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Untargeted lipidomics of ovine milk to analyse the influence of different diet regimens

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Research Article

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Abstract

In this work we report a lipidomics approach to study the effects of two diet systems on the composition of ovine milk. Milk from two groups of Sarda sheep grazing on 40% (P40) and 60% (P60) of pasture were analyzed by a UHPLC-QTOF-MS analytical platform and data submitted to multivariate statistical analysis. Pairwise partial least square discriminant analysis of the lipid profile of the data was carried out to classify samples and to find discriminant lipids. The two dietary groups were characterized by differences in triacylglycerols, phosphocholines and phosphatidylethanolamines levels. Discriminants of the P40 group were TG and PC containing in their backbone saturated medium chain FA thus suggesting greater *de novo* fatty synthesis in the mammary gland. On the other hand, the P60 group was characterized by TG and PC formed by unsaturated long chain FA originating from the diet or from lipid mobilization.

Milk and dairy products obtained from small ruminants such as sheep and goats are gaining consumer interest for their nutritional value and for being a source of vitamins, fats, amino acids and probiotic compounds. In the Mediterranean area, sheep feeding managements are optimized to sustain milk production and the feeding is traditionally based on the extensive and semi-extensive exploitation of natural and/or cultivated pastures. Typically the chosen feeding system is based on environmental and climatic considerations as well as land availability and profitability factors. In Italy, and in particular in Sardinia, pasture feeding is anciently practiced thanks to the temperate climate and fertile soils factors that allow sustainable and competitive grass production. However, pasture availability is seasonal and often scarce, as in many Mediterranean areas, making supplementation necessary, at least at some times during the productive season (Molle *et al*., 2021). A drawback of the pasture-based feeding system is the seasonality of production (Scano, 2019).

Milk composition is known to be dependent on animal breed, stage of lactation and, in particular, the feeding system. Grazing systems relying on semi-natural pasture vegetation and forage species and their phenological stage are known to strongly affect the fatty acid profile of milk (Addis *et al*., 2005; Morand-Fehr *et al*., 2007; Buccioni *et al*., 2012; Renna *et al*., 2020) and the functional and sensory characteristics of dairy products (Schönfeldt, Hall, and Smit, 2012). The effects of different forage diets (grazing *vs.* hay) on the fatty acid profile and gene expression in the mammary gland of the Churra Tensina sheep breed were studied (Dervishi *et al*., 2012). These authors reported that the forage type affected the levels conjugated linoleic acid (CLA) and long-chain saturated fatty acid (LCFA) content, with higher percentages in grazing when compared to hay feeding animals.

The effects of different feeding systems cannot be studied solely from the analysis of milk gross compositional traits or fatty acid profile but rather by means of chemical fingerprinting obtained from hyphenated analytical techniques combined with chemometrics approaches. Lipidomics is an -omics science that allows the investigation of the structural and functional complexity of lipids in biological systems. Liquid chromatography and high-resolution mass spectrometry are the analytical approaches most used in lipid research. With this analytical approach and in particular with MS/MS experiments important structural elucidation of complex lipids such triacylglycerols (TG), phosphatidylcholines (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), ceramides and sphingomyelins (SM) can be obtained. Recently, the change of goat lipidome and compositional characteristics of milk from two feeding treatments, grazing *vs.* hay-feeding in confinement, were reported (Argov-Argaman *et al*., 2021). Milk fat and protein contents were higher in grazing goats. Except for saturated fatty

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acids, dietary management affected fatty acid composition, in particular polyunsaturated fatty acids content. Omega 6 and omega 3 fatty acids increased and omega6/omega3 ratio decreased in goat milk under grazing when compared with confined managements (Argov-Argaman *et al.*, 2021).

In this work with a lipidomics approach we studied the impact of two different diets on the sheep milk lipidome. Animal diets consisted of 40% and 60% of ewe energy requirement derived from pasture integrated with hay and concentrate. Bulk milk samples were analyzed by a high-resolution mass spectrometric (UHPLC-QTOF-MS) platform. MS data were submitted to multivariate statistical analysis to differentiate the two diet regimens and to identify discriminant milk lipid compounds. MS/MS data were used for the annotation and confirmation purposes of complex lipids.

Materials and methods

Animals and diets

Forty-eight mature Sarda ewes were divided into two groups balanced for age, body weight, body condition score, lambing date and milk production and randomly assigned to the diets. The diets were either 40% (P40) or 60% (P60) of ewe energy requirement derived from pasture, with 4 and 6 h/day of grazing, respectively. Pasture was based on Persian clover, sulla and burr medic cultivated in mixture with Italian ryegrass. Diet supplementation consisted of maize and faba beans at 600 and 300 g/head day split into two meals at milking, and overnight 800 and 600 g/head day of a ryegrass or hay, for P40 and P60 respectively. After the morning milking the groups were at pasture while for the remaining time they were kept indoors in separate pens. Samples of bulk milk were collected for 10 weeks, from March to June 2020, for 3 d every two weeks for a total of 15 bulk milk samples for each group obtained by pooling milk from 24 ewes.

Sample preparation

150 µl of milk samples were transferred to Eppendorf tubes containing 10 µl of the internal mixture of standards (Splash, Lipidomics, Sigma Aldrich Milan, Italy) and added with 525 µl of methanol and 525 µl of MTBE. Samples were then centrifuged at 4000 rpm for 15 min. Subsequently, a second round of centrifuge was performed at 12 000 rpm for 5 min. Before transferring to autosampler vials, the supernatant was filtered through a 0.22 µm MS nylon syringe filter.

Lipidomics analysis

The supernatant of milk samples was analyzed with a LC-QTOF-MS coupled with an Agilent 1290 Infinity II LC system. An aliquot of 1.0 µl from each sample was injected in a Kinetex 5 µm EVO C18 100 A, 150 mm × 2.1 µm column (Agilent Technologies, Palo Alto, CA). The column was maintained at 50 °C at a flow rate of 0.4 ml/min. The mobile phase for positive ionization mode consisted of (A) 10 mM ammonium formate solution in 60% of milliQ water and 40% of acetonitrile and (B) 10 mM ammonium formate solution containing 90% of isopropanol and 10% of acetonitrile. In positive ionization mode, the chromatographic separation was obtained with the following gradient: initially 60% of A, then a linear decrease from 60% to 50% of A in 2 min then at 1% in 5 min staying at this percentage for 1.9 min and then brought back to the initial conditions in 1 min. The mobile phase for negative ionization mode differed only for the use of 10 mM ammonium acetate instead of ammonium formate (A and B). The analytical setup used was equipped with an Agilent jet stream technology source which was operated in both positive and negative ion modes with the following parameters: gas temperature, 200 °C; gas flow (nitrogen) 10 l/min; nebulizer gas (nitrogen), 50 psig; sheath gas temperature, 300 °C; sheath gas flow, 12 l/min; capillary voltage 3500 V for positive and 3000 V for negative; nozzle voltage 0 V; fragmentor 150 V; skimmer 65 V, octapole RF 7550 V; mass range, 40–1700 *m/z*; capillary voltage, 3.5 kV; collision energy 20 eV in positive and 25 eV in negative mode, mass precursor per cycle = 3; threshold for MS/MS 5000 counts.

Data analysis

The LCQTOF-MS data were uploaded to the web platform XCMS (Tautenhahn *et al.*, 2012) and the resulting data matrix was submitted to multivariate statistical analysis as implemented in SIMCA-P+ software (version 14.1, Umetrics, Umeå, Sweden). Prior to this, QTOF-MS features were mean centered and scaled to unit variance column-wise. Principal component analysis, partial least-squares-discriminant analysis (PLS-DA) and its orthogonal variant (OPLS-DA) were performed (Manis *et al.*, 2021). The quality of the models was evaluated based on the cumulative parameters R^2Y and Q^2Y, the latter estimated by cross validation. The variable importance in projection (VIP) scores in the predictive component were analyzed and only those metabolites having VIP values >1 were considered as discriminants between the classes.

Results

Untargeted lipidomics

Gross composition of the P40 and P60 groups of bulk milk samples was studied and the results are reported in online Supplementary Table S1. P60 showed a higher milk yield, but lower proteins and fat content.

Data from XCMS in positive and negative ionization modes were submitted to multivariate statistical analysis. Principal component analysis allowed us to explore the data structure in a visual way to understand peculiar characteristics such as groupings of samples and interrelated variables. From the analysis of the derived score plots of both ionization modes no clusters of milk samples, based on different diets, were observed (online Supplementary Fig. S1). Attempts to differentiate milk samples based on the P40 and P60 groups were carried out by performing a PLS-DA for both positive and negative ionization modes. The P40 and P60 milk samples were correctly classified (statistical parameters: R^2Y of 0.96 and Q^2Y of 0.45) by the statistical model built using the positive ionization mode results. Unfortunately, the PLS-DA model of the negative ionization mode data was not able to correctly classify samples. Furthermore, an OPLS-DA of positive ionization results was performed with the aim to underline differences between the two classes. The corresponding score plot is shown in Fig. 1, and Table 1 reports the discriminant lipids and their VIP values.

After the analysis in the positive ionization mode the P40 group showed higher levels of the following complex lipids:

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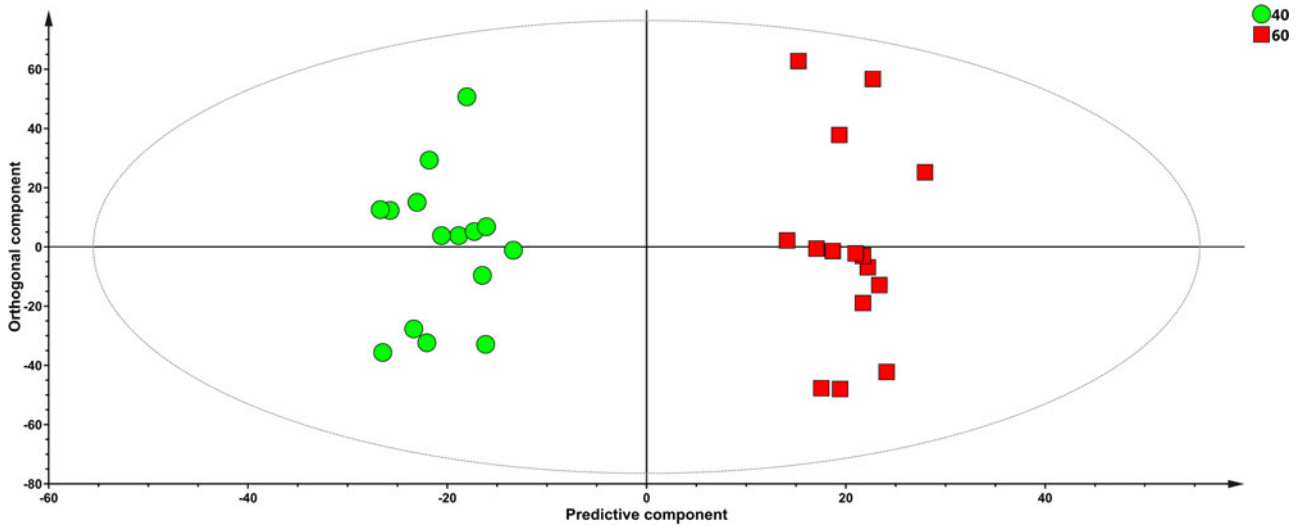

Fig. 1. OPLS-DA score plot of milk lipids acquired in the positive ionization mode (P40 green circles, P60 red boxes).

Table 1. OPLS-DA discriminant lipid compounds with the variable importance in the projection (VIP) values.

P40			P60		
Lipid	Sum composition	VIP value	Lipid	Sum composition	VIP value
LPC 18:1	18:1	2.59	PC 32:1	PC 16:1_16:0	2.89
LPE 18:1	18:1	2.15	PC 33:1	PC 15:0_18:1	2.02
PC 28:0	PC 12:0_16:0	1.67	PC 34:3	PC 16:1_18:2	2.02
PC 30:0	PC 14:0_16:0	2.03	PC 36:1	PC 18:1_18:0	2.63
PC 34:0	PC 16:0_18:0	2.51	PC 36:2	PC 18:1_18:1	2.08
PE 35:1		2.11	PC 36:3	PC 16:0_20:3	1.77
PE 36:1	PE 18:1_18:0	1.68	PC 36:4	PC 18:2_18:2	2.79
PE 36:3	PE 18:1_18:3	2.57	PC 38:4	PC 18:0_20:4	3.08
PS 36:1	PS 18:0_18:1	2.92	PE 34:3	PE 16:0_18:3	2.03
SM 41:2		3.10	SM 39:1		2.51
SM 42:3	SM 18:3_24:0	3.16	SM 40:0	SM 18:0_22:0	2.68
TG 40:5	TG 4:0_18:2_18:3	2.46	SM 32:0		1.77
TG 42:0	TG 10:0_12:0_18:0	1.84	SM 36:1	SM 16:0_20:1	2.43
TG 43:0	TG 10:0_18:0_15:0	2.45	TG 41:0	TG 12:0_12:0_17:0	3.36
TG 44:0	TG 16:0_10:0_18:0	1.92	TG 48:1	TG 18:1_14:0_16:0	2.28
TG 46:0	TG 10:0_18:0_18:0	1.74	TG 50:2	TG 16:0_16:0_18:2	2.48
TG 47:0	TG 16:0_15:0_16:0	2.46	TG 51:0	TG 16:0_17:0_18:0	2.30
TG 48:0	TG 16:0_14:0_18:0	2.76	TG 51:2	TG 15:0_18:1_18:1	1.74
TG 48:3	TG 18:1_12:0_18:2	3.08	TG 52:1	TG 16:0_18:0_18:1	1.79
TG 49:0	TG 17:0_14:0_18:0	2.25	TG 52:2	TG 16:0_18:2_18:0	2.22
			TG 54:2	TG 18:1_18:0_18:1	2.25
			TG 54:3	TG 18:1_18:1_18:1	1.96
			TG 55:1	TG 21:0_16:0_18:1	2.96

Saturated fatty acid PC such as PC(28:0), PC(30:0), PC(34:0) were found upregulated along with the unsaturated fatty acid PE (PE (35:1), PE(36:1) PE(36:3)) and lyso-derivative compounds LPC (18:1) and LPE(18:1). The most highly discriminant compounds for the P40 group were: sphingomyelins SM(41:2) and SM(42:3) with a VIP value of 3.10 and 3.16 respectively. Among

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discriminants in the P40 group, the triacylglycerols class showed TG with a carbon number (CN) from TG (40:5) to TG (49:0) and, in detail, the TG (48:3) showed the highest VIP value.

Conversely, the P60 group showed more unsaturated PC such as PC(32:1), PC(33:1), PC(34:3), PC(36:1), PC(36:2), PC(36:3), PC(36:4). Interestingly, in the P60 group we found more TG with a high CN from TG (50:2) to TG (55:1) and a higher overall unsaturation degree (Table 1). The P60 was also characterized by highest level of the following SMs, SM (32:0), SM(36:1), SM(39:1) and SM(40:0).

Discussion

From the UHPLC-QTOF-MS analysis we detected several lipid classes, and with the OPLS-DA model we were able to annotate discriminant lipids that can be used as potential biomarkers to differentiate different nutritional regimens. From a physiological point of view, milk TG are synthetized from FA that are derived from *de novo* mammary gland synthesis, dietary lipids, and endogenous fat stores (adipose or hepatic lipids). In our model we noticed that the discriminants TG for the P40 group preferentially incorporated medium and short saturated chain FA (C4–C16) that are produced in the mammary gland by *de novo* synthesis. On the contrary, TGs of the P60 group incorporated longer chain fatty acid with more unsaturated FA. In the mammary gland, these TGs are synthetized from polyunsaturated FA, that originate from the diet or from lipid mobilization of the adipose tissue, or from the activity of stearoyl desaturase (Leroux *et al.*, 2003).

Furthermore, the same trend can be observed for PC discriminants. The P40 group is characterized by PC bearing medium chain saturated FA (i.e. lauric acid, myristic acid, and palmitic acid) thus suggesting an increase of the *de novo* synthesis of FA. Instead, the PC of the P60 group are characterized by unsaturated LCFA. Interestingly, discriminant lipids of the P40 group were lysophosphatidylcholine (18:1) and lysophosphatidylethanolamine (18:1) produced by the hydrolysis of the corresponding precursors PC and PE.

Several studies have shown that the challenges induced by biotic factors (such as changes in vegetation and heat) may be a source of animal stress, eliciting a greater degree of behavioral plasticity and adaptability in grazing animals (Villalba and Manteca, 2019). The results obtained from our experiments show a downregulation in the P60 group of different lipid classes such as phosphocoline, lysophosphocoline and phosphoethanolamines. These results are in accordance with Liu's suggesting an intracellular stress condition of the animals under study that is reflected in the chemical composition of the milk. The condition of intracellular stress can inhibit the initial stages of the choline/phosphocholine biosynthesis process (Liu *et al.*, 2017).

In conclusion, pairwise partial least square discriminant analysis of the lipid profile of ovine milk was carried out to to find discriminant lipids characteristic of lesser (P40) or greater (P60) herbage grazing. The two dietary groups were characterized by differences in triacylglycerols, phosphocholines and phosphatidylethanolamines levels. Discriminants of the P40 group were TG and PC containing in their backbone saturated medium chain FA thus suggesting greater *de novo* fatty synthesis in the mammary gland. On the other hand, the P60 group was characterized by TG and PC formed by unsaturated long chain FA originating from the diet or from lipid mobilization.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022029921000583

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