Advanced analysis of NZ sheep milk lipids

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Background

Samples of sheep milk were assayed by Callaghan innovation within the **Boosting Exports of the Emerging NZ Dairy Sheep Industry** programme.

The following assays have been performed to date:

- determination of total lipid content (48 samples),
- fatty acids profile (48 samples),
- neutral lipids classes (48 samples),
  common phospholipids profile (48 samples),
- plasmalogen forms of phospholipids (48 samples),
  medium-chain triacylglycerols (48 samples),
- β-palmitate (OPO) content (5 samples),
- detection of glycated phospholipids (5 samples),
- glucosyl- and lactosylceramide content (48 samples),
- major gangliosides (5 samples).

Results: Total lipids content (n=43)

Sheep milk samples	Total lipids, avg. weight % ± st.d. (range)
Early lactation	5.6 ± 2.0 (4.1-9.2)
Mid lactation	5.4 ± 1.4 (4.0-8.3)
Late lactation	7.4 ± 1.2 (5.9-15.1)
Average for 43 samples	7.2 ± 2.5 (4.0-15.1)

Literature data: 3.6 -10.0% (Raynal-Ljutovac *et al.* 2008)

Major factors affecting the quantitative and qualitative composition of the milk: lactation stage, season, breed, genotype, and feeding (*ibid.*)

Overall, sheep milk total lipids content is higher than that of cow milk (3.0%), and human milk (1.4%), as reported by Prosser *et al.* (2010).

Fatty acids – a summary

Fatty acids, in weight% of total lipids	Ovine - our data	Bovine (Devle *et al.* 2012)	Human - Japanese (Jensen 1999)	Human - Sudanese (Jensen 1999)	Human - French (Jensen 1999)
	Avg ± St.Dev. (Min – Max)	Avg.	Min - Max	Min - Max	Min - Max
Saturated	66.1 ± 2.9 (57.3 - 71.8)	68.4	37.2 - 48.6	25.1 - 70.1	27.0 - 57.3
Short-chain (4 to 6 carbons)	6.0 ± 1.0 (4.3 - 8.3)	3.2	0.1 - 0.1	0.0	0.0 - 0.2
Medium-chain (6 to 12 carbons)	25.7 ± 4.2 (16.6 - 35.0)	10.1	4.1 - 8.7	1.6 - 13.9	2.4 - 12.3
Monoenoic	24.0 ± 2.7 (18.9 - 32.8)	25.7	30.5 - 34.3	21.5 - 39.2	24.4 - 35.8
Dienoic	4.1 ± 0.5 (2.4 - 5.6)	1.8	12.1 - 14.5	9.3 - 32.7	8.9 - 22.8
18:2 CLA	1.7 ± 0.4 (0.9 - 3.3)	0.4	0.0	0.0	0.0
Trienoic	1.0 ± 0.2 (0.6 - 1.5)	0.6	1.1 - 1.5	0.2 - 2.7	0.3 - 1.6
Total PUFA	5.0 ± 0.6 (3.1 - 6.2)	2.4	13.2 - 16.0	9.5 - 35.4	9.2 - 24.5

OPO and regiospecific distribution of SFA

OPO: a lipid component of ongoing commercial interest, 1,3-dioleoyl-2-palmitoylglycerol, the major triacylglycerol (TG) species in human milk, comprising 11.8% of the total TGs (Bar-Yoseph *et al*. 2013). Sheep milk?

Our results: OPO level varied from 0.25 to 0.44% of total lipids.

Saturated Fatty Acids (SFA):

Cow milk: about 35 mol% of saturated fatty acids are located at *sn*-2 position (Parodi 1979).

Human milk: 45 to 47 mol% of saturated fatty acids are located at *sn*-2 position (Haddad *et al*. 2012).

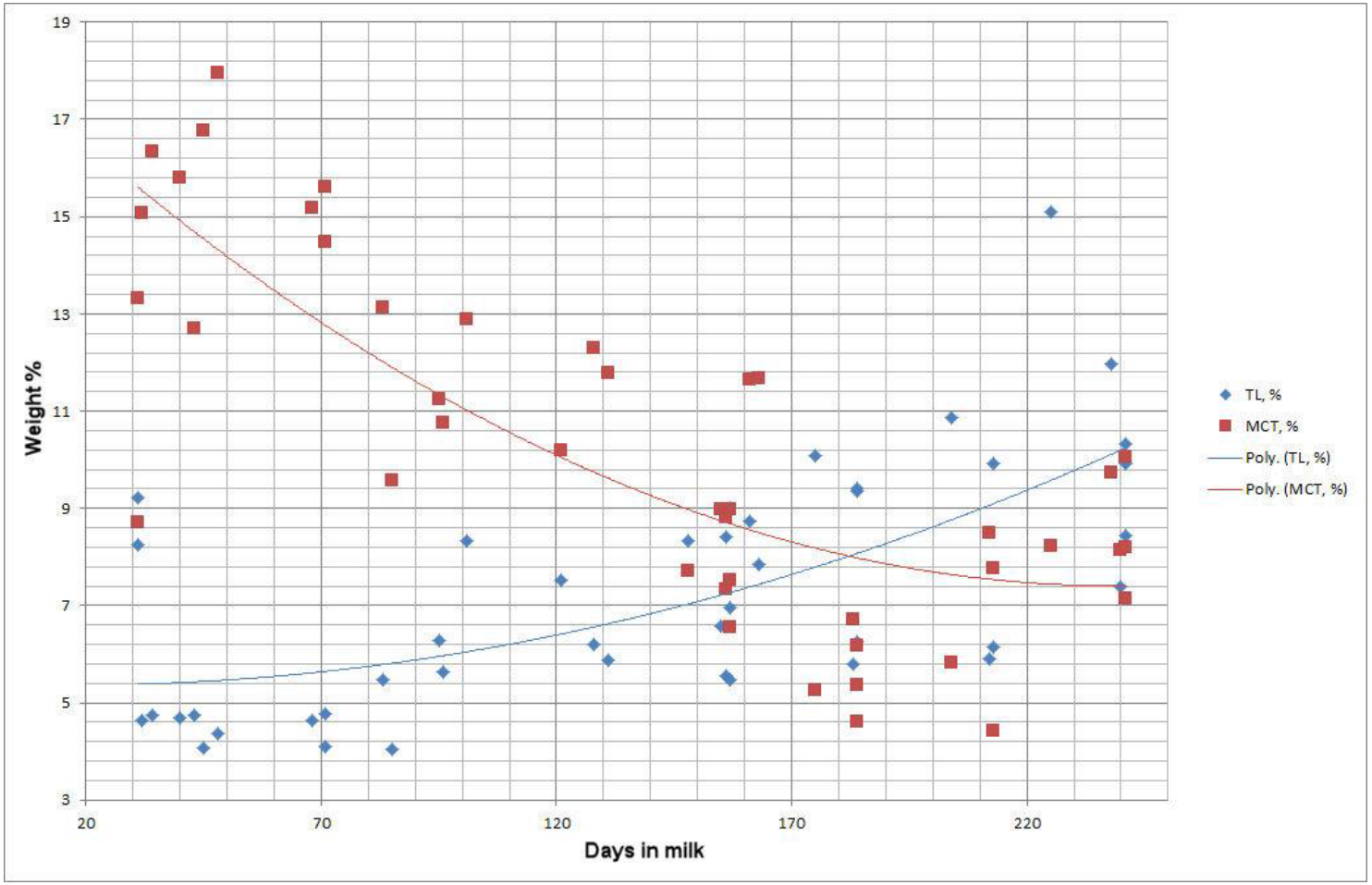
Sheep milk (our data): 30 to 32 mol% of saturated fatty acids are located at *sn*-2 position.

Medium-chain triacylglycerols, MCT

Triglyceride composition (in weight % of total triglycerides) of the milk fats of cow and sheep, literature data *vs.* current study.

Sample	30 carbons and below	36 carbons and below
Cow (Breckenridge & Kuksis 1967)	1.2-1.6	16.0-27.6
Sheep (Goudjil *et al.* 2003; *Mele et al.* 2011; Smiddy *et al.* 2012)	1.8-7.5	13.5-32.9
Early lactation	8.7-18.0	45.8-58.8
Mid lactation	9.6-15.6	44.1-54.4
Late lactation	8.8-12.3	44.2-50.4
Average ± St.Dev.	10.2±3.6	45.7±6.9

Medium-chain triacylglycerols, MCT



Sheep milk samples: Total lipids content in weight % of the sample, and MCT (30 carbons and below) content in weight % of triacylglycerols *vs*. Days in milk

Polar lipids: phospholipids

Sample	Early	Mid	Late	Average	Cow, Anchor Blue top
Phospholipids in total lipids, (% w/w)	0.60-0.86	0.72-1.10	0.74-0.90	0.84±0.12	1.27
Phospholipids (g/ 100g sample)	0.03-0.08	0.04-0.06	0.04-0.07	0.06±0.02	0.04
Total Lipid (g/ 100g sample)	4.1-9.2	4.0-8.3	5.9-8.7	7.2±2.5	3.2

Sphingomyelin: 17.4±7.0 mg/100 ml (7.7 – 37.2 mg/100ml)

Anchor Blue Top milk – 7.7 mg/100 ml

Ethanolamine plasmalogen: 1.5±0.7 mg/100 ml (0.7 – 3.8 mg/100ml)

Anchor Blue Top milk – 0.4 mg/100 ml

Human milk – 2.7 mg/100 ml (Garcia *et al.* 2012)

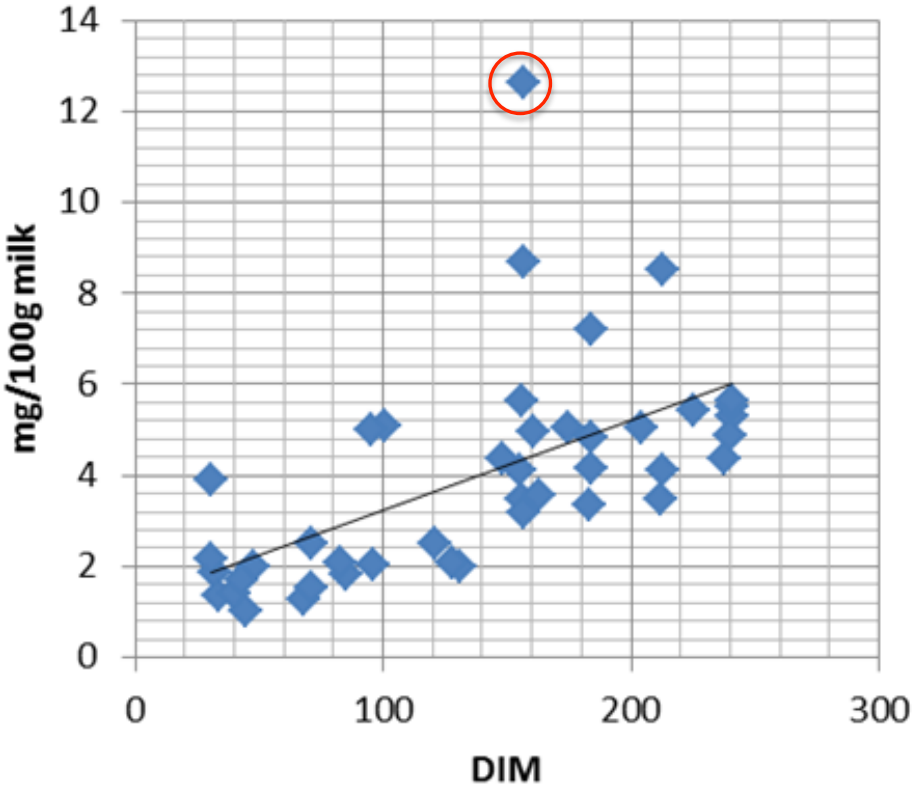
Polar lipids: glycated PL, gangliosides

No glycated phospholipids were detected in the samples studied.

No measurable levels of gangliosides were observed in the samples studied.

Literature data: The total ganglioside content of sheep milk reaches 6–7% of that of bovine milk (Puente *et al.* 1995), while content of gangliosides in human milk is about 2.3 times higher than that of bovine milk (Bode *et al*. 2004).

Polar lipids: glycosphingolipids



For a comparison, the combined glucosyl- plus lactosyceramide content in bovine milk with a total lipid content of 4% was reported to be about 2 mg/100 mL milk (Hladick & Michaelec 1966).

Lipid analysis detects pre-clinical mastitis

Unusual phospholipid profile:

AAPC – 3.4% of total phospholipids (not found in normal milk)

AAPE - 1.1% of total phospholipids (not found in normal milk)

PG - 1.1% of total phospholipids (not found in normal milk)

CL - 3.4% of total phospholipids (not found in normal milk)

EPLAS - 6.2% (*vs*. 2.1-4.2% of total phospholipids in normal milk)

Glycosphingolipids:

LacCer – 9.0 mg/100g milk (*vs*. 0.7-5.8 in normal milk)

GlcCer+LacCer – 12.6 mg/100g milk (*vs*. 1.0-8.7 in normal milk)

Results

- Sheep milk contains higher levels of certain beneficial lipid components when compared with cow milk lipids, e.g.
    - Medium-chain triacylglycerols (MCT)
    - Phospholipids - sphingomyelin and plasmalogens
    - Neutral sphingoglycolipids
- Lipid content and profile change with lactation stages, and geographical location.
- New Zealand sheep milk was found to have generally a higher content of total lipids and medium-chain triacylglycerols than that reported for European sheep milk.
- Sheep milk lipids might find their application in novel products (e.g. food for special needs – for elderly, sports food, etc.)

Thank you!

Extraction of lipids

The original method of Svennerholm & Fredman (1980) was modified as follows:

8g sheep milk in a 40mL centrifuge tube was mixed with 16 mL of methanol and 8 mL of chloroform, and sonicated for 10 min then spun at 2850 rcf for 10 minutes. The pellet was further extracted with chloroform (6 mL)/ methanol (6 mL), and twice with chloroform (6 mL)/methanol (3 mL) with the use of ultrasonication. The centrifuged lipid extracts from each extraction were combined and transferred to a separating funnel and washed with water (19 mL) and 10% aq. KCl (3 mL).

The lower layer was dried and constituted the crude lipid extract. The aqueous layer was also analysed for gangliosides content.

GC analyses

Fatty acids methyl esters (FAME) were prepared from total lipid extracts as described by Carreau & Dubacq (1978). GC analysis of the FAME was performed on a Trace GC Ultra (Thermo Fisher Scientific, USA) gas chromatograph equipped with a flame ionisation detector (FID) and RTX70 (60 m × 0.25 mm i.d., 0.25 µm) capillary column (SGE, Australia). Helium was used as carrier gas and a split ratio of 1:100 was maintained. Injector and detector temperatures were both 260 °C. Oven temperature was held at 60 °C for 4 minutes, then increased by 15 °C/min to 165 °C (held for 1minute) followed by an increase of 2 °C/min to 225 °C (held fro 20 minutes). Individual peaks of FAME were identified by comparison with standards of FAME.

GC analysis of acylglycerols was performed on a Trace GC Ultra (Thermo Fisher Scientific, USA) gas chromatograph equipped with a flame ionisation detector (FID) and TAP CB UniMetal (25 m × 0.25 mm i.d., 0.1 µm) capillary column (Agilent, USA). Samples were injected in splitless mode. Helium was used as the carrier gas. Injector and detector temperatures were 350 °C and 370 °C, respectively. The oven temperature was raised from 60 °C to 200 °C at a rate of 120 °C/min, then increased to 320 °C at a rate of 20 °C/min, followed by a rise to 360 °C at a rate of 1 °C/min, and maintained at that temperature for 6 minutes.

OPO assay

The method used for OPO analysis is a two step procedure starting with a preparative isolation of an OPO-containing fraction from the crude lipid mixture, by non-aqueous RP-HPLC. Subsequent Ag+-HPLC allows fine separation and quantification of OPO independently of its regioisomer, 1,2-dioleoyl-3-palmitoylglycerol (OOP), and the isomers of stearoyl-linoleoyl-palmitoyl glycerol that might be present in the sheep milk lipid. Approximately 100 mg of crude lipid sample was injected in three runs on the preparative non-aqueous RP-HPLC (solvent acetone:acetonitrile). The OPO-enriched fraction was collected. The enriched fraction was then dissolved in n-hexane and analysed by Ag+-HPLC with ELSD detection (solvent acetonitrile in hexane). A standard curve was prepared using OPO at 0.2 mg/mL in n-hexane.

Analysis of phospholipids

Phospholipid classes were analysed using ^{31}P NMR in a sodium cholate detergent system at pH 7 (MacKenzie *et al*. 2009). Glyphosate was used as an internal standard for quantification.

Amadori products of sugars (e.g. lactose, glucose) were detected upon increasing the pH to 9 and reanalysing by ^{31}P NMR.

Deacylation with monomethylamine allowed the detection and quantification of ethanolamine plasmalogen (EPLAS).

^{13}C-NMR experiment

NMR Spectroscopy. In a typical experiment 20–45 mg of a sample was dissolved in 0.75 mL of $CDCl_3$. ^{13}C NMR spectra were recorded at 125.7 MHz with WALTZ16 proton composite pulse decoupling on a Bruker Avance III NMR spectrometer equipped with an automatic tuning 5 mm multinuclear probe at 303 K. The pulse program ZGIG, which is supplied with Bruker TopSpin2.6 spectrometer software, was used for suppression of nuclear Overhauser effects *via* inverse gate proton decoupling.

Acquisition parameters were: 90° ^{13}C observe pulse 11.0 μsec, spectral width 2011 Hz (16 ppm) centered on the carbonyl region at 171 ppm, 32 768 real and imaginary f.i.d. data points collected during the 8.146 seconds f.i.d. data acquisition time, followed by a 22 seconds recovery delay time to ensure a pulse repetition time of more than 5 times the longest expected longitudinal relaxation time T_1. 800 scans were collected requiring almost 7 hours per spectrum.

Since this part of the study involved quantitation, some comments on digital handling at the receiver stage are warranted. Although ADC oversampling and the standard Bruker ‘sharp’ digital filter were used in conjunction with a 125 kHz analogue filter, routine ‘on the fly’ linear prediction (as implemented by the Bruker ‘baseopt’ parameter) was not used to correct the initial f.i.d. data points to align them to the true time zero of the f.i.d. near the center of the excitation observe pulse. The f.i.d. was zero filled once to 32 768 real data points, and a 0.06 Hz exponential weighting function applied before Fourier transformation.

Analysis of Glucosyl and Lactosyl Ceramides

The analysis of glucosyl and lactosyl ceramides levels in the powders was performed using HPLC. The method is a modification of the method of Trung Le *et al*. (2011) using the same solvents but employing a Hilic column and a 0.22 mL/min flow rate. A 100 mg portion of the crude lipid sample was dissolved in a minimal volume of chloroform and applied to a silica SPE column pre-equilibrated with hexane: diethyl ether (100:3). The column was washed with 6 mL hexane: diethyl ether (100:3) followed by 3 mL chloroform: acetone (3:1). The cerebroside fraction was eluted with 3 mL chloroform: methanol: water (65:30:8) and the ganglioside fraction with a further 6 mL of the same solvent. The cerebroside fraction was dissolved in 0.5 mL chloroform: methanol (2:1) and used directly for HPLC analysis. Quantification was by reference to a standard curve prepared from a combination standard solution made up from pure glucosyl ceramide and lactosyl ceramide.

Analysis of Gangliosides

The analysis of the gangliosides in the powders was performed by HPLC using the same method as described above for the glucosyl and lactosyl ceramides. Both the crude lipid fraction from the extraction step described above and the upper aqueous layer were used for the analysis. The ganglioside enriched fraction from silica SPE (see above) of the lipid extract was dissolved in 0.5 mL chloroform: methanol (2:1) and used directly for GD3/GM3 analysis. The aqueous layer from the original lipid extraction was subjected to a SPE clean up step. A portion of the aqueous material (approximately 10% of total) was loaded onto a pre-equilibrated C-18 SPE column in methanol: water 1:1. The column was washed with methanol: water 1:3 and the ganglioside fraction eluted with methanol followed by methanol: chloroform (1:1). The ganglioside fraction was evaporated to dryness and taken up in methanol: water (1:1) for GD3 analysis. GM3 analysis required a further SPE step using a strong anion exchange SPE column. A portion of the lipid sample was dissolved in methanol and loaded onto a pre-equilibrated SPE SAX column. The column was washed with methanol and the ganglioside eluted with 0.2 M ammonium acetate in methanol. Quantification was by reference to a standard curve prepared from a combination standard solution made up from pure GD3 and GM3.