NAD | 0 | 0 | 0–46.6 | 29.6 ± 10.8 | 0 | 0 | 8.1–10.9 |
| *Uridine | 0–51.4 | 21.0 ± 20.9 | 112.3–414.3 | 230.6 ± 109.2 | 0–86.4 | 14.8 ± 22.3 | 238.2–398.7 |
| Organic acids | | | | | | | |
| *Acetate | 34.8–128.7 | 60.8 ± 27.7 | 8.4–37.1 | 21.0 ± 9.2 | 44.0–406.6 | 121.7 ± 110.8 | 148.4–151.2 |
| *cis*-Aconitate | 25.2–55.4 | 36.7 ± 11.6 | 16.8–88.9 | 42.5 ± 23.3 | 0–32.1 | 13.1 ± 9.0 | 36.2–74.1 |
| Citrate | 10,729.7–16,851.3 | 12,788.6 ± 2015,5 | 7095.4–22,182.4 | 13,724.8 ± 5942.4 | 8460.7–15,710.0 | 10,940.5 ± 1664.6 | 5683.8–10,773.3 |
| Formate | 6.7–236.3 | 35.5 ± 75.4 | 4.5–13.1 | 7.6 ± 2.5 | 7.9–42.5 | 18.0 ± 8.0 | 10.5–14.3 |
| *Fumarate | 26.6–48.0 | 36.8 ± 7.8 | 43.4–246.0 | 116.3 ± 78.3 | 3.8–29.4 | 11.2 ± 6.5 | 0 |
| *Hippurate | 0 | 0 | 25.5–98.1 | 57.6 ± 23.9 | 22.0–238.9 | 65.8 ± 48.2 | 117.5–137.9 |
| Lactate | 51.9–130.0 | 95.0 ± 30.3 | 123.1–826.6 | 323.7 ± 180.6 | 118.0–1824.5 | 716.8 ± 466.2 | 2430.2–3512.0 |
| 2-Oxoglutarate | 0 | 0 | 0–786.5 | 273.8 ± 310.9 | 0–190.1 | 33.1 ± 65.3 | 0 |

(*continued on next page*)

**Table 1** (*continued*)

| Group/metabolite | Dromedary | | Giraffe | | White rhinoceros | | Blesbok |
|---|---|---|---|---|---|---|---|
| | Range | Average ± STD | Range | Average ± STD | Range | Average ± STD | Range |
| *Pyruvate | 84.9–194.2 | 140.2 ± 34.1 | 37.6–170.6 | 88.2 ± 49.8 | 0–29.9 | 7.0 ± 9.5 | 0–28.1 |
| Succinate | 33.1–68.8 | 43.6 ± 10.8 | 51.3–263.9 | 134.5 ± 65.8 | 9.0–66.9 | 31.0 ± 16.6 | |
| Unassigned signal | | | | | | | |
| 9.35 ppm | 0 | 0 | 34.7–88.7 | 68.3 ± 19.5 | 0 | 0 | 0 |
| 9.04 ppm | 0–7.0 | 3.3 ± 2.6 | 0 | 0 | 0 | 0 | 0 |
| 9.00 ppm | 0 | 0 | 34.2–87.8 | 63.8 ± 16.1 | 0 | 0 | 0 |
| 8.62 ppm | 0–24.6 | 9.3 ± 9.3 | 0 | 0 | 0 | 0 | 0 |
| 8.55 ppm | 0–18.2 | 5.2 ± 6.9 | 0 | 0 | 0 | 0 | 0 |
| 8.51 ppm | 0–20.2 | 7.3 ± 6.6 | 0 | 0 | 0 | 0 | 0 |
| 8.35 ppm | 0 | 0 | 0–64.4 | 40.5 ± 19.4 | 0 | 0 | 190.6–229.5 |
| 8.33 ppm | 0–12.8 | 4.1 ± 5.2 | 0 | 0 | 0 | 0 | 0 |
| 8.30 ppm | 0–13.8 | 4.8 ± 5.6 | 0 | 0 | 0 | 0 | 0 |
| 8.29 ppm | 0–17.6 | 5.3 ± 6.8 | 26.3–71.2 | 49.9 ± 15.1 | 0 | 0 | 0 |
| 8.25 ppm | 0 | 0 | 13.2–38.2 | 28.2 ± 8.0 | 0 | 0 | 218.8–235.0 |
| 8.21 ppm | 4.7–56.3 | 35.9 12.3 | 0 | 0 | 0 | 0 | 0 |
| 8.19 ppm | 0 | 0 | 0 | 0 | 10.6–241.9 | 43.6 ± 60.7 | 0 |
| 8.15 ppm | 17.3–164.7 | 89.4 ± 51.9 | 0 | 0 | 190.6–1286.9 | 934.0 ± 246.3 | 0 |
| 8.11 ppm | 190.2–395.0 | 275.3 ± 73.2 | 0–33.0 | 8.7 ± 13.3 | 174.3–530.5 | 401.4 ± 76.1 | 0 |
| 7.95 ppm | 40.6–170.6 | 114.7 ± 37.6 | 0 | 0 | 0 | 0 | 0 |
| 7.94 ppm | 48.5–499.3 | 229.3 ± 175.0 | 0 | 0 | 0 | 0 | 0 |
| 7.30 ppm | 60.7–128.0 | 92.7 ± 27.6 | 0 | 0 | 0 | 0 | 0 |
| 7.12 ppm | 40.7–115.6 | 76.3 ± 25.8 | 0 | 0 | 0 | 0 | 31.1–61.9 |
| 6.21 ppm | 0 | 0 | 0–88.4 | 54.8 ± 25.9 | 0 | 0 | 0 |
| 6.20 ppm | 28.0–71.6 | 48.0 ± 15.4 | 0–8.1 | 5.8 ± 2.5 | 0–24.0 | 8.8 ± 7.5 | 8.5–18.4 |
| 4.59 ppm | 0 | 0 | 0 | 0 | 0–516.2 | 136.9 ± 150.6 | 193.6–200.5 |
| 3.16 ppm | 473.4–693.6 | 589.3 ± 56.3 | 752.3–1415.9 | 965.3 ± 279.1 | 657.0–1470.8 | 975.2 ± 245.9 | 210.1–279.6 |

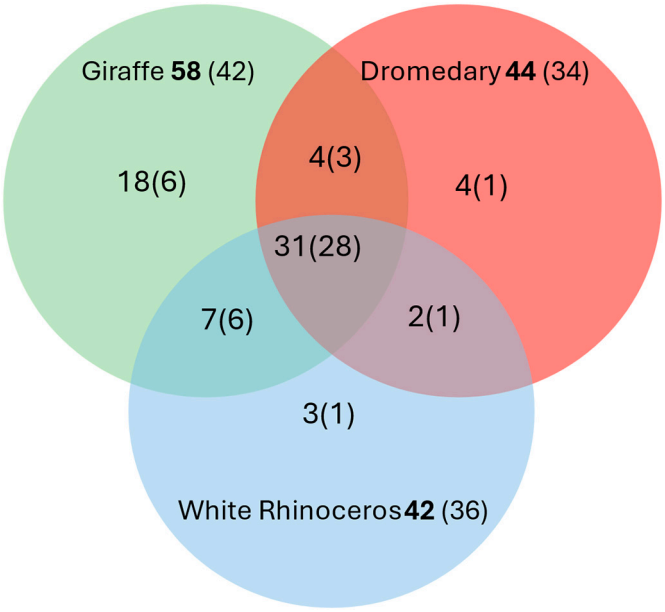



**Fig. 1.** Venn diagram of metabolites in the milk of dromedary (D), giraffe (G) and white rhinoceros (R). Numbers in bold indicate the total metabolites detected, in brackets are the annotated and partially identified metabolites.

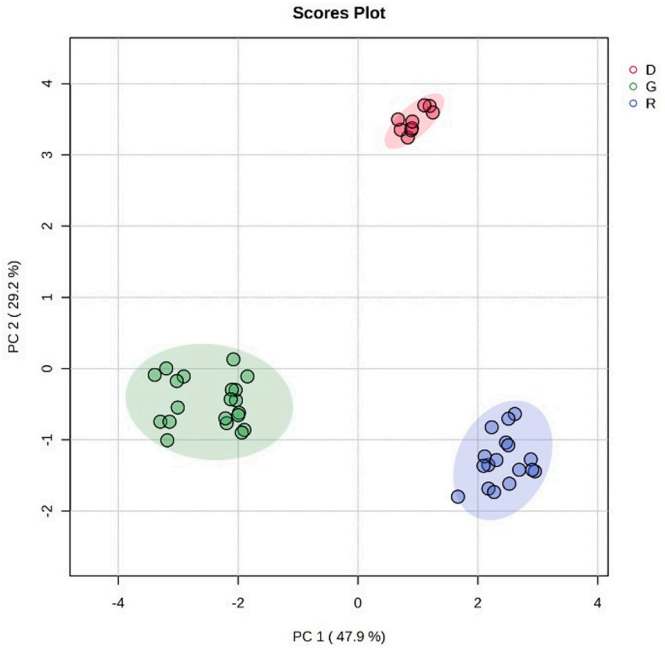



**Fig. 2.** Multivariate statistical analysis of metabolites in the milk of dromedary (D), giraffe (G) and white rhinoceros (R).

### *3.3. KEGG pathway enrichment analysis of significantly different metabolites*

The identified significantly different metabolites were subjected to KEGG enrichment to identify possible metabolic pathways in which they are involved. The top 12 KEGG pathways, based on *P* values, that were identified according to the milk metabolite contents are presented in Fig. 4. A KEGG enrichment was carried out based on the collective metabolites of all three species identified (Fig. 4A), as well as based on metabolites of individual species (Fig. 4B–D). The 12 pathways of the collective were pyruvate metabolism, glycolysis/gluconeogenesis, glyoxylate and dicarboxylate metabolism, citrate cycle, arginine biosynthesis, arginine and proline metabolism, tyrosine metabolism, glycine, serine and threonine metabolism, tyrosine metabolism, nicotinate and nicotinamide metabolism, glycerophospholipid metabolism and pyrimidine metabolism. Individual KEGG enrichment also identified alanine, aspartate and glutamate metabolism and nitrogen metabolism in dromedary and white rhinoceros milk, as well as galactose metabolism in the latter. Metabolic pathways exclusive to giraffe milk were glycine, serine and threonine metabolism, and amino sugar and nucleotide sugar metabolism. A condensed map of these metabolic pathways was compiled (Fig. 5) and each pathway was colour coded according to its preferred activity in singular or multiple species.

## 4. Discussion

The metabolomic analysis of dromedary, giraffe and white rhinoceros milk resulted in the identification of 46 metabolites across seven classes. A large number of molecules were identified as saccharides that could not be completely annotated. Valuable information could still be extracted from these saccharides. Compared to other metabolomic research of milk, the number of annotated metabolites was low. $^{1}$H NMR based analyses of milk from donkey (Garhwal et al., 2023) described 36 metabolites and 45 from cow (Settachaimongkon et al., 2021), while as many as 606 metabolites were detected in milk from donkey (Li et al., 2020), and 587 polar metabolite and 233 lipid features in a comparative study of bovine, ovine and goat milk (Cabrera et al., 2023) with chromatographic separation and MS detection. Our results are in agreement with others (Rysova et al., 2020; Settachaimongkon et al., 2021; Garhwal et al., 2023), that lipid-related metabolites seem to be lacking in $^{1}$H NMR-based analysis. Only a few metabolites of the dromedary milk were shared with an extensive analysis by coupled plasma-mass spectrometry (ICP-MS) and GC–MS (Ahamad et al., 2017).

The significantly different metabolites showed that dromedary milk lacked *N*-phenylacetylglycine, UDP-*N*-acetylglucosamine, some other UDP-saccharides and hippurate. It had the highest content of 3′siallyllactose and was the only one of the four species that contained 3-fucosyllactose, although in only two of the five individuals. These two oligosaccharides were previously described in the milk of the Bactrian camel (*Camelus bactrianus*) (Fukuda et al., 2010) and 3′siallyllactose in that of dromedary (Alhaj et al., 2013).

Giraffe milk lacked creatinine, glutamate and 3-fucosyllactose, had the highest content of creatine and phosphocreatine, UDP-saccharides and was the only milk of the four species that contained *N*-phenylacetylglycine and UDP-*N*-acetylglucosamine. Although *N*-acetylglucosamine was not detected, the UDP-variant was separated from it by one step in the metabolic pathway (KEGG, 2024). Giraffe milk therefore contains *N*-acetyl sugars, similar as bovine milk (Rysova et al., 2020). The two groups of giraffe milk samples shown by the PCA plot were representative of the two reserves where the giraffes resided. The extreme drought conditions that the Rooipoort reserve experienced during the collection times might be the cause. The vegetation species composition between Sandveld Nature Reserve (Free State Province) and Rooipoort Nature Reserve (Northern Cape Province) differed notably during the study period, likely influencing giraffe diets and contributing to their separation in the ordination analysis. Sandveld supported more diverse and abundant vegetation, including species such as *Vachellia karroo*, *Ziziphus mucronata*, and *Searsia lancea*, which provide high-quality browse with greater moisture content and nutrient availability (Mucina and Rutherford, 2006). In contrast, Rooipoort experienced drier conditions, with more sparse, drought-resistant species like *Vachellia erioloba* and *Boscia albitrunca*, offering lower moisture and potentially fewer secondary metabolites. These differences likely shaped giraffe dietary intake, as individuals in Sandveld accessed richer forage, while Rooipoort giraffes adapted to more limited, arid browse only available during the wet season. This variation in forage quality and availability likely influenced physiological markers and body

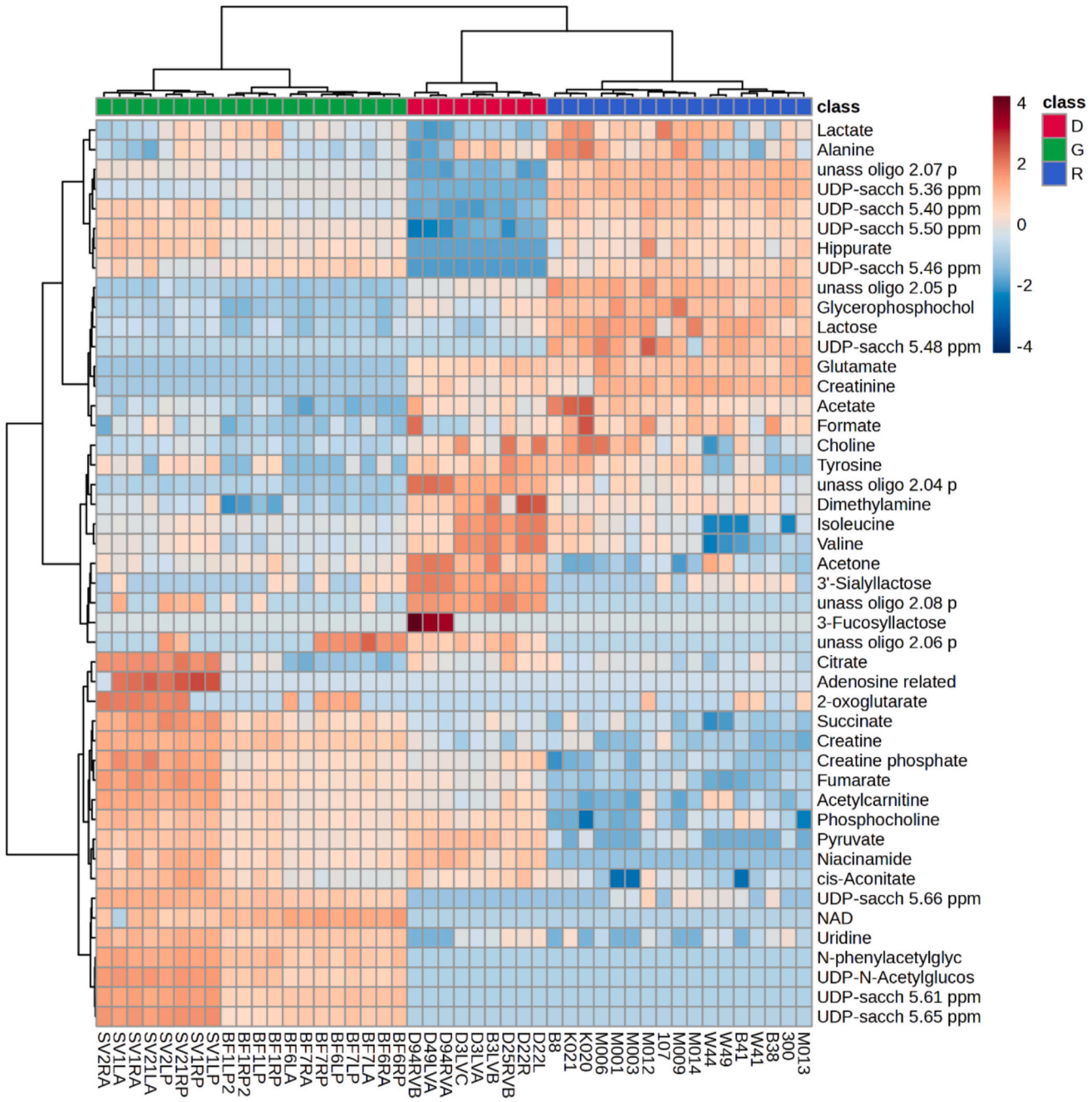



**Fig. 3.** Heat-map visualization and hierarchical clustering of metabolite profiles of the milk of dromedary (D), giraffe (G) and white rhinoceros (R). The dendrogram represents sample clusters based on Pearson's correlation coefficient with average linkage. Shading of blue to red indicates increasing content of the corresponding compound.

condition, explaining the distinct population clustering in the ordination diagram. In a different study, it was shown that the difference of habitat of reserves affects the gut metabolome and chemical composition as determined in the faeces of giraffes (Grobbelaar et al., 2024, 2025). Recognizing these habitat-driven dietary shifts enhances our understanding of regional giraffe ecology and health. Since the two giraffes in the Sandveld reserve did not form a statistical representative sample, we did not pursue any further data interpretation.

White rhinoceros milk was devoid of nicotinamide, *N*-phenylacetylglycine, fucosyllactose, UDP-*N*-acetylglucosamine and some UDP-sugars, and had the highest content of lactose, lactate and glycerophosphocholine. It also contained the lowest number of metabolites with unassigned signals and the composition of UDP-sugars differed from the other three species. The 3′-siallyllactose was detected in low concentrations in the milk of only eight white rhinoceros individuals. This oligosaccharide had been found in the colostrum of all individuals of this species, its concentration and presence decreased towards and after the transitional milk phase (Osthoff and Nieuwoudt, 2024).

One or both samples of blesbok milk lacked amines, creatine and phosphocreatine, valine, *N*-phenylacetylglycine, 3-fucosyllactose, UDP-*N*-acetylglucosamine and almost all the UDP-sugars, several unassigned oligosaccharides, acetone, fumarate and pyruvate and had the highest content of creatinine. It also contained a low number of the metabolites of unassigned signals. The blesbok is a ruminant, as is the giraffe, however, their milk metabolomes differ in many of the parameters. The only common parameters are the absence of 2-fucosyllactose and high

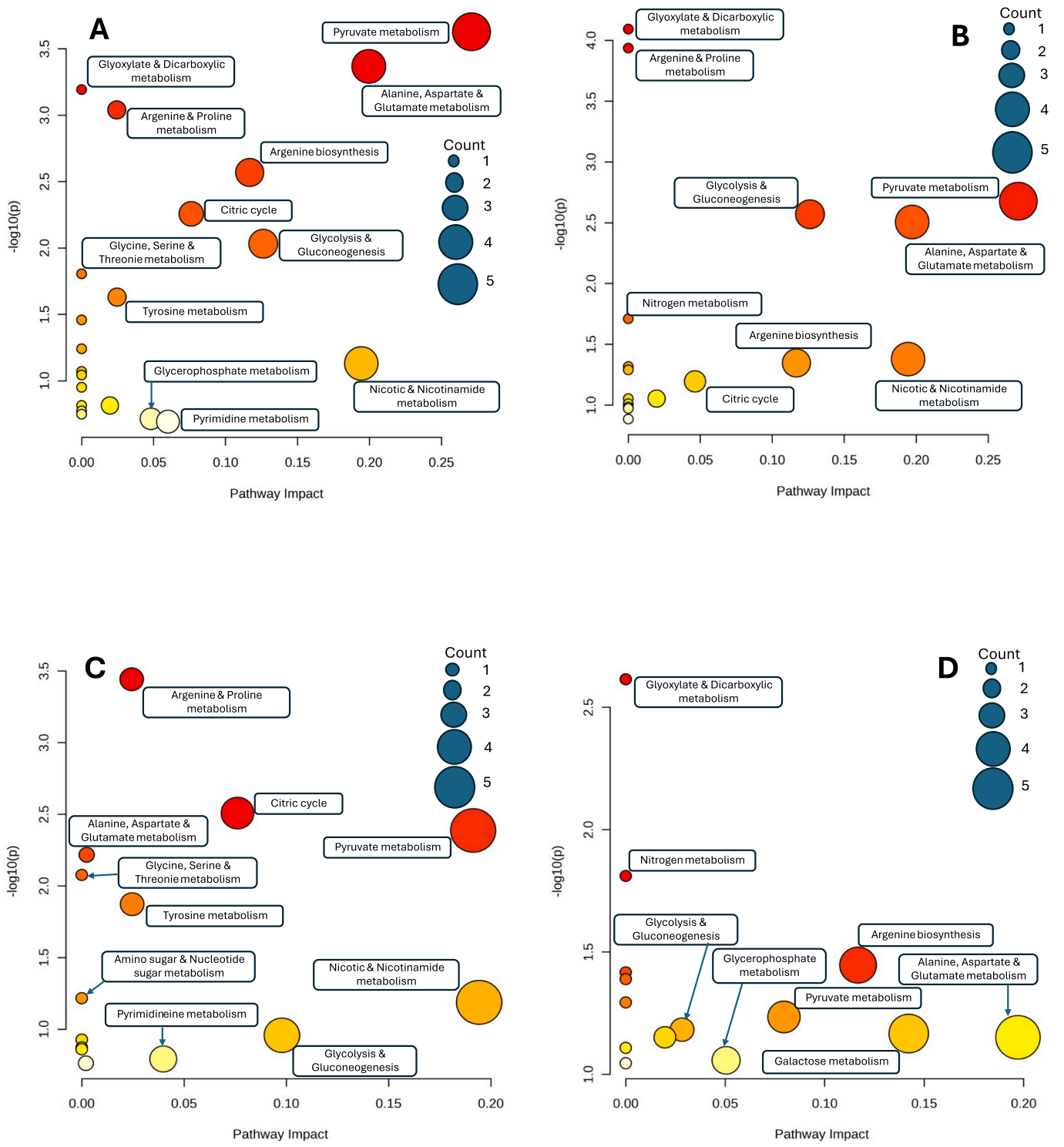



**Fig. 4.** KEGG pathway enrichment analysis of different metabolites in the comparisons of metabolites in the milk of all three species (A), dromedary (B), giraffe (C) and white rhinoceros (D).

content of uridine. Although the differences in milk metabolome between dromedary, giraffe and white rhinoceros seem clear, the discrepancy shown between giraffe and blesbok, both Ruminantia, shows that more comparative research between species within and across different taxa is required to obtain a clearer picture of phylogenetic properties of metabolites. One reason might arise from the diet; the giraffe is a browser and the blesbok a grazer (Hofmann, 1989).

The $^{1}$H NMR milk metabolome of dromedary, giraffe and white rhinoceros involved 16 metabolic pathways and they were compiled in a compact map (Fig. 5). Of these 16, only five out of six were also shared with yak milk, being galactose metabolism, glycine, serine and threonine metabolism, arginine and proline metabolism, arginine biosynthesis, pyrimidine metabolism, and glyoxylate and dicarboxylate metabolism (Li et al., 2023). Nine pathways are shared with human milk, being arginine biosynthesis, glycolysis/gluconeogenesis, arginine and proline metabolism, citrate cycle, amino sugar and nucleotide sugar metabolism, galactose metabolism, nitrogen metabolism, pyrimidine metabolism, glyoxylate and dicarboxylate metabolism, and nicotinate

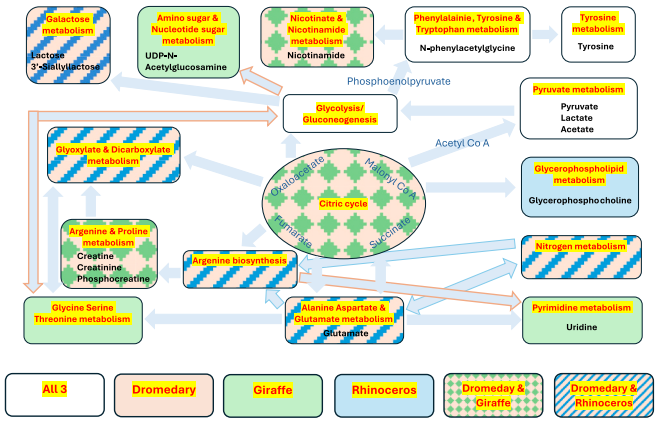



**Fig. 5.** Condensed map of metabolic pathways (red on yellow) that were selected by KEGG enrichment analysis in which the key metabolites (black) of dromedary, giraffe and white rhinoceros milk are involved. Molecules in blue represent intermediate steps. The colour codes of the boxes indicate shared pathways and pathways of preference of individual species. Preferred pathways in giraffe indicated by arrows with pink outline. Preferred pathways in dromedary and white rhinoceros indicated by arrows with blue outline.

and nicotinamide metabolism (Li et al., 2023). Donkey milk analysed by GC–MS (Li et al., 2020), found only three pathways, namely citrate cycle, galactose metabolism, and glycerophospholipid metabolism, in common with our study. A complete comparison of our work with the latter two metabolomes is not possible, since they were carried out using GC–MS. Shared with $^{1}$H NMR analysis of donkey colostrum and milk, seven metabolic pathways were common to our findings, being glyoxylate and dicarboxylate metabolism, citrate cycle, alanine, aspartate and glutamate metabolism, glycine, serine and threonine metabolism, glyceropgospholipid metabolism, arginine and proline metabolism, arginine biosynthesis, (Garhwal et al., 2023).

The individual pathways in Fig. 5 were colour coded to indicate pathways shared by the milk gland cells of all three species and preferred pathways of one species or shared by two. Although the central citric acid cycle was indicated by the KEGG enrichment as being shared by dromedary and giraffe, the high citrate concentration in the milk of all three species (Table 1) indicates that it is highly active in all three species. Not so the glycerophospholipid metabolism, since glycerophosphocholine occurred in the highest concentration in white rhinoceros, indicating that this is possibly a preferred pathway in the species. Likewise, the absence of glutamate from giraffe milk implied that the alanine, aspartate and glutamate metabolism might be less active in the giraffe. In this case, the latter metabolic pathway seems not to supply substrates for the glycine, serine and threonine metabolism, and the pyrimidine metabolism directly. In the giraffe, substrates therefore seem to be sourced through other routes, respectively the glycolysis/gluconeogenesis and arginine synthesis pathways. Glutamate also seemed to be the result of an interconnected high activity of the alanine, aspartate and glutamate metabolism, nitrogen metabolism, and arginine metabolism in dromedary and white rhinoceros. The absence of *N*-phenylacetyglucosamine from the milk of dromedary and rhinoceros suggested that amino sugar and nucleotide sugar metabolism is active in the giraffe only. The high content of lactose in white rhinoceros milk and 3'sialyllactose in dromedary milk are indicative of high activity of galactose metabolism in these two species.

In an inter-species metabolomic study by $^{1}$H NMR and LC-MS, metabolic pathway analysis of different metabolites showed that glycerophospholipid metabolism as well as valine, leucine, and isoleucine biosynthesis were shared in ruminants (Jersey, buffalo, yak, and goat), and biosynthesis of unsaturated fatty acids was shared in the non-ruminants (camel and horse) (Yang et al., 2016). Our research did not support this; firstly, valine, leucine, and isoleucine biosynthesis, and the biosynthesis of unsaturated fatty acids were not identified as important metabolic pathways in any of our species; secondly, glycerophospholipid metabolism was identified as important pathway in the non-ruminant white rhinoceros.

At this stage it is also not possible to explain the specialization of different metabolic pathways in the milk between the species, not within our study, nor in comparison with the literature. Other than metabolomic studies of serum and urine, a base line metabolome has not yet been compiled for milk against which differences can be measured. Although milk obtains certain minerals and nutrients from the compounds absorbed in the digestive tract via the blood, there is no exchange in return of components that were synthesized in the milk glands (Van Zyl et al., 2023), so that comparisons of serum metabolites would also not be of aid.

To conclude, the milk metabolome differed between the four species that were investigated, not just by concentration but also by the absence of certain metabolites and the presence of others. The greatest differences between the milk metabolites were in their carbohydrate component, followed by the amino acid derivatives, nucleic acid derivatives and organic acids, and 15 statistically important metabolites were identified for KEGG metabolic pathway enrichment. The important metabolic pathways revealed specialization of metabolic pathways within species. In the white rhinoceros the glycerophospholipid metabolism seemed highly active, while the galactose metabolism seemed active in the white rhinoceros as well as the dromedary. In the giraffe mammary cells, the amino sugar and nucleotide sugar metabolism were active and the alanine, aspartate and glutamate metabolism were less active. We found it difficult to compare our results with that of other research reports of similar or closely related species, even of $^{1}$H NMR based data, and conflicting conclusions were also observed. Metabolomic data of milk from more species is therefore required before any conclusions can be drawn of the role of metabolism on the phylogenetic difference in chemical composition of milk between taxa. It would also be required that the same analytical methods should be employed.

## CRediT authorship contribution statement

**G. Osthoff:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **L. Schmidt:** Investigation, Formal analysis, Data curation. **A.S. W. Tordiffe:** Writing – review & editing, Methodology, Conceptualization. **F. Deacon:** Supervision, Resources, Project administration.

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## Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Gernot Osthoff reports a relationship with University of the Free State. Gernot Osthoff reports a relationship with National Research Foundation that includes: funding grants. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Data availability

Osthoff, Gernot; Schmidt, Lauren; Tordiffe, Adrian; Deacon, Francois (2025), "Non-targeted metabolomics by $^{1}$H nuclear magnetic resonance spectroscopy; an interspecies comparison of milk between dromedary, giraffe and white rhinoceros with observations on blesbok", Mendeley Data, V1, https://data.mendeley.com/datasets/psdf59wfgb/1.

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