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## ► To cite this version:

Mylène Delosière, Laurence Bernard, Didier Viala, Hélène Fougère, Muriel Bonnet. Milk and plasma proteomes from cows facing diet-induced milk fat depression are related to immunity, lipid metabolism and inflammation. *Animal*, 2023, 17 (6), pp.100822. 10.1016/j.animal.2023.100822 . hal-04170800

**HAL Id: hal-04170800**

**<https://hal.science/hal-04170800>**

Submitted on 25 Jul 2023

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# Animal

## The international journal of animal biosciences



### Milk and plasma proteomes from cows facing diet-induced milk fat depression are related to immunity, lipid metabolism and inflammation



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#### ARTICLE INFO

##### Article history:

Received 28 July 2022

Revised 6 April 2023

Accepted 7 April 2023

Available online 21 April 2023

##### Keywords:

Dairy cow

Dietary lipid supplement

Mammary lipid secretion

Milk

Proteomics

#### ABSTRACT

Milk proteins are a source of bioactive molecules for calves and humans that may also reflect the physiology and metabolism of dairy cows. Dietary lipid supplements are classically used to modulate the lipid content and composition of bovine milk, with potential impacts on the nutrient's homeostasis and the systemic inflammation of cows that remains to be more explored. This study aimed at identifying discriminant proteins and their associated pathways in twelve Holstein cows ( $87 \pm 7$  days in milk), multiparous and non-pregnant, fed for 28 d a diet either, supplemented with 5% DM intake of corn oil and with 50% additional starch from wheat in the concentrate (**COS**,  $n = 6$ ) chosen to induce a milk fat depression, or with 3% DM intake of hydrogenated palm oil (**HPO**,  $n = 6$ ) known to increase milk fat content. Intake, milk yield and milk composition were measured. On d 27 of the experimental periods, milk and blood samples were collected and label-free quantitative proteomics was performed on proteins extracted from plasma, milk fat globule membrane (**MFGM**) and skimmed milk (**SM**). The proteomes from COS and HPO samples were composed of 98, 158 and 70 unique proteins, respectively, in plasma, MFGM and SM. Of these, the combination of a univariate and a multivariate partial least square discriminant analyses reveals that 15 proteins in plasma, 24 in MFGM and 14 in SM signed the differences between COS and HPO diets. The 15 plasma proteins were related to the immune system, acute-phase response, regulation of lipid transport and insulin sensitivity. The 24 MFGM proteins were related to the lipid biosynthetic process and secretion. The 14 SM proteins were linked mainly to immune response, inflammation and lipid transport. This study proposes discriminant milk and plasma proteomes, depending on diet-induced divergence in milk fat secretion, that are related to nutrient homeostasis, inflammation, immunity and lipid metabolism. The present results also suggest a higher state of inflammation with the COS diet.

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#### Implications

We compared the plasma and milk proteomes of two groups of cows fed diets supplemented with two types of lipids and diverging in milk fat production. The plasma and milk's proteins discriminating the two groups of cows are related to immunity, lipid metabolism and inflammation, clarifying the impact of lipid nutrition on the metabolism and physiology of the dairy cow. Targeted protein changes in biological fluids could be used as low invasive indicators of the dairy cows' metabolic and physiological status after a period of lipid supplementation in particular in the case of milk fat depression to diagnose and prevent inflammation.

#### Introduction

Content and composition of milk are rapidly and reversibly modulated by dietary factors with considerable effects observed. Research efforts focused on a better comprehension of the nutritional regulation of milk fat content and fatty acid (**FA**) composition over these last decades (Chilliard et al., 2008). In particular, the effects of diets supplemented with lipid sources have been largely investigated to improve the dietary energy density and milk FA profile in dairy cows (Doreau et al., 2012). Indeed, certain dietary conditions in dairy cows such as diets rich in starch (high grain/low forage) and supplemented with polyunsaturated fatty acids (**PUFAs**) of plant or marine origin (fish oil or algae) a so-called classical diet-induced milk fat depression (**MFD**; Bauman and Griinari, 2001). Conversely, diets supplemented with high 16:0 FA lipids increased milk yield and milk fat percentage (Mosley et al.,

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2007). The dietary unsaturated FAs in ruminants are extensively biohydrogenated in the rumen leading to the synthesis of numerous and specific intermediates (i.e., conjugated linoleic acid and trans-C18:1), and increasing saturated fatty acids (i.e., C18:0). Both dietary lipids and their ruminal intermediates, some of them evidenced for their biological properties (e.g. trans-10, cis-12- conjugated linoleic acid, trans-10 18:1), were shown to modify the metabolism of tissues related to dairy cow performance, such as the mammary gland (Shingfield et al., 2010), the adipose tissue (Zachut, 2015; Qin et al., 2018) or the liver (Veshkini et al., 2022, 2022b). At the whole-body level, recent reviews delineated the effects of dietary lipids and FA on performances, energy and endocrine status, or health of dairy cows (Ingvarsen and Moyes, 2013; Kuhla et al., 2016; Veshkini et al., 2022a). These studies suggest an interplay between these biological functions that remains to be more explored. In a recent experiment, Fougère et al. have managed by the diet the milk fat secretion of dairy cows, energy balance (Fougère et al., 2018; 2021) and digestive processes (enteric methane emissions, ruminal fermentation, total tract digestibility) (Martin et al., 2021). In this experiment, we compared plasma or milk proteomes from cows fed either a diet rich in corn oil and starch (COS), recognised to induce a strong MFD, or a diet supplemented with hydrogenated palm oil (HPO), recognised to increase milk fat secretion, with the aim to identify pathways modified at the body or mammary levels, as the results of nutrient profiles or whole-body adaptations relative to these extreme diets. To the best of our knowledge, this is the first report of both milk and plasma discriminant proteomes of cows divergent by the milk lipid content and consequently by the energy status and nutrient availability at the body level, as the results of dietary lipid supplements. Changes in fluid proteomes in this extreme model of cows provide insights that link the nutrition, the plasma nutrient profile with the animal's endocrine status, immunity and lipid metabolism.

## Material and methods

### Animals, diets and sampling

The present work was realised on samples from twelve multiparous Holstein cows at  $87 \pm 7$  days in milk (mean  $\pm$  SD) fed with a diet composed of grass hay ad libitum with concentrates containing corn oil (5.0% total DM intake (DMI)) and with 50% additional starch from wheat (COS) and a diet composed of grass hay ad libitum with concentrates containing hydrogenated palm oil (3.0% total DMI) (HPO) (Table 1). Initially, four groups of three cows were constituted according to DIM, milk production, milk fat and milk protein content and four treatments were randomly assigned to each group in a replicated  $4 \times 4$  Latin square design over four 28-d experimental periods (Fougère et al., 2018). Only two groups of three cows from the periods 3 and 4 (to keep closer lactation stages) and fed with COS and HPO diets (two extreme dietary conditions in terms of milk fat content) were used to constitute two sets (one per treatment) of six cows leading to a total of 12 experimental units. The amounts of feed offered were adjusted regarding the refusals to maintain the targeted dietary forage-to-concentrate ratio (45:55 on a DM basis). The animals had access to fresh water ad libitum. The milk yields of individual animals were recorded over six milking at 0800 and 1530 h on d 21, 22 and 24. Milk samples were individually collected: one part was treated with bronopol-B2 preservative (LIAL, Aurillac, France) to measure fat, protein and lactose content, another part (unpreserved) was stored at  $-80^\circ\text{C}$  for protein extraction and identification analyses. Blood (9 mL) was sampled by jugular vein puncture using tubes containing ethylenediaminetetraacetic acid (Terumo Vacutainer-LML, Nemours, France) before the morning feeding on d 27 of each experimental

**Table 1**

Ingredients and chemical composition of the basal concentrates supplemented with corn oil and starch (COS) or hydrogenated palm oil (HPO) and of the grassland hay diets offered to lactating multiparous dairy cows (source: Fougère et al., 2018).

Items	Concentrate		Grassland Hay
	COS	HPO	
Ingredients, g/kg of DM			
Wheat starch	395		
Corn	394	500	
Soy	150	147	
Dehydrated Alfalfa	.	294	
Molasse Cane	35	39	
Dicalcium Phosphate	2	2	
Carbonate Flour	19	13	
Salt	3	3	
Mineral and vitamin complement	2	2	
Corn Oil <sup>1</sup>	50		
Hydrogenated palm oil <sup>2</sup>		30	
Chemical composition, g/kg of DM			
Organic Matter	964	932	921
CP	264	265	142
NDF	125	206	625
ADF	42	116	351
Starch	507	337	
Ether extract	47	39	15

<sup>1</sup> Corn oil (Olvea, Saint Léonard, France) was added to the concentrate at 5% of total DM intake and contained [g/kg of total fatty acids (FAs)]: 16:0 (114), 18:0 (16.4), cis-9 18:1 (297), cis-11 18:1 (6.30), 18:2n-6 (535), 18:3n-3 (7.57), 20:0 (3.48), 22:0 (1.0), 24:0 (1.5), and total FA (1 000 g/kg).

<sup>2</sup> Hydrogenated palm oil (Provimi, Crevin, France) was added to the concentrate at 3% of total DMI and contained (g/kg of total FA): 12:0 (5.09), 14:0 (12.4), 15:0 (0.51), 16:0 (463), 17:0 (1.26), 18:0 (474), cis-9 18:1 (11.8), cis-11 18:1 (0.71), cis-9, cis-12 18:2 (0.78), 20:0 (3.39), 20:4n-6 (3.58), and total FA (995 g/kg).

period. Immediately after collection, the samples were cooled on crushed ice and centrifuged (1 100g for 15 min at  $4^\circ\text{C}$ ). The plasma was harvested and stored at  $-80^\circ\text{C}$  until analyses.

### Milk and plasma samples processing

All the reagents were purchased from Sigma-Aldrich, Saint Louis, USA (MO). Commercial kits were used to automatically determined by spectrophotometry (ARENA 20XT, Thermo Fisher Scientific, Cergy Pontoise, France): plasma glucose (Glucose GOD-POD, Thermo Scientific, Asnières Sur Seine, France), FAs (fatty acids HR2, Sobioda, Montbonnot Saint Martin, France),  $\beta$ -Hydroxybutyrate ( $\beta$ -Hydroxybutyrate Kit, Thermo Scientific, Asnières Sur Seine, France), urea (Urea Kit, Thermo Scientific, Asnières Sur Seine, France), acetate (Acetic Acid, Thermo Scientific, Asnières Sur Seine, France) and insulin concentrations (Insulin-CT RIA, CIS Bio International, Gif Sur Yvette, France) as described by Pires et al. (2019). The rumen fermentation parameters were measured from the collected ruminal fluid samples as described in detail by Martin et al. (2021); the Volatile-Fatty Acids concentrations were determined by gas chromatography using a flame ionisation detector, and ammonia was measured by colorimetry using a Technicon autoanalyzer II system as described by Morgavi et al. (2008).

The raw milk defrosted slightly during one night at  $4^\circ\text{C}$  and milk components were equilibrated by incubation during 1 h at  $37^\circ\text{C}$ . The defrosted and equilibrated raw milk was centrifuged at 3 500g for 15 min at  $4^\circ\text{C}$  to separate the cream and the skimmed milk fractions. Acetic acid was added to the skim milk (SM) reaching pH 4.6 to precipitate and discard the caseins. The homogenate was centrifuged at 10 000g for 10 min at  $4^\circ\text{C}$  and the supernatant recovered until protein concentration determination. The milk fat globules membranes (MFGM) were isolated from the cream according to the protocol of Ji et al. (2017) with slight modifications. Briefly, the cream layer was washed twice using phosphate buffered solution (4.3 mM  $\text{Na}_2\text{HPO}_4$ , 2.7 mM KCl, 1.8 mM  $\text{KH}_2\text{PO}_4$  and 137 mM NaCl) for 10 min at  $37^\circ\text{C}$  and once using distilled

water. Each washing step was followed by centrifugation at 4 000g for 15 min at 4 °C with elimination of the aqueous phases. The sample was then stored at 4 °C overnight to harden the milk fat. Ten ml of distilled water was added to the cream sample and stirred for 10 min at 50 °C with water bath. Then, a sonification for 10 min allowed releasing the fat from the fat globules. The fat fraction was separated from the MFGM materials by centrifugation at 12 000g for 30 min at 4 °C. After removing the top fat fraction and aqueous phase, the MFGM protein pellets were suspended in 0.2 mL reducing buffer (Tris 150 mM pH 8; Sodium dodecyl sulphate 4%; Dithiothreitol 1 mM) and incubated for two periods of 5 min at 75 °C in an oven, interrupted by a homogenisation step using an ultrasonic bath for 5 min. After a centrifugation at 16 000g for 30 min at 20 °C, the upper 2/3 of the aqueous phase was recovered until protein concentration determination.

Plasma samples were thawed slowly in the fridge (2–8 °C). Enrichment of low-abundance plasma proteins was performed using the ProteoMiner Small-Capacity columns (#163-3006, Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. After twice wash of column, 0.2 mL of plasma were deposited on column and incubated for 2 h at room temperature (RT) with rotate mixing. Then, spin columns were washed twice with wash buffer and once with purified milli-Q water (1 min at RT) followed by centrifugations at 1 000g to eliminate unbound proteins. Then, proteins enriched from medium- and low-abundance were incubated two-fold for 15 min with 20 µL of elution buffer (1 min, RT) and centrifuged at 1 000g. Supernatant was recovered until protein concentration determination. After extraction from the three biological fluids, protein concentrations were determined using Pierce™ BCA (bicinchoninic acid) protein assay kit (Fisher Scientific, Hampton, NH, USA). Briefly, 100 µg of proteins were mixed with reducing sodium dodecyl sulphate polyacrylamide gel electrophoresis loading buffer (Sodium dodecyl sulphate 2%, β-mercaptoethanol 5%, glycerol 10%, Tris-HCl pH 6.8, 62.5 mM, and 0.005% bromophenol blue) and incubated 10 min at RT and 10 min boiling. Reduced proteins were concentrated at the start of the separation gel part (12% polyacrylamide) in a Mini Protean II (Bio-Rad Hercules, CA, USA) at 120 V for 15 min and stained using Coomassie blue R250. For each sample, one stained band was excised from the gel with a scalpel and cut into pieces of 1 mm side. Gel pieces were dehydrated with ethanol 96% (15 min). The disulfide bonds were reduced in dithiothreitol (Dithiothreitol; 10 mM, 1 h at 56 °C) and alkylated in iodoacetamide (55 mM, 30 min, protected from light). Gel pieces were destained with a washing step (30 min) with 5% and two washing steps with 50% acetonitrile in ammonium bicarbonate (25 mM). Finally, all bands were dehydrated with 100% acetonitrile for 10 min and dried in a SpeedVac. In-gel digestion was performed with sequencing grade trypsin (Promega, Madison, Wisconsin, USA) 1:50 [w/w] enzyme/protein ratio in 25 mM ammonium bicarbonate with an overnight incubation at 37 °C. Tryptic peptides were extracted from gel by adding 60 µL of 0.1% formic acid in acetonitrile with 10 min ultrasonication. Supernatants were transferred into vials, and peptides were dried in a SpeedVac. Peptides were recovered in 40 µL of 5% acetonitrile and 0.05% trifluoroacetic acid solution and transferred into glass vials (Interchim, Montluçon, France) prior to nanoscale liquid chromatography coupled to tandem mass spectrometry (**nano-LC-MS/MS**) analysis.

#### Protein identification by nanoscale liquid chromatography coupled to tandem mass spectrometry

Peptides were analysed by nano-LC-MS/MS using an Ultimate 3000 system coupled to an LTQ Orbitrap Velos mass spectrometer with a nano-electrospray ion source (Thermo Fisher Scientific). Three microliters of hydrolysate from each sample were concen-

trated and desalted at a flow rate of 30 µl/min on a C18 precolumn 5 cm length × 100 µm (Acclaim PepMap 100 C18, 5 µm, 100 Å nanoViper) equilibrated with trifluoroacetic acid 0.05% in water. Then, the concentration column was switched online with a nano-flow analytical C18 column (Acclaim PepMap 100–75 µm inner diameter × 25 cm length; C18 – 3 µm – 100 Å – SN 10942001) equilibrated with a 96% solvent A (99.9% H<sub>2</sub>O, 0.1% formic acid) flow at 300 nL/min. The peptides were then separated according to their hydrophobicity with a 114 min gradient of solvent B (99.9% acetonitrile, 0.1% formic acid) from 4 to 25%. Eluates were electrosprayed in positive-ion mode at 1.6 kV through a nano-electrospray ion source heated to 250 °C. The LTQ Orbitrap Velos mass spectrometer was used in CID top 10 modes (i.e. 1 full scan MS and the 10 major peaks in the full scan were selected for MS/MS) accordingly with the previously used by Boudon et al. (2020). The parameters of mass spectrometry analysis were as follows: Full-enhanced-scan MS spectra realised in the FTMS ion trap at a resolution of 30 000 (tolerance 10 ppm) acquired with 1 microscan (*m/z* 300–1 400), dynamic exclusion used with two repeat counts, 20 s repeat duration and 60 s exclusion duration. For MS/MS, isolation width for ion precursor was fixed at two mass to charge ratio, single charged species were rejected; fragmentation used 35% normalised collision energy as the default activation of 0.25 and 10 ms activation time.

The raw data processing of peptide MS/MS spectra was performed in Progenesis QI software (version 4.2, Nonlinear Dynamics, Newcastle upon Tyne, UK) consisting in an automatic alignment with respect to a reference run defined by the software considering a maximum allowable ion charged set to 5. The mass generating function list containing the detected ions was directly exported to Mascot v2.5 (<https://www.matrixscience.com>) interrogation engine and searched against a *Bos taurus* decoy database (i.e. ref\_bos\_taurus, 23 971 sequences – Update 08/17/2017). The following parameters were considered for the search: precursor mass tolerance of 10 ppm and fragment mass tolerance of 0.5 Da, a maximum of two missed cleavage sites of trypsin, carbamidomethylation (C) and oxidation (M) set as variable modifications. Protein identification was validated when at least two peptides originating from one protein showed statistically significant identity above Mascot scores > 29 with a False Discovery Rate of 1% (adjusted significance threshold  $P < 0.0125$ ). Ions score is  $-10 \log(P)$ , where  $P$  is the probability that the observed match is a random event. Individual ions scores > 29 indicate identity or extensive homology. The identified peptides from the database search were imported back to Progenesis QI. All unique validated peptides of an identified protein were included, and the total cumulative abundance was calculated by summing the abundances of all peptides allocated to the respective protein. The resulting UniProtKB protein identifiers were converted to corresponding gene names (**GN**) using the Retrieve/ID mapping tool of the UniProt web service. Proteins were identified with a minimum of two peptides and a  $P$ -value  $\leq 0.05$  and subjected to subsequent analyses.

#### Statistical analysis

Statistical analyses were performed with R Studio environment (R version 4.2.1 (2022-06-23)) in order to compare dairy cows either fed with the HPO or COS diets. Data were obtained from six cows per diet (COS and HPO diets), reared over two periods (P3 and P4 differing by 28 d of lactation). DMI, milk production and composition were statistically evaluated by a linear model testing the diet, the period (as fixed effects) and their interaction using the model  $\leftarrow \text{lm}(\text{data} \sim \text{treatment} * \text{period})$ . The  $P$ -values  $< 0.05$  were considered as statistically significant. The diet and the period effects were evaluated on the protein abundances determined from the MFGM, the SM and the plasma of cows. Raw pro-



teomics data with less than 50% of samples missing intensities were deleted, and data with more than 50% of samples missing intensities were imputed by the k-neighbour imputation method with  $k = 5$  (impute package, version 1.70.0). Then, proteomic intensities were transformed into  $\log(\text{data})$ . We looked for a proteomic signature that discriminate COS and HPO groups by applying two complementary statistical analyses: a univariate differential analysis to identify differential protein one by one, and a supervised multivariate analysis to identify a group of proteins that together discriminate the two dietary groups (Fig. 1). The parametric univariate differential analysis was performed with a fit linear model testing the diet and period fixed effects (limma package, version 3.52.2), using the design\_matrix  $\leftarrow$  model.matrix (~treatment + period), with an adjusted correction (adj.  $P$ -value) by the Benjamini-Hochberg test considering the false discovery rate in multiple tests. The proteins with adj.  $P$ -value  $< 0.1$  and fold-change  $> 1.5$  were considered as differentially abundant.

A supervised sparse partial least square discriminant analysis (sPLS-DA) was applied on proteomics data corrected for the period effect using the mixOmics package in R (version 6.20.0, Rohart et al., 2017). For each milk fraction and plasma, we used 30% of protein datasets to build the sPLS-DA based on two components, which correspond to 30, 48 and 20 proteins from plasma, MFGM and SM datasets. The multivariate sPLS-DA analysis then selects the most predictive or discriminative proteins to separate the two groups of cows, some of them were more abundant in HPO, other in COS and their contribution represent weights assigned by the sPLS-DA to each protein on a component (Supplementary Figures). The Plot loading function provides visualisation of the weight of variables on the component, where colours indicate the group for which the selected protein has a maximal mean value (Supplementary Figures). For each sPLS-DA, the first component was the most discriminant between HPO and COS. The proteins contributing to the first component were compiled into lists with their respective contribution. Contributions are coefficients assigned to each variable to define each component. Greater absolute values in contributions mean that a given protein has a greater importance.

Lists of proteins composed by the result of both univariate and multivariate analysis were mined for biological meaning by a gene ontology (GO) enrichment analysis, performed using ProteINSIDE webservice online tool version 2.0 (available at [https://umrh-bioinfo.clermont.inrae.fr/ProteINSIDE\\_2/index.php?page=upload.php](https://umrh-bioinfo.clermont.inrae.fr/ProteINSIDE_2/index.php?page=upload.php), Kaspric et al., 2015) based on *Bos taurus* and a search of biological reactions on Reactome based on *Homo sapiens*.

## Results

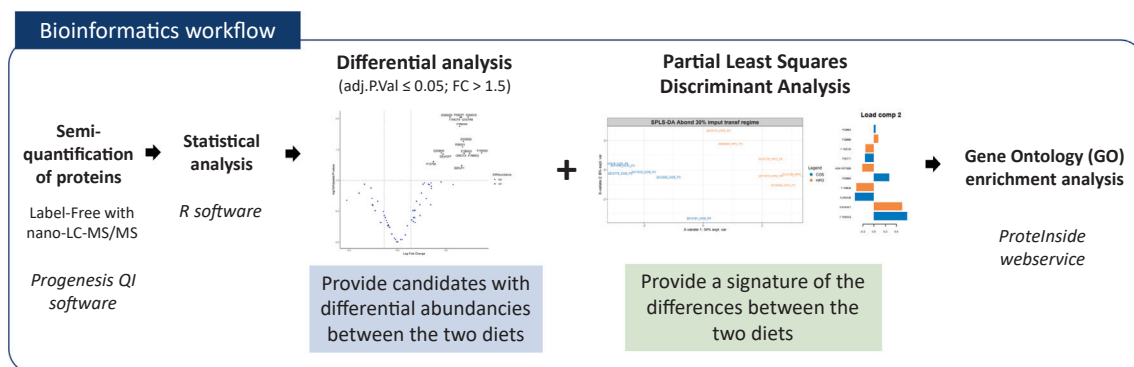
### Intake, milk production, energy balance and nutrient availability

The COS compared to the HPO diet decreased the total DMI ( $-18\%$ , Table 1) and oriented the ruminal fermentations towards the production of propionate ( $+34\%$ ) at the expense of butyrate ( $-40\%$ ) and to a lesser extent of acetate ( $-5\%$ , tendency at  $P = 0.09$ ; Table 2). Within milk, the concentration and yield of fat ( $-55$  and  $-63\%$ ) and protein ( $-14$  and  $-9\%$ ), as well as the yield of lactose ( $-22\%$ ), were decreased by the COS when compared to the HPO diets (Table 3). At the body level, the energy balance was higher in COS than in HPO diet ( $+23\%$ ), while the plasma concentration of  $\beta$ -hydroxybutyrate ( $-57\%$ ), urea ( $-33\%$ ), acetate ( $-47\%$ ) were decreased, and those of Non-Esterified Fatty Acids, glucose and insulin were not modified (Table 2).

### Plasma proteins

When a univariate differential analysis was applied to the abundance of the 98 proteins identified within plasma (Supplementary Table S1 available in the Data INRAE repository at <https://doi.org/10.15454/AL30HS>), no protein had an abundance that reached a significant difference between COS and HPO (Fig. 2A). However, among the 98 proteins, 15 proteins were able to discriminate the COS and HPO groups according to a multivariate analysis (Table 4 and Supplementary Fig. S1). Among them, three proteins (SERPINF2, TXNDC8, LBP) were associated with HPO diet effect and 12 proteins (CGN1, APOA4, ADIPOQ, AHSG, FN1, A2M, SERPINA3-5, LOC107131209, ALB, CRIM1, SERPING1, GPLD1) with the COS diet effect.

Due to the low number of proteins related to the HPO diet, the GO analysis was done for the 12 proteins related to COS diet (Supplementary Table S2 available in the Data INRAE repository at <https://doi.org/10.15454/AL30HS>). The 12 proteins were annotated by terms within the biological process (BP) category that emphasised the regulation of proteolysis; immune system process for SERPING1, LOC107131209, APOA4, FN1, GPLD1, ADIPOQ; acute-phase response for FN1 and AHSG; cholesterol efflux or regulation of lipid transport for APOA4 and ADIPOQ; regulation of cellular response to insulin stimulus for GLPD1. Moreover, LOC107131209 and AHSG were annotated by terms from the Reactome database related to the insulin pathways: Regulation of IGF transport and uptake by IGF Binding Proteins (IGFBPs) and Regulation of Complement cascade.



**Fig. 1.** Schematic diagram of the bioinformatics workflow reporting the protein identification and semi-quantification (performed with LC-Progenesis software coupled with the Mascot search engine), the statistical analysis based on both differential analysis and sparse Partial least squares discriminant analysis (sPLS-DA) (performed with R software) and the Gene Ontology (GO) enrichment analysis (performed with ProteINSIDE webservice) on proteins from dairy cow's fluids. Abbreviations: LC-MS/MS = liquid chromatography coupled to tandem mass spectrometry; adj.  $P$ -Val = adjusted  $P$ -value; FC = Fold change.

**Table 2**

Intake, plasma and rumen fermentation parameters of lactating multiparous dairy cows (n = 6) receiving dietary supplements of corn oil and starch (COS) or hydrogenated palm oil (HPO) during two periods (P3 and P4). Values are means for n = 6 cows per diet and per period.

Items	Diet		Period		SEM	P-value		
	COS	HPO	P3	P4		Diet	Period	Diet × Period
DIM <sup>1</sup> (day)	85	87	80	92	7	0.88	0.47	0.53
Intake								
Grassland hay <sup>2</sup> (kg)	7.8	9.8	9.3	8.2	0.5	0.04	0.11	0.04
Concentrate + lipid supplement <sup>3</sup> (kg)	10	12	11.6	10.4	0.5	0.03	0.03	0.01
Total DMI <sup>4</sup> (kg)	17.8	21.8	20.9	18.6	0.9	0.03	0.06	0.02
Energy balance (%)	121.3	98.5	113.4	106.4	5.0	0.01	0.35	0.13
Feed efficiency <sup>5</sup>	0.83	1.24	1.07	0.99	0.08	<0.01	0.44	0.68
Plasma parameters								
NEFA <sup>6</sup> (mmol/L)	0.126	0.121	0.12	0.13	0.01	0.77	0.50	0.41
β-hydroxybutyrate (mmol/L)	0.324	0.755	0.52	0.56	0.07	<0.01	0.52	0.51
Glucose (g/L)	0.712	0.745	0.69	0.76	0.02	0.51	0.17	0.69
Urea (g/L)	0.125	0.186	0.152	0.159	0.01	0.03	0.81	0.86
Acetate (mmol/L)	0.238	0.445	0.351	0.332	0.04	<0.01	0.67	0.14
Insulin (μUI/mL)	18.8	22.1	21.0	19.8	1.3	0.22	0.68	0.72
Rumen fermentation parameters								
Propionate (%/total VFA <sup>7</sup> )	22.5	14.8	18.3	18.9	1.36	<0.01	0.71	0.59
Acetate (%/total VFA)	67.9	71.5	69.9	69.5	0.99	0.09	0.82	0.64
Butyrate (%/total VFA)	6.3	10.6	8.7	8.1	0.68	<0.01	0.17	0.29
Ammonia (mmol/L)	2.8	4.6	3.1	4.3	0.51	0.06	0.21	0.35

<sup>1</sup> DIM: Days in Milk.

<sup>2</sup> For Grassland hay (kg), the mean values were 9.1 and 6.4 in P3 and P4 for COS and 9.6 and 10.0 in P3 and P4 for HPO groups.

<sup>3</sup> For Concentrate + lipid supplement (kg), the mean values were 11.3 and 8.7 in P3 and P4 for COS and 11.9 and 12.2 in P3 and P4 for HPO groups.

<sup>4</sup> DMI: DM intake. The mean values were 20.5 and 15.1 in P3 and P4 for COS and 21.5 and 22.1 in P3 and P4 for HPO groups.

<sup>5</sup> Ratio: kg standard milk production/kg DMI.

<sup>6</sup> NEFAs: Non-Esterified Fatty Acids.

<sup>7</sup> VFAs: Volatile-Fatty Acids.

**Table 3**

Milk production of lactating multiparous dairy cows (n = 6) receiving dietary supplements of corn oil and starch (COS) or hydrogenated palm oil (HPO) during two periods (P3 and P4). Values are means for n = 6 cows per diet and per period.

Items	Diet		Period		SEM	P-value		
	COS	HPO	P3	P4		Diet	Period	Diet × Period
Yield								
Milk (kg/d)	22.2	27.4	27.3	22.2	1.4	0.03	0.04	0.26
Fat (g/d)	388.2	1 061.5	768.5	681.2	104.8	<0.01	0.12	0.48
Protein (g/d)	752.9	827.6	861.6	718.9	33.5	0.20	0.03	0.57
Lactose (g/d)	1 107.2	1 434.1	1 388.6	1 152.7	77.2	0.01	0.06	0.40
Concentration								
Fat (g/L)	17.3	38.8	27.7	28.4	3.3	<0.01	0.60	0.42
Protein (g/L)	34.5	30.2	31.5	33.2	1.0	0.03	0.33	0.18
Lactose (g/L)	50.0	52.4	50.5	51.5	0.7	0.09	0.47	0.92

### Milk fat globule membrane proteins

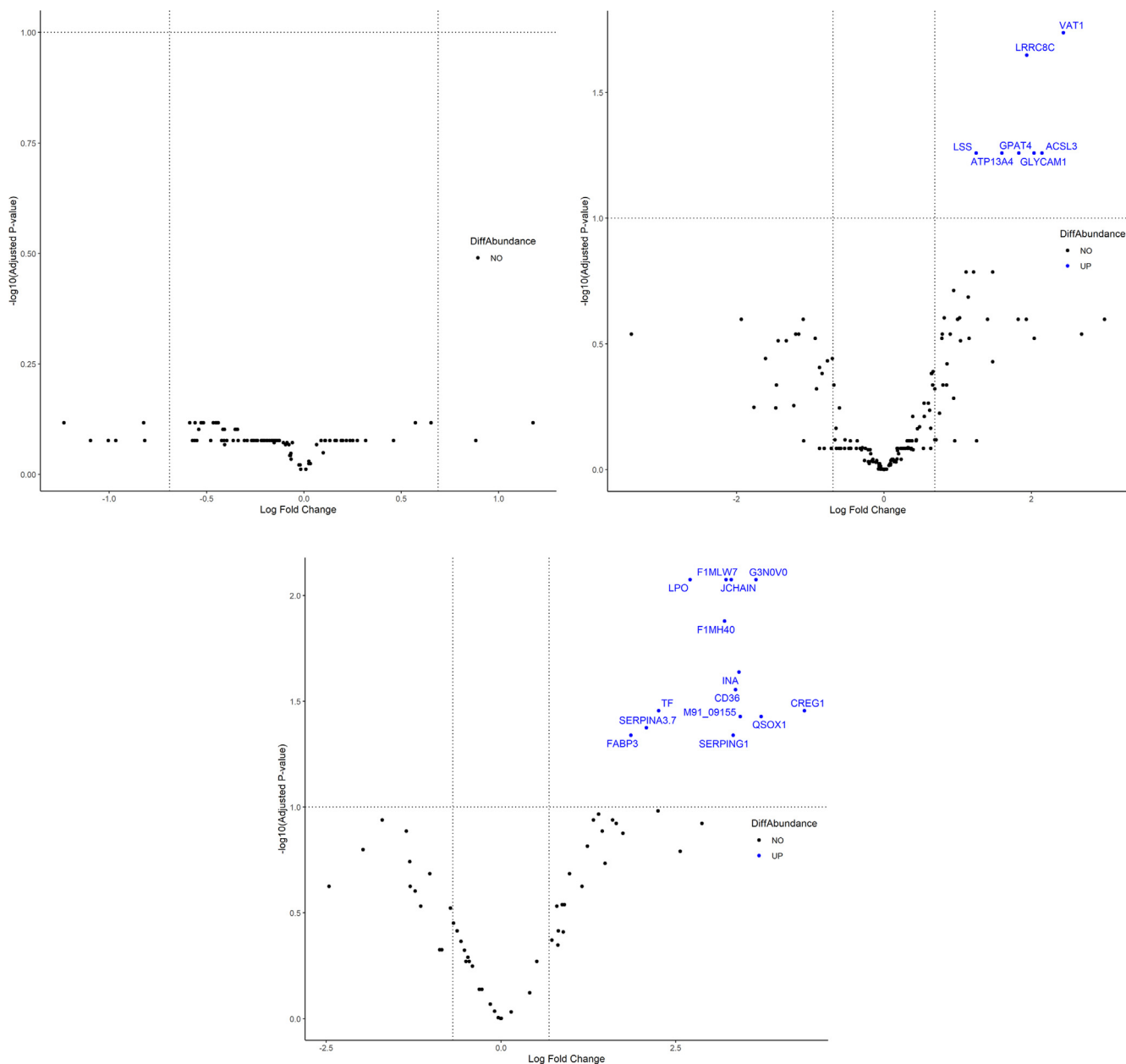
Among the 158 proteins identified from the MFGM fraction (Supplementary Table S1 available in the Data INRAE repository at <https://doi.org/10.15454/AL30HS>), seven proteins (VAT1, ACSL3, GLYCAM1, LRRC8C, GPAT4, ATP13A4, LSS), when considered one by one in a univariate analysis, were significantly less abundant (Fold Change range between 2.4 and 5.4, *P*-value adjusted by BH test < 0.1) in milk from cows fed the COS compared to the HPO diet (Table 5 and Fig. 2B). Furthermore, the sPLS-DA selected 24 proteins that discriminated the COS- and HPO-fed cows, including the seven proteins differentially less abundant (univariate analysis) in the COS than HPO groups (Table 5 and Supplementary Fig. S2). Most of these proteins were related to the HPO diet, except DHRS3, CLTC and LOC100337079 that were related to the COS diet.

GO analysis of 21 MFGM proteins related to HPO diet (Supplementary Table S3 available in the Data INRAE repository at <https://doi.org/10.15454/AL30HS>) was annotated by GO terms within the BP category or terms provided by the Reactome database that were mainly related to the lipid metabolism. The proteins

ACSL3, ALOX15, GPAM, GPAT4, DHRS3, and LSS were annotated by terms such as glycerolipid biosynthetic process, and metabolism of lipids. Of these, three proteins (ACSL3, ALOX15, and LSS) are localised in the milk fat globule membrane and were annotated by the GO term lipid droplet in the cellular component (CC) category, and two (GPAM and GPAT4) were annotated by the term glycerol-3-phosphate O-acyltransferase activity within the molecular function (MF) category. To sum up, the GO analyses of the MFGM proteins that discriminate the COS and HPO diet highlighted their biological functions within the lipid or glycerolipid biosynthetic process as well as the lipid droplet organisation.

### Skimmed milk proteins

Among the 70 proteins identified in SM fraction (Supplementary Table S1 available in the Data INRAE repository at <https://doi.org/10.15454/AL30HS>), when considered one by one in a univariate analysis, 14 proteins (LPO, JCHAIN, F1MLW7, G3N0V0, IGK, TF, INA, FABP3, SERPINA3-7, CD36, CREG1, QSOX1, M91\_09155, and SERPING1) were significantly more abundant (FC between 3.6 and



**Fig. 2.** Volcano plots report the differentially abundant proteins between COS and HPO diets in plasma (A), MFGM (B) and SM (C) of dairy cows. The abundance increases are significant with  $P$ -value  $< 0.1$  and FC  $> 1.5$ . Abbreviations: COS = corn oil and starch; HPO = hydrogenated palm oil; MFGM = Milk Fat Globule Membrane; SM = Skimmed milk; FC = Fold change.

20.3,  $P$ -value adjusted by BH test  $< 0.1$ ) in milk from cows fed HPO compared to COS diet (Table 6 and Fig. 2C). Furthermore, the sPLS-DA has selected 10 proteins that discriminated the COS- and HPO-fed cows, all were differentially abundant between the COS and HPO groups (Table 6 and Supplementary Fig. S3). According to GO analysis of the 14 proteins (Supplementary Table S4 available in the Data INRAE repository at <https://doi.org/10.15454/AL30HS>), seven proteins (G3NOV0, JCHAIN, SERPING1, TF, LPO, CD36, and F1MLW7) were annotated by GO terms within the BP category related to response to bacterium and the immune function with terms such as immune system process or humoral immune response. Supplementary Table S5 available in the Data INRAE repository at <https://doi.org/10.15454/AL30HS> reported the raw abundance ratios of discriminant proteins.

### Discussion

This study investigated the plasma and milk proteomes of cows fed with diet rich in corn oil and starch (COS) increasing C18:2n-6 in milk by comparison with cows fed with hydrogenated palm oil (HPO) increasing C16:0 in milk. These two groups of cows were chosen among a previous larger experiment because they experienced the most divergent milk fat secretion, with expected consequences on the DMI and the energy balance (Fougère et al., 2018; 2021). Indeed, as previously reported, the COS diet lowered DMI and caused a dramatic decrease in milk fat content ( $-45\%$ ) and yield ( $-10\%$ ) when compared to the control group. At the opposite, HPO had no adverse effects on milk yield and DMI but induced a 13% increase in milk fat content, when compared to the control

**Table 4**

Dairy cow plasma proteins from multivariate analysis that signed differences between the COS and HPO diets.

Accession	Gene Name	Protein Name	Multivariate analysis	
			Contribution* to COS	Contribution* to HPO
P28800	<i>SERPINF2</i>	Alpha-2-antiplasmin		0.649
A0JNM2	<i>TXNDC8</i>	Thioredoxin		0.148
F1MNN7	<i>LBP</i>	Lipopolysaccharide-binding protein		0.088
F1MFY6	<i>CGN1 (70% homology)</i>	Conglutinin	−0.536	
F1N3Q7	<i>APOA4</i>	Apolipoprotein A-IV	−0.340	
Q3Y5Z3	<i>ADIPOQ</i>	Adiponectin	−0.293	
P12763	<i>AHSG</i>	Alpha-2-HS-glycoprotein	−0.146	
G5E5A8	<i>FN1</i>	Fibronectin	−0.143	
Q7SIH1	<i>A2M</i>	Alpha-2-macroglobulin	−0.090	
A2I7N1	<i>SERPINA3-5</i>	Serpin A3-5	−0.074	
F1MVK1	<i>LOC107131209</i>	C4a anaphylatoxin	−0.038	
A0A140T897	<i>ALB</i>	Albumin	−0.037	
F1MW38	<i>CRIM1</i>	Cysteine-rich transmembrane BMP regulator 1	−0.037	
P80109	<i>GPLD1</i>	Phosphatidylinositol-glycan-specific phospholipase D	−0.025	
E1BMJ0	<i>SERPING1</i>	Serpin family G member 1	−0.001	

Abbreviations: COS = corn oil and starch; HPO = hydrogenated palm oil.

\* Contributions indicate the importance of each protein on the component 1 that discriminates the COS and HPO diets.

**Table 5**

Dairy cow milk fat globule membrane proteins from multivariate and univariate analyses that signed differences between the COS and HPO diets.

Accession	Gene Name	Protein Name	Multivariate		Univariate		
			Contribution* to COS	Contribution* to HPO	FC**	RSD	adj. P-value***
F1MUP9	<i>VAT1</i>	Vesicle amine transport 1		−0.368	5.4	0.737	0.018
F1MEX9	<i>ACSL3</i>	Acyl-CoA synthetase long-chain family member 3		−0.242	4.4	0.987	0.055
P80195	<i>GLYCAM1</i>	Glycosylation-dependent cell adhesion molecule 1		−0.275	4.1	0.855	0.055
A5PK13	<i>LRRC8C</i>	Volume-regulated anion channel subunit LRRC8C		−0.363	3.8	0.599	0.022
A3FPG8	<i>GPAT4</i>	Glycerol-3-phosphate acyltransferase 4		−0.265	3.6	0.792	0.055
F1N272	<i>ATP13A4</i>	Cation-transporting ATPase		−0.267	3.0	0.688	0.055
F1MTJ9	<i>LSS</i>	Terpene cyclase/mutase family member		−0.351	2.4	0.406	0.055
Q0IIG8	<i>RAB18</i>	Ras-related protein Rab-18		−0.265			
P08239	<i>GNAO1</i>	Guanine nucleotide-binding protein G(o) subunit alpha		−0.255			
Q5GJ77	<i>GPAM</i>	Glycerol-3-phosphate acyltransferase 1, mitochondrial		−0.222			
P10790	<i>FABP3</i>	Fatty acid-binding protein, heart		−0.209			
Q2KIS4	<i>DHRS1</i>	Dehydrogenase/reductase		−0.158			
F1MHC2	<i>STXBP2</i>	Syntaxin binding protein 2		−0.152			
Q95LA9	<i>TLR2</i>	Toll-like receptor 2		−0.142			
F1N6D4	<i>SLC34A2</i>	Sodium-dependent phosphate transport protein 2B		−0.113			
Q2TBH2	<i>RRAS</i>	RAS related		−0.086			
Q3ZCA7	<i>GNAI3</i>	G protein subunit alpha i3		−0.082			
E1BQ28	<i>SLC39A8</i>	Solute carrier family 39 member 8		−0.055			
A7MBA9	<i>PSEN1</i>	Presenilin		−0.053			
E1BA29	<i>GNAQ</i>	G protein subunit alpha q		−0.040			
P27479	<i>ALOX15</i>	Polyunsaturated fatty acid lipoxygenase ALOX15		−0.017			
F1N334	<i>DHRS3</i>	Short-chain dehydrogenase/reductase 3	0.054				
F1MPU0	<i>CLTC</i>	Clathrin heavy chain	0.012				
G3MW08	<i>LOC100337076</i>	Uncharacterised protein	0.010				

Abbreviations: COS = corn oil and starch; HPO = hydrogenated palm oil.

\* Contributions indicate the importance of each protein on the component 1 that discriminates the COS and HPO diets.

\*\* FC: Fold Change ratio.

\*\*\* adj. P-value: adjusted P-value.

group (Fougère et al., 2018). Thus, in the present study, when COS-fed cows were compared to HPO-fed cows, they experienced strong decreases in milk fat content (−55%) and yield (−63%), as well as in DMI (−18%). Because of the lowered energy secretion in the milk, COS-fed cows had a higher energy balance (+23%) than the HPO-fed cows. Moreover, at the rumen level, the COS diet has induced a shift of ruminal fermentation towards propionate synthesis, at the expense of butyrate and acetate, and a lower total tract digestibility of fibre. This was accompanied by a putative lowering ruminal pH or ruminal functionality with COS rich in starch compared to HPO (Martin et al., 2021). In addition, the COS diet induced a concomitant shift of the ruminal PUFA biohydrogenation pathway from *trans*-11 towards *trans*-10 leading to the synthesis of intermediates with antilipogenic effects (Fougère and Bernard, 2019). We thus hypothesised that the comparison of COS- relative to HPO-fed cows was an extreme model to look for molecular sig-

natures, measurable within the body fluids (both plasma and milk), of energy status, energy partitioning or immune/inflammation processes depending on the nutrition and its consequence in energy balance and plasma nutrient profile. The major finding of the present work was the identification of the molecular signatures that discriminate the COS from HPO cows, and they were related to the interplay between nutrient availability and lipid metabolism or inflammation or innate immunity, depending on the origin of proteins, i.e. plasma, SM or MFGM.

*Plasma discriminant proteome revealed an interplay between nutrient availability and endocrine system or immune function*

The multivariate molecular signature that discriminated the cows fed with COS diet from cows fed the HPO diet was composed of 15 plasma proteins. We then identified, by mining, proteins



**Table 6**

Dairy cow skimmed milk proteins from multivariate and univariate analyses that signed differences between the COS and HPO diets.

Accession	Gene Name	Protein Name	Multivariate	Univariate		
			Contribution* to HPO	FC**	RSD	adj. P-value***
G3MXZ0	LPO	Lactoperoxidase	-0.528	6.5	0.971	0.008
Q3SYR8	JCHAIN	Immunoglobulin J chain	-0.461	9.8	1.276	0.008
F1MLW7	F1MLW7	Ig-like domain-containing protein	-0.444	9.3	1.271	0.008
G3NOV0	G3NOV0	(Similar to) Immunoglobulin-like	-0.401	12.6	1.506	0.008
F1MH40	IGK	(Similar to) IGK Protein	-0.294	9.2	1.465	0.013
G3XG63	TF	Beta-1 metal-binding globulin	-0.178	4.8	1.145	0.035
Q08DH7	INA	(Similar to) Alpha-internexin	-0.124	10.6	1.809	0.023
P10790	FABP3	Fatty acid-binding protein. heart	-0.103	3.6	1.005	0.046
G8JKW7	SERPINA3-7	Serpin A3-7	-0.062	4.2	1.163	0.042
P26201	CD36	Platelet glycoprotein 4	-0.056	10.3	1.886	0.028
F1MX50	CREG1	Cellular repressor of E1A stimulateds 1		20.3	2.772	0.035
F1MM32	QSOX1	Sulfhydryl oxidase		13.2	2.419	0.037
G5E513	M91_09155	(Similar to) Ig-like domain-containing protein ( <i>Bos mutus</i> )		10.7	2.207	0.037
E1BMJ0	SERPING1	Serpin family G member 1		10.0	2.316	0.046

Abbreviations: COS = corn oil and starch; HPO = hydrogenated palm oil.

\* Contributions indicate the importance of each protein on the component 1 that discriminates the COS and HPO diets.

\*\* FC: Fold Change ratio.

\*\*\* adj. P-value: adjusted P-value.

mainly related to (1) energy partitioning, nutrient availability and insulin sensitivity (ADIPOQ, APOA4, GPLD1, AHSG), or (2) the immune-inflammatory processes with eight proteins related to the acute-phase response (LBP, A2M, LOC107131209, ALB, ASHG, FN1, SERPING1, CGN1).

The relatively higher abundances of adiponectin (ADIPOQ), Phosphatidylinositol-glycan-specific phospholipase D (GPLD1), Apolipoprotein A-IV (APOA4) and Alpha-2-HS-glycoprotein (AHSG) in the plasma of COS- than HPO-fed cows are consistent with the decreased DMI, the nutrient availability or the putative higher insulin sensitivity of non-mammary tissues of the COS group. Indeed, APOA4 is a major protein component of lymphatic chylomicrons which is produced and secreted by enterocytes in response to fat consumption. Compared to the HPO-fed cows, the higher abundance of APOA4 in the plasma of COS-fed cows suggests a higher secretion of chylomicrons. This is in line with the stimulated intestinal secretion of chylomicrons by unsaturated long-chain fatty acids reported in sheep, while saturated long-chain fatty acids specifically cause the intestinal production of very low-density lipoprotein (Harrison et al., 1974). APOA4 was also reported as a gastrointestinal satiety signal that reduces food intake through the cholecystokinin system in rodents (Lo et al., 2012). In the present study, the increase of plasma APOA4 and the concomitant decrease in DMI when the COS was compared to the HPO group sustain this hypothesis even if additional research is needed to determine whether APOA4 is of physiological importance for dietary fat-induced satiety in ruminants.

Elsewhere, since large amounts of glucose are required to produce lactose, a high milk production requests high glucose availability in dairy cows. The glucose uptake by the mammary gland is prioritised and achieved by a reduction of the insulin sensitivity in peripheral organs (such as adipose tissue and skeletal muscle), whereas the mammary gland is independent of insulin. The higher plasma abundances of ADIPOQ, GPLD1 and AHSG observed in this experiment with COS compared to HPO diet could alleviate insulin resistance in peripheral tissues other than the mammary gland. Indeed, ADIPOQ is an adipokine with insulin-sensitising effects that was proposed to be associated with glucose and lipid metabolisms in ruminants (Sauerwein and Häußler, 2016). The higher concentration of ADIPOQ in the plasma of COS- than HPO-fed cows might reflect a prioritisation for energy partitioning towards body tissues rather than mammary gland, as it was proposed when primiparous and multiparous cows were compared for their adaptive ability to face early lactation (Sauerwein and Häußler, 2016).

Interestingly, in this latter model, low  $\beta$ -hydroxybutyrate concentrations were concomitant with high adiponectin plasma levels, as in the present study, in the cows fed COS diet relative to HPO. Thus, the highest abundance of ADIPOQ in the COS group is consistent with the involvement of  $\beta$ -hydroxybutyrate rather than energy balance in the regulation of ADIPOQ synthesis by bovine subcutaneous adipose tissue (Hosseini et al., 2012).

Much less knowledge is available for GPLD1 and AHSG, especially in ruminants. Regarding GPLD1, the dependence of serum GPLD1 activity on the hyperglycemic/hyper insulinemic state was proposed (Muller et al., 2021), and GPLD1 (with APOA4) was described as a plasma biomarker associated with human prediabetes (von Toerne et al., 2016). Moreover, AHSG also called Fetuin-A, apart from being an acute-phase proteins (APPs), is a 64-kDa liver-derived circulating glycoprotein that functions as a major carrier of free FA in plasma. Consistently, higher levels of plasma AHSG in obese or diabetic humans as well as in high-fat-fed animals were reported, together with a relationship between lipid and AHSG to decrease insulin sensitivity (Dasgupta et al., 2010). Among the mediators of plasma AHSG abundance, palmitic acid-enriched diet was reported to decrease the plasma concentration of AHSG in rabbits (Donis et al., 2021), while the AHSG concentration was not modified by the level of DMI or the energy balance in mid-lactating cows, or decreased in early lactating cows in negative energy balance (Strieder-Barboza et al., 2018; Veshkini et al., 2022a). The link between AHSG or GPLD1 plasma concentration and insulin signalling or nutrient availability and uptake in lactating cows warrants further investigations. We may hypothesise that these proteins have mediated an increased glucose uptake in non-mammary tissues, while the lactose yield in milk was decreased in the COS compared to the HPO group, probably explaining the lack of differences in glycaemia between the two groups.

The second cluster of proteins that we have identified in plasma is APP. In plasma from mammals, approximately 40 APPs are described that share a common protective role from injury (e.g., infection or tissue damage). Their concentrations increase (positive APP) or decrease (negative APP) at least by 25% in response to inflammation (Serrano et al., 2018). In the present study, increased abundance of both positive (A2M, LOC107131209, SERPING1, CGN1) and negative (ALB, AHSG, FN1) APP or APP-related proteins were observed in COS-fed compared to HPO-fed cows. In addition, the abundance of LBP, which is considered as a negative APP with anti-inflammatory properties, was decreased. The modifications in the abundance of several APP or APP-related proteins between the

COS and HPO diets may reflect that circulating saturated and unsaturated FAs (Delavaud et al., 2022) may have large immunomodulatory effects in dairy cows even if the relationship between lipids and immune response is not fully understood (Ingvarsten and Moyes, 2013). Whether this multivariate molecular signature reflects an activation of specific- or innate-immune responses depending on the nutrient availability remains to be investigated.

To conclude, we provided a discriminant plasma proteome of mid-lactating cows divergent in DMI, energy balance, plasma nutrient availability as the result of lipid-diet supplementation. The discriminant proteome on these two sets of dairy cows constitutes a very original contribution that illustrates the interplay between the nutrient availability and the endocrine system or the immune function, and is in line with previous reports in periparturient cows (Veshkini et al., 2022a).

*Milk discriminant proteome was related to lipid metabolism/secretion and to inflammatory/immune processes*

Among the 158 proteins identified in MFGM, 76% were common with the extensive list of 970 gene names (GN) identified in cows MFGM by Reinhardt and Lippolis (2008) and 63% were common with the 246 GN-specific cows MFGM compiled by Delosière et al. (2019) outlining their specificity of this milk fraction. Among the 70 SM proteins, 70 and 63% were common with the extensive whey GN list identified by Reinhardt et al. (2013) and the SM GN listed in silico (Delosière et al., 2020) on dairy cows outlining their specificity of SM.

The molecular signature that differentiates HPO and COS treatments was composed of 24 and 15 proteins identified respectively in MFGM and SM fractions, of these 12 (GPAM, GPAT4, ACSL3, PSEN1, STXB2, TLR2, GNAI3, GLYCAM1, ALOX15, LSS, DHRS3, and FABP3) were annotated by GO terms related to lipid metabolism and secretion. We particularly underlined six of them that were more abundant in milk MFGM or SM from HPO compared to COS group: GPAT4, GLYCAM1, ACSL3, GPAM and FABP3 in the MFGM and CD36 and FABP3 in SM. This protein list is consistent with the highlighted key genes involved in ruminant milk fat synthesis reviewed by Mu et al. (2021). Indeed, milk lipids are present in the form of milk fat globules (MFG) that contains a core of non-polar lipids (mainly triacylglycerols that represents more than 98% of milk fat) coated into the MFGM composed of polar lipids, mainly phospholipids, glycolipids, and minor specific proteins. The milk FAs constituting the triacylglycerols fraction have two origins: they are *de novo* synthesised in the mammary epithelial cells by cytosolic enzymatic complex (ACACA and FASN) and taken up by mammary epithelial cells from circulating FA as free FA by various transporters or released by the enzyme lipoprotein lipase from TAG circulating under the form of chylomicrons and very low-density lipoprotein. Among the sets of proteins involved in milk fat synthesis and secretion pathways (Bernard et al., 2005), lipoprotein lipase and FASN were detected in the MFGM of both COS and HPO treatments, but without noticeably changed abundance at the protein (present study) and mRNA (Fougère and Bernard, 2019) levels. Conversely, GPAT4, ACSL3, and GPAM related to “lipid biosynthetic process”, “FA metabolic process” and “secretion” reactions were highly expressed in MFGM from HPO compared to COS. Thus, ACSL3 belongs to the long-chain-fatty-acid-CoA ligase proteins and plays a key role in lipid biosynthesis, with ACSL3 gene identified among 20 promising genes associated with milk FA traits in Chinese Holstein (Li et al., 2014). Regarding GPAT4 and GPAM, both are involved in the esterification of the FA to glycerol for the milk triacylglycerols synthesis. Similarly, FABP3 in MFGM and SM, and CD36 in SM, which are proteins that participate in the uptake and transport of long-chain FA

(Bionaz and Loor, 2008), were more expressed in HPO compared to COS. This is in line with a much higher activity of lipid transport and synthesis within the mammary gland in the animals under HPO dietary treatment, as evidenced by the increase of milk fat secretion. All these findings were consistent with the maintenance of milk fat secretion under HPO dietary conditions compared to the dramatic decrease observed with COS (Fougère et al., 2018). Conversely, during diet-induced MFD, a decreased mammary expression of genes for key enzymes and proteins involved in FA uptake, synthesis, transport and esterification was reported (Shingfield et al., 2010), suggesting a decrease in the encoded proteins. However, some studies did not show an effect on the expression of all mammary lipogenic genes that were studied during diet-induced MFD, which could be explained by post-transcriptional or post-translational regulation of these genes (Fougère and Bernard, 2019; Harvatine et al., 2018). Consistent with these findings, in the present study, the lower abundance of milk proteins involved in lipid synthesis observed in COS compared to HPO diet reveals a decreased mammary capacity for lipid synthesis due to a down-regulation of the synthesis of enzymes and proteins involved in milk fat synthesis and secretion.

Otherwise, GLYCAM1 associated to the MFGM, significantly more abundant in HPO, was correlated (results not shown) to the mammary mRNA abundances of Sp1 and TLR4 ( $R = -0.77$  and  $R = -0.64$ ,  $P < 0.05$ , respectively) and mTor ( $R = +0.69$ ,  $P < 0.05$ ) quantified on the same animals from the same study (Fougère and Bernard, 2019). This positive relationship between GLYCAM1 and mTor can be related to the mediating role of the mTOR/4EBP1/PPARG axis in insulin-induced lipogenesis that regulates lipogenic gene expression in bovine mammary epithelial cell cultures (Guo et al., 2019). Moreover, mTOR complex 1 is involved not only in lipid metabolism but also in immune responses in the body (Cheon and Cho, 2021). Therefore, mTOR expression was decreased in the mammary gland of high concentrate-fed dairy cows that experienced inflammation due to subacute ruminal acidosis (Zhang et al., 2016). The lowest abundances of GLYCAM1 in milk, a protein known to bind to the L-selectin receptor during inflammation, thereby promoting leukocytes recruitment to sites of inflammation, as well as the lowest mTOR mRNA abundance in the mammary gland of COS-fed cows thus suggest an inflammation. In line with this, the negative relationships between GLYCAM1 together with the mammary mRNA abundance of SP1 and TLR4 (Fougère and Bernard, 2019) involved in many cellular processes, including apoptosis and immune responses, probably reveal the induced ruminal acidosis and subsequent systemic inflammation due to COS treatment (Ma et al., 2022; Meng et al., 2022).

Otherwise, CD36, F1MLW7, G3NOV0, JCHAIN, QSOX1, IGK, SERPING1, M91\_09155, TF, and SERPING1 proteins are more abundant in the SM of HPO-fed cows compared to COS. These proteins annotated with immunity-related terms highlight the effect of various lipid supplements on immune pathways in dairy cows that would warrant further clarification. In addition to the positive APP SERPING1, a cluster of immunoglobulins is highly represented in SM from cows fed HPO diet (JCHAIN, F1MLW7, G3NOV0, IGK, M91\_09155). Immunoglobulins are milk-soluble proteins that provide immune defences and antibodies for the mammary gland and the young mammals. The significant GO BP terms that have annotated the SM proteins largely relate to “immune response” and “defence response to bacterium” with domination of IgA and IgM terms. These secreted immunoglobulins support the idea of immune defence mechanisms in mammary gland with HPO diets. On the other hand, HPO increased the abundance of lactoperoxidase compared to COS highlighting the lower antimicrobial capacity of the mammary gland of cows fed COS diet. The enzyme LPO with hydrogen peroxide ( $H_2O_2$ ) and thiocyanate ( $SCN^-$ ) forms the lactoperoxidase system, a natural antimicrobial system of milk.

Moreover, LPO as an antiviral protein (Perraudin, 1991) may protect the udder from infection and promote growth in newborn calves.

Finally, the milk proteomic signature that differentiates the HPO and COS treatments highlights and supports the maintenance of lipid synthesis, lipid droplet formation and secretion observed with HPO conversely to COS explaining the large decrease in milk fat secretion in the latter case. Probably that the occurrence of inflammation under COS treatment contributed to alter the physiological function of the mammary gland, and exacerbated the depression of milk fat synthesis in response to the ruminal synthesis of antilipogenic intermediates (Fougère et al., 2018).

## Conclusions

This study provides new knowledge on the impact of nutrition, mainly used as a strategy to improve the nutritional value of milk, on the metabolism of the whole dairy cow. Indeed, a decrease in milk fat synthesis, by reducing milk energy secretion, improves the energy balance of lactating ruminants and the efficiency of nutrient utilisation at the body level. Therefore, nutritional strategies inducing short-term MFD could be considered to spare energy for milk fat synthesis in favour of fat body stores, which could improve subsequent lactation and reproduction of dairy animals and in the longer term their longevity. However, diets inducing MFD cause alterations in ruminal metabolism that may lead to subacute ruminal acidosis. The results of this study suggest a higher state of inflammation with the COS diet that induced MFD compared to the HPO diet, which may have altered the physiological function of the mammary gland with a higher risk of mastitis.

## Supplementary material

Supplementary material to this article can be found online at <https://doi.org/10.1016/j.animal.2023.100822>.

## Ethics approval

The Auvergne Rhône-Alpes Ethics Committee for Experiments on Animals approved the experimental procedures (France, DGGRI agreement APAFIS#3277-2015121411432527 v5). These procedures were compliant with the guidelines established by the European Union Directive 2010/63/EU.

## Data and model availability statement

The dataset generated for this study is publicly available with a CC-BY licence and can be found in the Data INRAE repository (<https://entrepot.recherche.data.gouv.fr/dataverse/inrae>): Delosière, Mylène; Bernard, Laurence; Viala, Didier; Fougère, Hélène; Bonnet, Muriel, 2023, "Datasets reporting s-PLSDA results and gene ontology enrichment of proteins identified in plasma and milk of dairy cows fed lipid supplements.", <https://doi.org/10.15454/AL30HS>, Recherche Data Gouv, V2, UNF:6:YvW3DjFQTfBkY9hpgXqRg== [fileUNF].

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## Author contributions

**Mylène Delosière:** Proteome Methodology, Data curation, Formal analysis, Visualisation, Writing – original draft.

**Laurence Bernard:** Conceptualisation and management of the NutriLip projet, Supervision, Visualisation, Writing – original draft.

**Didier Viala:** Proteome Analysis, Data Curation, Writing – review and editing.

**Hélène Fougère:** Management of the animal experiment, and plasma or milk sampling, Writing – review and editing.

**Muriel Bonnet:** Conceptualisation of the present study, Funding acquisition, Supervision, Visualisation, Writing – original draft. All authors agree to be accountable for the content of the work.

## Declaration of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Acknowledgments

The experimental cow model is issued from PhD thesis of [Hélène Fougère \(2020\)](https://tel.archives-ouvertes.fr/tel-02094454), <https://tel.archives-ouvertes.fr/tel-02094454>. The authors gratefully acknowledge the staff of Unité Expérimentale Herbipôle de Theix (INRAE, Saint-Genès-Champanelle, France; <https://doi.org/10.15454/1.5572318050509348E12>) for their diligent care of the experimental animals; Sylvie Bardou-Valette, Martine Tourret and Arnaud Delavaud from UMR 1213 Herbivores (INRAE) for their assistance with the experiment management, sample collection and laboratory analysis; Christophe Chambon from the Plateforme d'Exploration du Métabolisme composante Protéomique (INRAE, Saint-Genès-Champanelle, France; <https://doi.org/10.15454/1.557239511356972E12>) for the proteome analysis; Andréa Rau and Maxime Guilleton for their help in coding of statistical analysis.

## Financial support statement

The experimental trial was financed by APIS-GENE as part of the NutriLip project. Milk proteome analyses were funded by INRAE. The authors acknowledge the support received from the International Research Center on sustainable AgroEcosystems (ISITE CAP2025).

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