Milk metabolite phenotyping profiling of bovine, ovine, buffalo, caprine and donkey by LC-MS/MS QTOF analysis

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Overview

- The lipidome fraction of milk from five animal species (bovine, buffalo, caprine, donkey and ovine) was analyzed by HRMS QTOF LC-MS/MS. This fraction is arguably the major factor that determines the organoleptic quality as well as the commercial price of the milk.
- Triacylglycerides (TAGs) dominate the milk lipidome fraction and significant differences in distribution and intensity of TAGs were found between animals.
- TAGs were identified by MS and DIA-MS/MS using FA neutral mass information migrated from LipidBlast.

1. Introduction

Global milk production is almost entirely derived from bovine (81%), buffaloes (15%), goats (2%), sheep (1%) and camels (0.4%) [FAOstat May 2021]. Dependent on the region and country the presence and importance of each species varies significantly and are influenced by market demand, dietary traditions and the socio-economic characteristics of individual households (e.g., poorer families tend to rely more on small ruminants). The nutritional composition of milk is highly complex and varies between species, for example:

- Approximately 3 to 4 percent of the solid content of bovine milk is fat, dominated by triglycerides (TAGs); protein and lactose are present at 3.5 and 5 percent respectively. Goat milk has a similar fat composition to bovine milk.
- Buffalo milk has a markedly higher fat content compared to bovine milk and has a fat-to-protein ratio of 2:1 with a corresponding higher casein-to-protein ratio.
- Ovine milk has a higher fat and protein contents than both caprine and bovine milk.
- Equine milk from both donkey and horse have similar compositions and like human milk is relatively low in protein. Compared to other diary species, equine milk contains low levels of fat and protein.

In this metabolomics study, high resolution mass spectrometry LC-MS/MS was used to identify lipid profiles in milk from bovine, buffalo, caprine, donkey and ovine.

2. Methods

Milk from five animal species (bovine, ovine, buffalo, caprine and donkey) were prepared by the Folch method (200 µL sample + 50 µL water + 1000 µL solvent - chloroform:methanol 2:1 v/v). The lower, lipid-containing phase, was transferred to a glass vial and placed into a speed vac and evaporated to dryness. Samples were reconstituted in 1mL butanol:methanol:chloroform (3:5:4), then diluted 1:500 with inclusion of Lipidomix SPLASH standard (330707), injection volume 5 µL. Samples were measured by LC-MS/MS QTOF (LCMS-9030, Shimadzu Corporation).

The method acquired a single time-of-flight (TOF) MS scan (m/z 50−1800) followed by 50 DIA-MS/MS mass scans in positive ion; each MS/MS mass scan had a precursor isolation width of 35 Da and a collision energy spread of 5−55V, resulting in a cycle time of 1.1 s. Insight software was used for data processing and data analysis.

3. Results

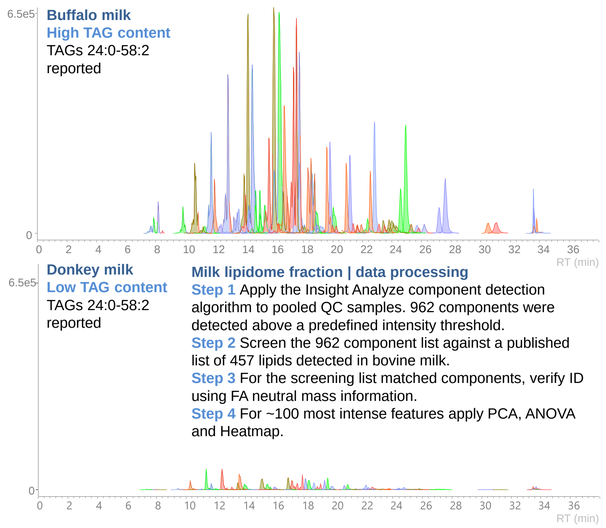

Figure 1. Normalized mass chromatograms for ~100 of the most intense components detected in buffalo and donkey milk samples (TAG 24:0-58:2 are shown).

As an assessment of system performance, the peak area variance of the SPLASH internal standard was less than 10% for the batch analysis (n=89, total batch time 90h);

- 15:0-18:1(d7)-15:0 TAG rt 22.8 mins, peak area variance 3.2%.
- 15:0-18:1(d7) DAG rt 12.8 mins, peak area variance 9.3%.

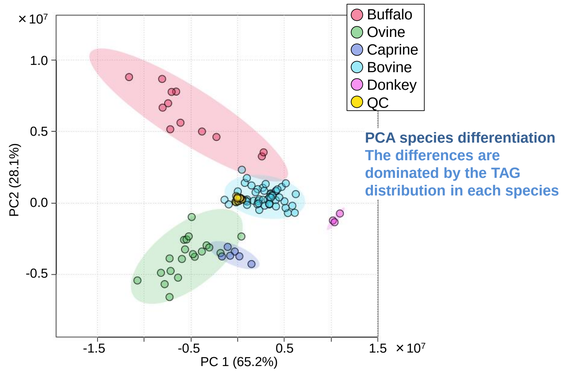

Figure 2. Multivariate analysis using PCA showing species differentiation using ~100 of the most intense lipid components detected and identified in the pooled QC sample.

3.1 Heat map analysis

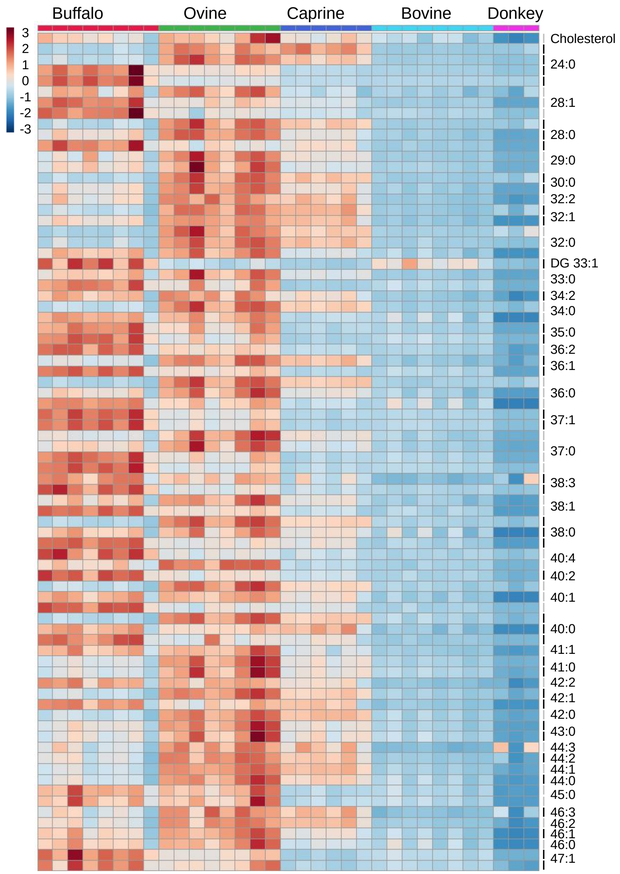

Figure 3. Heat map analysis generated by MetaboAnalyst highlighting the differences in the distribution of TAGs 24:0-47:1 in milk samples for buffalo, ovine, caprine, bovine and donkey (matched by region, breed and date - taken in the months of May and June). All TAGs larger than 47:1 were significantly higher in buffalo compared to other animal species (the range of TAGs shown is limited to 24:0-47:1 for display purposes only).

The heat map is dominated by TAGs (with the exception of cholesterol and DG 33:1) showing the peak area response for each component including each TAG isomer detected, for example TAG 36:0 has 3 isomers shown.

3.2 TAG distribution in different species

- As one example, TAG 36:0 has 3 isomers, the most dominant feature in buffalo milk was identified as **TG(4:0_16:0_16:0)** based upon the DIA-MS/MS spectrum

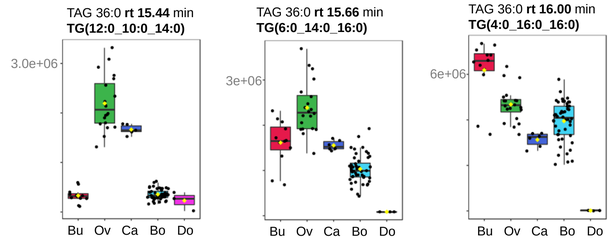

Figure 4. Box plots highlighting the differences in TAG 36:0 isomer distribution in buffalo (Bu), ovine (Ov), caprine (Ca), bovine (Bo) and donkey (Do) milk samples.

3.3 TAG identification

- Using a database tool (populated with FA neutral mass information migrated from LipidBlast) the TAG species were identified using the corresponding DIA-MS/MS product ion spectrum for each detected TAG precursor. The naming convention follows LipidMaps, for example:
  - Short form identification **TAG 36:0** rt 16 min; precursor m/z 639.55636.
  - Fragment ion m/z 551.50396 (chain length 4:0); *sn*-1 acyl loss.
  - Fragment ion m/z 383.31628 (chain length 16:0); *sn*-2 and *sn*-3 acyl loss.
  - Long form identification **TG(4:0_16:0_16:0)**.
- To increase TAG isomer resolution, the LC analysis time was extended.
- DIA-MS/MS resulted in a higher reporting confidence compared to DDA-MS/MS as confirmatory fragment ion chromatograms are fully aligned with the precursor ion mass chromatogram and can help confirm the TAG fatty acid *sn*-1/*sn*-2/*sn*-3 positions.

4. Conclusions

- TAG signals varied significantly between animal species both in terms of distribution and intensity. Donkey TAG content was markedly different to all other animal species, with very low TAG levels and fewer TAGs detected.
- TAG 24:0 and TAG 36:0 isomers can be used to authenticate milk samples (early eluting isomers were significantly higher in caprine and ovine compared to buffalo and bovine milk; conversely, later eluting isomers were significantly higher in buffalo.
- Buffalo TAG distribution and intensity was markedly higher than all other animal species particularly above TAG 47:1.