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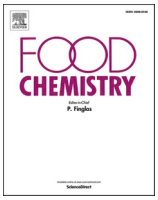

Lipidomics of sheep and goat Milk-based infant formulae during *in vitro* dynamic digestion

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ABSTRACT

Lipid hydrolysis process during IF digestion, particularly the characterization of the lipidome and the resulting lipid breakdown products, has not been thoroughly investigated. This study aimed to compare the lipid hydrolysis profiles during the *in vitro* dynamic digestion of IFs made from whole sheep and goat milk. Using a lipidomics platform and multivariate statistical analysis, we observed changes in complex lipid levels during digestion. In the gastric compartment, we noted a progressive hydrolysis of triacylglycerols, phosphatidylcholines, and sphingomyelins. Conversely, lipolysis breakdown products like monoacylglycerols (*e.g.*, MG(16:0), MG (18:0)), diacylglycerols, lysophosphatidylcholines (LPC 16:0, LPC 18:1, LPC 18:2), and free fatty acids increased in the intestinal compartment. The lipolysis trends were similar for both types of infant formulas, with long-chain fatty acid triglycerides (C > 46) exhibiting lower digestibility compared to medium-chain fatty acid triglycerides. Overall, these results indicate that sheep milk can be used as an ingredient in the manufacturing of IF.

1. Introduction

Human milk is recognized as a complete source of nutrition for newborns, fully meeting their physiological needs during development. When breastfeeding is not possible, infant formula (IF) serves as an appropriate alternative. Most commercial IF products are based on bovine milk, with lipids supplied by a blend of vegetable oils. Recently, IFs made from the milk of small ruminants like goats and sheep have been introduced. While goat milk-based IFs are widely accepted and commercially available (Mohapatra et al., 2019), the use of sheep milk in IFs is prohibited in the EU (Commission Directive 2013/46/EU) but permitted in other countries, including New Zealand and China. (Maryniak et al., 2022). In a previous study, we evaluated various sheep milk fractions as ingredients for manufacturing infant formulas (IFs). The resulting IFs exhibited a balanced nutritional profile suitable for neonatal growth (Lai et al., 2023). Among ruminants, whose milk is used as ingredient of IFs, sheep milk exhibits higher average fat levels compared to cow and goat milk (Park et al., 2007). Ruminant milk fat differs from human milk fat, with human milk generally having higher levels of long-chain fatty acids and lower levels of short-chain fatty acids (Wang et al., 2022). Therefore, in the production of IF, the milk fat is often supplemented with or entirely replaced by vegetable oils to better mimic the composition of human milk. IF lipids are essential in influencing the short-term and long-term health of infants. Ongoing monitoring of nutritional implications becomes essential when substituting bovine milk fat with vegetable oils (Berger et al., 2000). Indeed, the presence of various bioactive compounds in vegetable oils, including phytosterols, triacyclglycerols containing very long-chain fatty acids, warrants investigation to understand their impact on neonatal health.

Thus, considering the crucial role of lipids in human nutrition, lipidomics, *i.e.* the large-scale study of lipids using hyphenated analytical techniques (Han, 2016), may improve the knowledge of their biological role. In this context, we recently published a study using a lipidomics approach to examine variations in the lipid profiles of goat and sheep whole milk (Manis et al., 2023), highlighting differences in the complex lipid composition of the two types of milk. This lipidomic approach can also be used for the monitoring of lipid changes or their hydrolysis products throughout the entire digestion process.

Dynamic digestion systems are employed to simulate the digestive processes in the stomach and small intestine following food ingestion, allowing for the monitoring of proteolysis and lipolysis kinetics. (Ménard et al., 2014). The *in vitro* dynamic model exhibits variations in

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pH and enzyme concentrations which are dependent on the progression of time (Ménard et al., 2014). This contrasts with *in vitro* static digestion models, where pH and enzyme concentrations remain constant. *In vitro* dynamic systems adapted to simulate infant digestion have been proposed and validated, and a comparison with *in vivo* digestion of IF was performed (Ménard et al., 2015). Nonetheless, there has been limited research on *in vitro* lipid digestion. This was due to the absence of human gastric lipase analogs, but in 2006, rabbit gastric extract was proposed as a viable substitute for human gastric lipases, as indicated by Sams et al. (2016), and subsequently integrated into the INFOGEST protocol (Brodkorb et al., 2019).

In vitro lipid digestion studies are indispensable for advancing our knowledge of lipid digestion and absorption. Recently, de Figueiredo Furtado et al. (2021) reported that the extent of lipolysis during *in vitro* dynamic digestion of IF is influenced by its lipid composition, with IF containing medium-chain triacylglycerols showing a higher degree of lipolysis (de Figueiredo Furtado et al., 2021).

This study aimed to compare, *via* a lipidomic approach, the hydrolysis profiles of lipids during *in vitro* dynamic digestion of 0–6 months IFs based on sheep and goat whole milk. We hypothesized that sheep milk-based IF lipids would be digested similarly to those in goat-based IF. Thus, gastric and intestinal digested samples were collected at different time points and submitted to UHPLC-QTOF/MS platform coupled to multivariate statistical analysis. The complete characterization of complex lipids was achieved through mass spectrometric fragmentation experiments. The concentrations of annotated complex lipids were compared over time, and the fatty acid (as methyl esters) content of IFs, digested samples, and vegetable oils used in IF manufacturing, were measured.

2. Materials and methods

2.1. Chemicals and reagents

Analytical grade methanol, 2-propanol, acetonitrile, chloroform, potassium chloride, hexane, ammonium acetate, ammonium formate, and sodium methoxide were obtained from Sigma Aldrich (Milano, Italy), while deuterated lipid analytical standard SPLASH lipidomix was obtained by Avanti Polar (Birmingham, AL, USA). Bi-distilled water with conductivity of 0.072 μS/cm was obtained with a MilliQ purification system (Millipore, Milan, Italy). Porcine pepsin (P6887; 3090 U/mg), porcine pancreatin (P7545; 83 U of lipase/mg), bovine bile extract (B3883; 1 mmol/g), and the enzyme inhibitors pepstatin A (P5318), pefabloc (76307) and 4-bromophenyl boronic acid (B75956) were obtained from Sigma Aldrich (Milano, Italy). Rabbit gastric extract (RGE), 74 U/mg of lipase and 754 U/mg of pepsin was provided by Lipolytech (Marseille, France).

2.2. Infant formulae

Powdered IFs (0 to 6 months) were kindly provided by Blue River (Invercargill, New Zealand), together with the vegetable oil blend used as ingredient. Sheep and goat IFs were manufactured using whole milk (10–20%), whey (55–65%). The vegetable oils consist of a mixture of canola oil, sunflower oil, and coconut oil. Gross compositions of sheep and goat milk IF samples, expressed as % of dry matter, are reported in **Table S1**. Before introduction into the *in vitro* dynamic digestion system the IF powders were rehydrated with warm boiled drinking water, following instructions indicated in their labels.

2.3. In vitro dynamic digestion

Gastrointestinal digestions of goat and sheep IFs were performed in a *in vitro* dynamic system (DIDGI®, INRAE) as previously described and validated by Ménard et al. (2014). The parameters for gastric and intestinal phases were chosen to closely mimic the digestive conditions of full-term newborns fed with human milk at the postnatal age of four weeks and have been adapted from de Oliveira et al. (2017), with a modification in the gastric emptying half-time of 78 min, to simulate the emptying related to IFs (de Oliveira et al., 2016). The *in vitro* dynamic system was controlled by the StoRM® software (INRA, Grignon, France), which allows the regulation and monitoring of the digestive parameters, such as the gastric acidification curve, the gastric and intestinal emptying, and the enzymes flow. Gastro-intestinal digestive parameters are reported in **Table S2**. Digestion experiments were performed over three h collecting three independent samples for each IF. Samples were collected in both gastric (G) and intestinal (I) compartments at 40, 80, 120, and 180 min. Samples were labelled from G0 to I180 for sheep and goat IF (27 samples for each group). Protease inhibitors, 10 μL of 0.72 mM Pepstatin A per mL of gastric digested sample and 50 μL of 0.1 M Pefabloc ((4-(2-Aminoethyl)-benzolsulfonylfluorid-hydrochloride), and 50 μL of 0.1 M 4-bromophenyl boronic acid per mL intestinal *digesta*, were added to the samples before freezing at −20 °C.

2.4. UHPLC-QTOF/MS analysis

Samples were thawed on ice and 100 μL of the solutions were poured in 1.5 mL Eppendorf tubes and 250 μL of methanol and 125 μL of chloroform were added. After 1 h, another 380 μL of chloroform and 90 μL of KCl 0.1 M were added. The samples were vortexed and centrifuged for 10 min at 17700 relative centrifugal force or g force. Two hundred μL of the organic phase were transferred to glass vials and evaporated under a gentle nitrogen stream. The samples were then redissolved with 20 μL methanol/chloroform (1:1 *v/v*) and 980 μL of a solution of 2-propanol/acetonitrile/water (2:1:1 *v/v*). The samples were filtered with 0.22 μm PTFE filters. Two hundred μL of these solutions were then transferred to glass vials adding 10 μL of the SPLASH lipidomix deuterated internal standards. For the gastric and intestinal sheep and goat digested samples, quality control samples were prepared aliquoting together 10 μL of each sample.

The samples were analyzed with an Agilent 6540 IM-QTOF/MS (Agilent Technologies, Palo Alto, CA). Samples were injected in a 1290 Infinity II UHPLC (Agilent Technologies, Palo Alto, CA) and resolved on a Kinetex 150 mm × 2.1 mm C18 column (Agilent Technologies, Palo Alto, CA). The column was maintained at 50 °C at a flow rate of 0.4 mL/min. For the positive ionization mode, the injection volume was 1 μL and the mobile phase consisted of (A) water containing 10 mM ammonium formate, 0.1% formic acid and acetonitrile (3:2 *v/v*, pH = 4.66) and (B) isopropanol/acetonitrile (9:1 *v/v*) containing 10 mM ammonium formate and 0.1% formic acid (pH = 6.10) The chromatographic separation was obtained with the following gradient: initially 40% of B, then a linear increase of B to 50% in 2.1 min and then to a 70% of B in 12.1 min. Subsequently the mobile phase B was increased to 99% in 18.1 min and then brought back to the initial conditions in 2 min.

The injection volume for the negative ionization was 6 μL while the mobile phase differed for the use of 10 mM ammonium acetate instead of ammonium formate and acetic acid instead of formic acid. An Agilent jet stream source was operated 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, 50–1700 *m/z*; capillary voltage, 3.5 kV; collision energy 20 eV in positive and 25 eV in negative mode, mass precursor per cycle = 3. Following the guidelines and harmonized protocols for the untargeted lipidomics analysis, quality control samples were analyzed every five samples (Eriksson et al., 2008).

The relative concentrations of complex lipids were calculated as follows:

$$C_s = (A_s/A_i)\ \mathrm{x}\ C_i$$

where C_s is concentration, expressed as μg/mL, of the complex lipid while C_i is the concentration of the internal standard. A_s and A_i are the chromatographic peak area of the molecular specie in the sample and of the internal standard, respectively. LOD and LOQ values for complex lipids analysis are reported in **Table S3**.

Triacylglycerols (TG) digestibility was calculated as reported by Teng et al. (2020):

$$\text{TG digestion\%} = 1 - (C_t/C_0) \text{ x } 100$$

where C_t is the relative concentration of TG for the last timepoint (I180) while C_0 is referred to the TG concentration before digestion.

2.5. GC-FID fatty acid methyl esters analysis

Fatty acid methyl esters (FAMEs) analyses were performed on three independent samples on sheep and goat IF digested samples (from G0 to I180), and the blend of vegetable oils used to manufacture IF. The FAMEs were obtained by adding sodium methoxide-hexane to the samples which were then heated to 70 °C to trans-esterify the acyl lipids. The FAMEs were quantitated using a capillary gas chromatographic system coupled to a flame ionization detector (GC-FID 8860, Agilent Technologies, Palo Alto, CA). Briefly, 1 μL of each sample was injected in a DB-5 column (30 m × 0.25 μm). The helium flow was 1.0 mL/min and the injector was set at 20 mL/min split flow with the inlet temperature set at 250 °C. The initial oven temperature was set at 50 °C and was increased by 10 L/min to 175 °C and held for 10 min, then was further increased by 5 °C/min to 210 °C and held for 10 min. It was then further increased by 5 °C/min to 230 °C and finally increased by 10 °C/min to 300 °C, with a total time run of 61 min. Peak identification was performed by comparing peak retention time with Supelco 37 component FAME Mix. The relative concentrations of FAME were obtained by using the expression reported in section 2.4.

In our experiment conditions, when we injected the standard mixture of 37 methyl esters of fatty acids at different concentration, the LOQ and LOD values were found between 0.88 and 1.22 mg/L and 2.93 and 4.06 mg/L, respectively. Method sensitivity was calculated based on signal to noise ratio which is 3:1 and 10:1, respectively. Repeatability of the method was evaluated injecting six times the FAME standard mixture diluted 50 times to obtain FAME levels from 4 to 8 mg/L. The RSD of methyl esters fatty acid was found to be comprised between 0.9%–6.0%. The intermediate precision was evaluated by data generated on two different days. Injecting the same FAME mixture the difference of the mean from two days ranged from 0.01% to 3.40%.

2.6. Multivariate data analysis

UHPLC-QTOF/MS data were pre-processed with the Mass Profiler 10.0 software (Agilent Technologies, Palo Alto, CA) to perform mass deconvolution and peak alignment, yielding a matrix containing all features present across all samples. We obtained a data matrix of 387 × 59 dimensionality for the negative ionization mode, and one of 2693 × 59 for the positive ionization mode. A row wise internal scaling was performed by a constant-sum normalization. Features were subsequently processed with the SIMCA software 14.1 (Sartorius, Umeå Sweden). First, a Principal Component Analysis (PCA) was carried out to observe samples and variables distribution in the multivariate space based on their similarity. For samples classification, a partial least square- discriminant analysis (PLS-DA) was performed by implementing a dummy **Y**-variable for each class. Its orthogonal modification (OPLS-DA), where the systematic variations are separated into predictive (x -axis, interclass variability) and orthogonal (y – axis, intraclass variability), was carried out to facilitate the interpretation of group separation variability. Classificatory and predictive powers were verified based on the cumulative parameters R^2Y (goodness of fit) and Q^2Y (goodness of prediction, determined through cross validation). From the OPLS-DA, the discriminant metabolites along the predictive component were extracted based on the variable importance in projection (VIP) scores; values >1 were deemed as discriminant and their UHPLC-QTOF/MS features submitted to annotation procedure following the COSMOS Metabolomics Standard initiative (Salek et al., 2015). For assessing the DA models' reliability, we used the cross-validation ANOVA (CV-ANOVA) diagnostic tool, as implemented in SIMCA (Eriksson et al., 2008).

3. Results

3.1. Complex lipids analysis

Digested samples from goat and sheep IFs were collected at different timepoints during *in vitro* dynamic digestion and subjected to analysis. Data underwent unsupervised principal component analysis (PCA) to explore sample distribution in the multivariate space, revealing patterns, deviating features, and similarities. PCA score plots of goat and sheep IF are reported in Fig. 1. Based on the animal species, no discernible sample grouping was observed (**Fig. 1A** and **B,** for positive and negative mass spectrometric ionization modes, respectively). In the plots, when samples were color-coded based on different digestion phases, namely gastric and intestinal (**Fig. 1C** and **D,** for positive and negative mode, respectively), a tendency of samples to cluster is evident with a tighter grouping for the intestinal phase. These observations indicate that the lipid composition of samples significantly changes during the transition from the gastric to the intestinal tract, regardless of the animal species used as the milk source for IF production. To emphasize the distinctions in the complex lipid profiles between sheep and goat IF digested samples, discriminant analyses were conducted separately for the gastric and intestinal compartments. The pair-wise goat *vs* sheep OPLS-DA model in the positive ionization mode demonstrated robust classificatory power and predictive ability, therefore discriminant features were selected based on their VIP values and submitted to the annotation procedure. On the contrary, in the negative ionization mode, where FFAs are the prevalent features, the discriminant analyses were unsuccessful (data not shown). The results in the positive ionization mode are reported as score plots in **Fig. 2A** and **B**, along with the list of the most discriminant lipids (**Tables 1** and **2**), for the gastric and the intestinal compartments, respectively. Results indicate that sheep IF samples contain higher number of diacylglycerols (DGs) in the gastric compartment, while for goat IF samples they are higher in the intestinal tract. In both compartments, sheep IF showed higher levels of TGs bearing a palmitic acid in the *sn*-2 position when compared to goat IF, while the latter showed higher levels of phosphatidylcholines and phytosterols (Table 1).

Given the PCA results, which showed no distinct grouping for sheep and goat milk-based IF but instead a tight clustering for the two digestion compartments, we proceeded with a pairwise discriminant analysis of gastric *versus* intestinal samples. OPLS-DA score plots for positive and negative ionization modes are reported in **Fig. 3A** and **B**, respectively. OPLS-DA models showed a good classificatory power and a good predictive ability. The plots show a marked features separation of the gastric *vs* intestinal phases along the predictive component (x-axis) and a tighter clustering of samples of the intestinal tract, confirming the trend observed in the exploratory PCA (**Fig. 1C** and **D**). Discriminant features between gastric and intestinal samples are reported in **Table S4.** The gastric compartment is richer in triacylglycerols (TGs), phosphatidylcholines (PCs), and sphingomyelins (SMs); and following their hydrolysis, lysophosphatidylcholines (LPCs), diacylglycerols (DGs), monoacyglycerols (MGs), and the free fatty acids (FFAs) are more abundant in the intestinal tract.

To note, we were able to annotate exogenous compounds added during *in vitro* dynamic digestion (see paragraph 2.3.). In the intestinal compartment we found the bile acid glycine conjugate glycoursodeoxycholic acid, taurodeoxycholic acid, and cholic acid, commonly found in

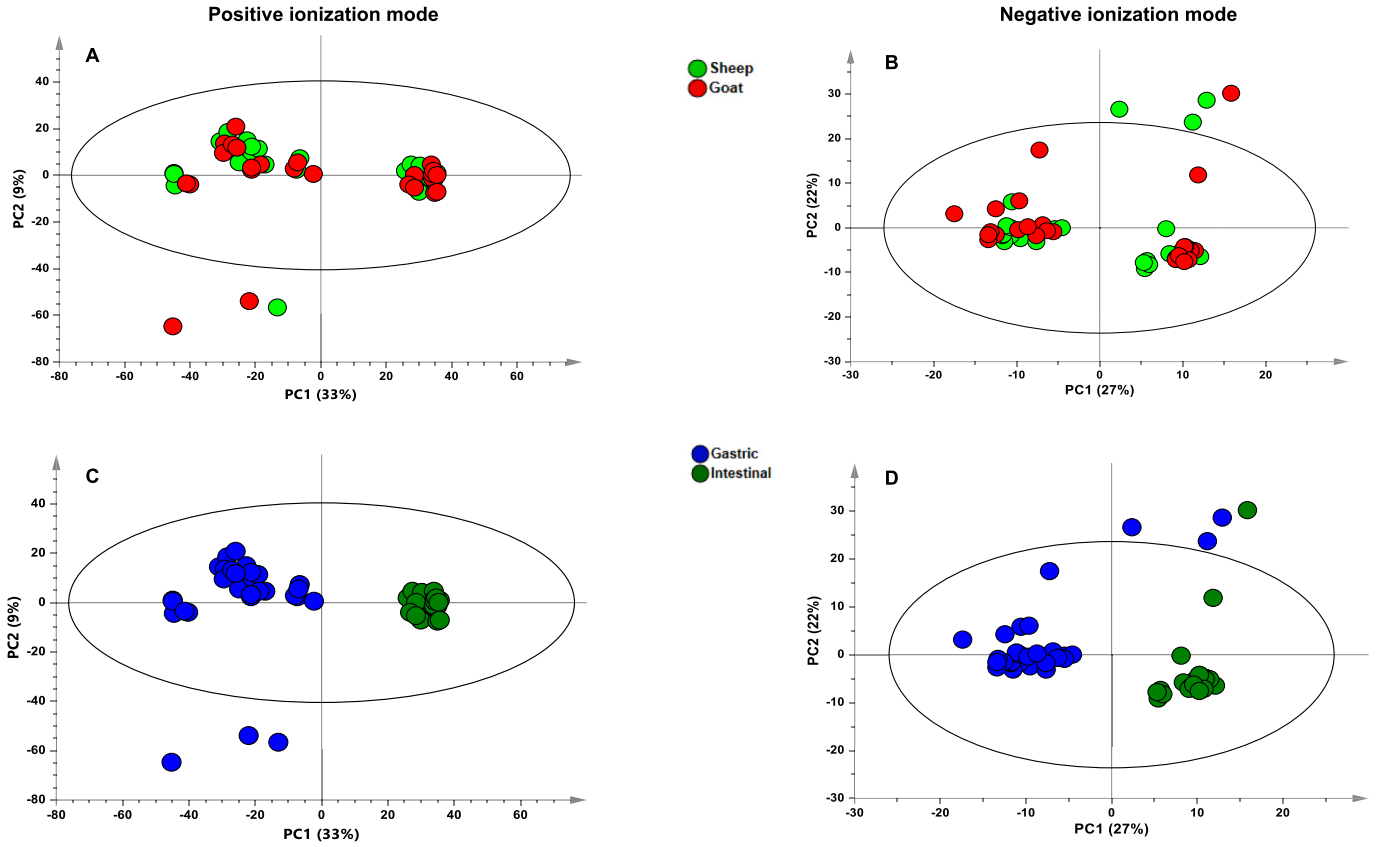

Fig. 1. Principal component analysis (PCA) score plots of sheep and goat IF digested samples during *in vitro* dynamic digestion ($n = 54$; 2 groups, 9 time points, 3 sample replicates), in the positive (**A** and **C**; $R^2X = 0.43$ and $Q^2X = 0.32$) and negative ionization mode (**B** and **D**; $R^2X = 0.49$ and $Q^2X = 0.37$). Samples were differently colored depending on milk origin of IF (sheep and goat) and digestion tract.

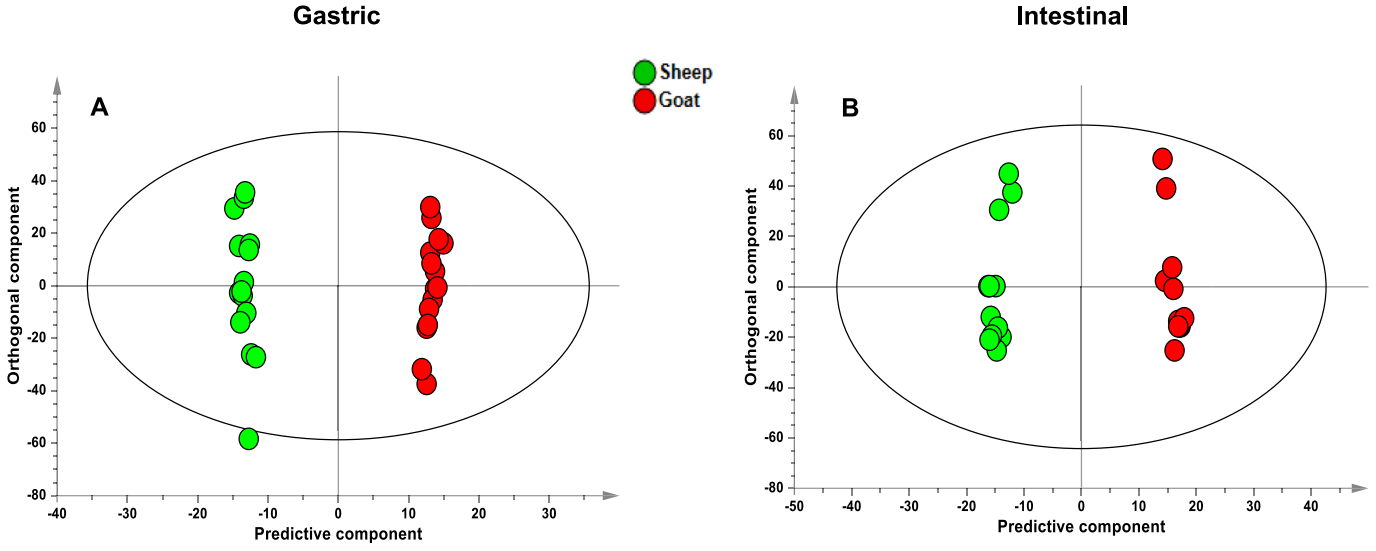

Fig. 2. Pair-wise sheep *vs.* goat OPLS-DA score plots of IF samples during *in vitro* dynamic digestion. Gastric (**A;** $R^2Y = 0.997$; $Q^2 = 0.872$, Components $=1 + 4$; $p < 0.001$) and intestinal (**B**; $R^2Y = 0.994$; $Q^2 = 0.798$, Components $=1 + 2$; $p < 0.001$) compartments, for the positive ionization mode.

the bile. Additionally, we also annotated pepstatin A and serine protease inhibitor 4-(2-Aminoethyl)-benzolsulfonylfluoride which were added after sampling to inhibit the activity of proteases (data not shown).

To study the hydrolysis mechanisms during digestion, in Fig. 4 **and Table S5** we report the variations in the levels of complex lipids at different timepoints. A gradual decrease in the levels of TGs, PCs, and SMs as the digestion progresses from the gastric to intestinal tracts can be seen. Interestingly, the levels of the long-chain fatty acid triacylglycerols, *i.e.* TG(54:5), TG(56:5), TG(60:12), did not change during dynamic digestion. Different lipids such as DGs, MGs, and LPCs were upregulated in the intestinal tract. In particular, LPC(16:0) and LPC (18:2) increased rapidly in the first time point of the intestinal tract

Table 1
Pair-wise sheep *vs.* goat IF OPLS-DA discriminant metabolites in the gastric comportment, along with their respective mass spectrometric characteristics in the positive ionization mode.

Chemical Class	Compound	Formula	Adduct	*m/z*		Error (ppm)	VIP[a] value	*G vs S*[b]
				Theoretical	Detected			
Phosphatidylcholines	PC 34:1 (18:1/16:0)	$C_{42}H_{82}NO_8P$	$[+H]^+$	760.5851	760.5835	−2.1	1.82	***G***
	PC 36:4 (16:0/20:4)	$C_{44}H_{80}NO_8P$	$[+H]^+$	782.5694	782.5681	−1.6	2.02	***G***
	PC 36:3 (18:1/18:2)	$C_{44}H_{82}NO_8P$	$[+H]^+$	784.5851	784.5848	−0.4	1.83	***G***
	PC 36:1 (16:1/20:0)	$C_{44}H_{86}NO_8$ P	$[+H]^+$	788.6164	788.6150	−1.7	1.59	***G***
Sphingomyelins	SM (d34:0)	$C_{39}H_{79}N_2O_6P$	$[+H]^+$	703.5749	703.5745	−0.6	2.48	***S***
Ergosterols	16:2-Glc-Campesterol	$C_{50}H_{84}O_7$	$[+H]^+$	797.6290	797.6253	−4.6	2.00	***G***
	22:2-Glc-Stigmasterol	$C_{57}H_{96}O_7$	$[+H]^+$	893.7229	893.7179	−5.6	1.84	***G***
	22:1-Glc-Stigmasterol	$C_{57}H_{98}O_7$	$[+H]^+$	895.7385	895.7360	−2.8	1.85	***G***
	16:3-Glc-Campesterol	$C_{50}H_{82}O_7$	$[+H]^+$	795.6133	795.6108	−3.1	1.58	***G***
Diacylglycerols	DG 36:3 (18:1/18:2)	$C_{39}H_{70}O_5$	$[NH_4]^+$	636.5562	636.5543	−3.0	1.75	***S***
	DG 42:11 (20:5/22:6)	$C_{45}H_{66}O_5$	$[NH_4]^+$	704.5249	704.5297	6.8	3.13	***S***
	DG 44:11 (22:5/22:6)	$C_{47}H_{70}O_5$	$[NH_4]^+$	732.5562	732.5612	6.4	3.14	***S***
Triacylglycerols	TG 46:3 (12:0/12:0/22:3)	$C_{49}H_{88}O_6$	$[NH_4]^+$	790.6919	790.6900	−2.4	1.82	***G***
	TG 47:1 (15:0/16:0/16:1)	$C_{50}H_{94}O_6$	$[NH_4]^+$	808.7389	808.7376	−1.6	3.54	***S***
	TG 47:0 (15:0/16:0/16:0)	$C_{50}H_{96}O_6$	$[NH_4]^+$	810.7545	810.7521	−4.3	2.52	***S***
	TG 49:2 (15:1/17:0/17:1)	$C_{52}H_{96}O_6$	$[NH_4]^+$	834.7545	834.7527	−2.1	3.54	***S***
	TG 49:1 (15:1/16:0/18:0)	$C_{52}H_{98}O_6$	$[NH_4]^+$	837.7702	837.7717	1.8	3.42	***S***
	TG 50:3 (14:1/17:1/19:1)	$C_{53}H_{96}O_6$	$[NH_4]^+$	846.7545	846.7536	−1.1	1.91	***G***
	TG 50:1 (15:1/17:0/18:0)	$C_{53}H_{100}O_6$	$[NH_4]^+$	850.7858	850.7850	−0.9	1.98	***G***
	TG 51:2 (16:0/16:1/19:1)	$C_{54}H_{100}O_6$	$[NH_4]^+$	862.7858	862.7846	−1.4	3.45	***S***
	TG 52:4 (15:1/15:1/22:2)	$C_{55}H_{98}O_6$	$[NH_4]^+$	872.7702	872.7682	−2.3	1.90	***G***
	TG 54:7 (18:2/18:2/18:3)	$C_{57}H_{96}O_6$	$[NH_4]^+$	894.7545	894.7529	−1.8	2.05	***G***
	TG 54:5 (18:1/18:2/18:2)	$C_{57}H_{100}O_6$	$[NH_4]^+$	898.7858	898.7817	−4.6	1.75	***G***

[a] Variable importance in the model. [b] Compounds detected at higher levels in digested samples from goat (G) or sheep (S) infant formula.

Table 2
Pair-wise sheep *vs.* goat IF OPLS-DA discriminant metabolites in the intestinal comportment, along with their respective mass spectrometric characteristics in the positive ionization mode.

Chemical Class	Compound	Formula	Adduct	*m/z*		Error (ppm)	VIP[a] value	*G vs S*[b]
				Theoretical	Detected			
Phosphocholines	PC 34:2 (16:0/18:2)	$C_{42}H_{80}NO_8P$	$[+H]^+$	758.5694	758.5687	−0.9	1.14	***G***
	PC 34:0 (10:0/24:0)	$C_{42}H_{84}NO_8P$	$[+H]^+$	762.6007	760.6002	−0.6	1.85	***G***
	PC 36:4 (16:0/20:4)	$C_{44}H_{80}NO_8P$	$[+H]^+$	782.5694	782.5681	−1.6	1.27	***G***
Sphingomyelins	SM d34:0	$C_{39}H_{79}N_2O_6P$	$[+H]^+$	703.5749	703.5745	−0.6	2.02	***G***
	SM d36:1	$C_{41}H_{83}N_2O_6P$	$[+H]^+$	731.6062	731.6048	−1.9	2.43	***G***
	SM d40:1	$C_{45}H_{91}N_2O_6P$	$[+H]^+$	787.6688	787.6669	−5.3	2.57	***G***
Ergosterols	22:2-Glc-Stigmasterol	$C_{57}H_{96}O_7$	$[+H]^+$	893.7229	893.7179	−5.6	1.85	***G***
	22:1-Glc-Stigmasterol	$C_{57}H_{98}O_7$	$[+H]^+$	895.7385	895.7360	−2.8	1.77	***G***
Monoacylglycerols	MG 16:0	$C_{19}H_{38}O_4$	$[+Na]^+$	353.2662	353.2672	−2.8	2.30	***G***
Diacylglycerols	DG 34:2 (16:0/18:2)	$C_{37}H_{68}O_5$	$[+NH_4]^+$	610.5405	610.5389	−2.6	1.5	***G***
	DG 36:3 (18:1/18:2)	$C_{39}H_{70}O_5$	$[+NH_4]^+$	636.5562	636.5543	−3.0	2.46	***G***
	DG 36:1 (18:1/18:0)	$C_{39}H_{74}O_5$	$[+NH_4]^+$	640.5875	640.5868	−1.1	1.45	***G***
	DG 44:10 (22:6/22:4)	$C_{47}H_{72}O_5$	$[+NH_4]^+$	734.5718	734.5724	0.8	1.36	***S***
Triacylglycerols	TG 36:0 (12:0/12:0/12:0)	$C_{39}H_{74}O_6$	$[+NH_4]^+$	656.5824	656.5809	−2.3	1.62	***G***
	TG 38:0	$C_{41}H_{78}O_6$	$[+NH_4]^+$	684.6137	684.6122	−2.2	1.66	***G***
	TG 44:1 (12:0/14:0/18:1)	$C_{47}H_{88}O_6$	$[+NH_4]^+$	766.6919	766.6885	−4.4	1.71	***G***
	TG 47:1 (15:0/16:0/16:1)	$C_{50}H_{94}O_6$	$[+NH_4]^+$	808.7389	808.7376	−1.6	3.02	***S***
	TG 49:1 (15:1/16:0/18:0)	$C_{52}H_{98}O_6$	$[+NH_4]^+$	837.7702	837.7717	1.8	2.90	***S***
	TG 50:0 (16:0/16:0/18:0)	$C_{53}H_{102}O_6$	$[+NH_4]^+$	852.8015	852.7976	−4.6	1.66	***G***
	TG 51:2 (16:0/16:1/19:1)	$C_{54}H_{100}O_6$	$[+NH_4]^+$	862.7858	862.7846	−1.4	3.01	***S***
	TG 52:4 (15:1/15:1/22:2)	$C_{55}H_{98}O_6$	$[+NH_4]^+$	872.7702	872.7682	−2.3	1.40	***G***

[a]Variable importance in the model. [b] Compounds detected at higher levels in digested samples from goat (G) or sheep (S) infant formula.

(I40), while LPC(18:1) decreased steadily since the beginning of digestion. Likewise, there was a rapid increase in the levels of oleic and linoleic free fatty acids in the intestinal tract. DGs, MGs, and FFAs were mainly produced during the intestinal step due to the activity of pancreatic lipase and exhibited their highest concentrations at I80. In Table 3 we reported the TGs calculated percent digestibility during *in vitro* dynamic digestion. We observed that percent digestibility was greater for TGs with a lower carbon number irrespectively from the unsaturation degree. A slightly higher TGs percent digestibility can be observed for longer TGs (carbon number $>$ 44) in sheep IF when compared to goat IF.

3.2. FAME analysis

The quantitative analysis of fatty acid methyl esters (FAME) was carried out for sheep and goat IFs (Fig. 5) and for the blend of vegetable oils used for the IF manufacturing. The fatty acid percent compositions of the IF samples are reported in **Table S6** while for the vegetable oil samples are reported in **Table S7**. A higher diversity of fatty acids was evident in the IFs compared to the vegetable oil blend. Specifically, caproleic acid (C10:1 *cis*-9), *trans*-vaccenic acid (C18:1 *trans*-11) and odd chain FA (OCFA) such as C15:0, C17:0, characteristic of ruminant's milk (Nudda et al., 2021; Scano et al., 2011) were detected in IF samples, and not in the blend of vegetable oils. The IFs also contained long chain

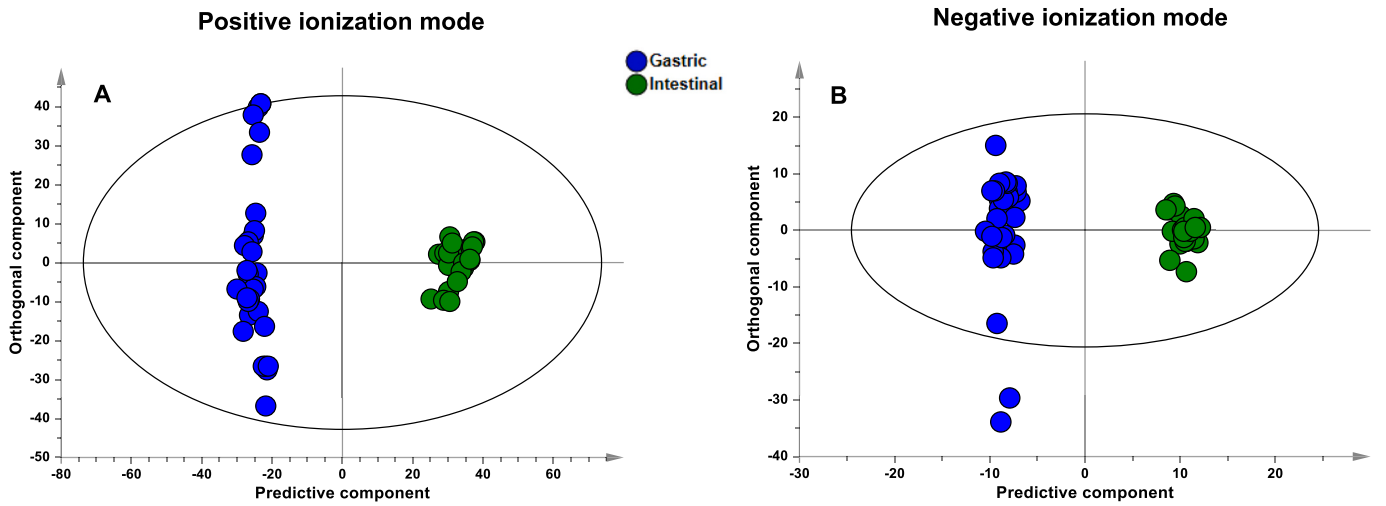

Fig. 3. Pair-wise gastric *vs* intestinal compartments OPLS-DA score plots of IF samples during *in vitro* dynamic digestion. Positive ionization mode (**A**: $R^2Y = 0.99$; $Q^2 = 0.83$, Component =1 + 1; $p < 0.001$) and negative ionization mode (**B:** $R^2Y = 0.97$; $Q^2 = 0.85$, Component =1 + 2; $p < 0.001$).

polyunsaturated fatty acids, including arachidonic acid (AA, C20:4, n-6) and docosahexaenoic acid (DHA, C22:6 n-3). After comparison, sheep IF showed higher levels of short-chain fatty acids (SCFA, C6-C10) and saturated fatty acids, whereas goat IF exhibited a slightly higher content of OCFA. The precursor of DHA, docosapentaenoic acid (DPA C22:5, n-3), and AA were more abundant in goat IF.

Finally, samples collected during the *in vitro* dynamic digestion process were analyzed for their FAME content. Fig. 5 illustrates the changes in fatty acid levels over time during digestion. As expected, fatty acid levels decreased as the digestion progressed from the gastric to the intestinal compartment.

4. Discussion

In the present study, an original and powerful lipidomic strategy was applied to the fine characterization of lipolysis during the *in vitro* gastrointestinal digestion of sheep and goat IFs. Digestion is composed by different steps, begins in the mouth, and continues in the gastric compartment and in the small intestine. During the *in vitro* dynamic digestion system, the stomach pH is gradually set to low values, and enzymes, such as pepsin and lipases, are added. Submicronic size lipid droplets are formed under the control of the acidic pH, ionic strength, and the action of pepsin on casein while no coalescence is usually observed. Furthermore, within the context of lipid digestion, Carriere et al. (1993) indicate that human gastric lipases are involved in hydrolyzing 10 to 25% of triacylglycerols, leading to the formation of diacylglycerols and free fatty acids. An *in vivo* study on preterm infants calculated a 12% of lipolysis degree of mother milk in the gastric compartment (de Oliveira et al., 2017). Lipolysis is gradually impeded by the accumulation of free fatty acids at the oil-water interface. In the intestine the bile salts emulsify the droplets, making fatty molecules available for pancreatic lipase hydrolysis.

In our work we observed, as hypothesized, a progressive hydrolysis of complex lipids, including TGs, PCs, and SMs, occurring from the initial stages of gastric digestion. Consistent with the findings of Teng et al. (2020), the levels of lipolysis breakdown products (DGs, MGs, and LPCs) increased in the intestinal tract. IFs lipids are primarily composed of triacylglycerols, which consist of three fatty acids esterified to a glycerol backbone. These fatty acids are stereospecifically numbered as *sn*-1, *sn*-2, and *sn*-3. When lipase acts on triacylglycerols, it breaks them down into diacylglycerols, free fatty acids, and monoacylglycerols. Human lipase activity in the gastrointestinal tract exhibits specificity for the regio distribution of fatty acids in triacylglycerols. Notably, short-chain fatty acids and medium-chain fatty acids (MCFA) at the *sn*-3 position are more readily hydrolyzed in the stomach. On the other hand, long-chain fatty acids on the *sn*-1 and *sn*-3 positions are preferentially hydrolyzed by pancreatic lipases. In ruminant milk, SCFA are typically esterified at the *sn*-1,3 positions of the glycerol backbone (Teng et al., 2020). This positioning makes them more susceptible to hydrolysis, allowing them to be quickly released. Infants are able to absorb short- and medium-chain saturated fatty acids more efficiently than longer-chain saturated fatty acids (Lindquist & Hernell, 2010). In our experiments we found that sheep IF is richer in SCFA when compared to goat IF, their esterification at the *sn-1,3* positions make them more susceptible to hydrolysis thus suggesting that sheep milk fat may be nutritionally advantageous in IF preparation (Prosser, 2021). Consistently with these observations, in the gastric compartment the level of fatty acids still bound to TGs (expressed as FAME) is lower for sheep IF digested samples, when compared to goat IF, suggesting a rapid hydrolysis of TGs bearing SCFA. In support of these results, we found that comparing IF digested samples in the gastric compartment, sheep IF was found richer in the levels of DGs, when compared to goat IF.

Pancreatic lipases are specific for *sn*-1 and *sn*-3 positions of TGs therefore releasing *sn-2* MG. In our experiments, we found higher levels of MG(16:0), and MG(18:0) in the intestinal tract. FAs in *sn-2* MG are preferentially absorbed in the intestine, which means that the bioavailability of a single fatty acid is affected by its position on the TGs. Fatty acids on the *sn-2* position of TG are of paramount nutritional interest in the formula milk manufacturing because human milk, as opposed to vegetable oils, is rich of palmitic acid in the *sn-2* position (Innis, 2011; Straarup et al., 2006). To mimic the human milk composition, efforts have been made to enrich IFs with TGs bearing palmitic acid in *sn-2* (Bhutada et al., 2022). In this regard, we found that digested samples of sheep IF showed higher level of this type of TG, when compared to goat milk IF. TGs are efficiently hydrolyzed in the gastric phase and at a higher rate for medium chain compared with the long chain fatty acids. These findings are in accordance with *in vitro* experiment (Bernbäck et al., 1989; de Oliveira et al., 2017; Jensen et al., 1994).

In the case of digestion of IFs, FAME levels are an index of the quantity of fatty acids that remain associated with complex lipids and consequently susceptible to hydrolysis. The transesterification procedure conducted prior to FAME analysis does not enable the detection of free fatty acids (FFA). In examining the fatty acid profile of IFs, we identified *trans*-vaccenic acid and caproleic acid, which are absent in the vegetable oil blend. These compounds can be regarded as valuable indicators of the presence of milk fat in IFs. To estimate the proportion of fat milk used in the manufacturing of IF, we considered the levels of

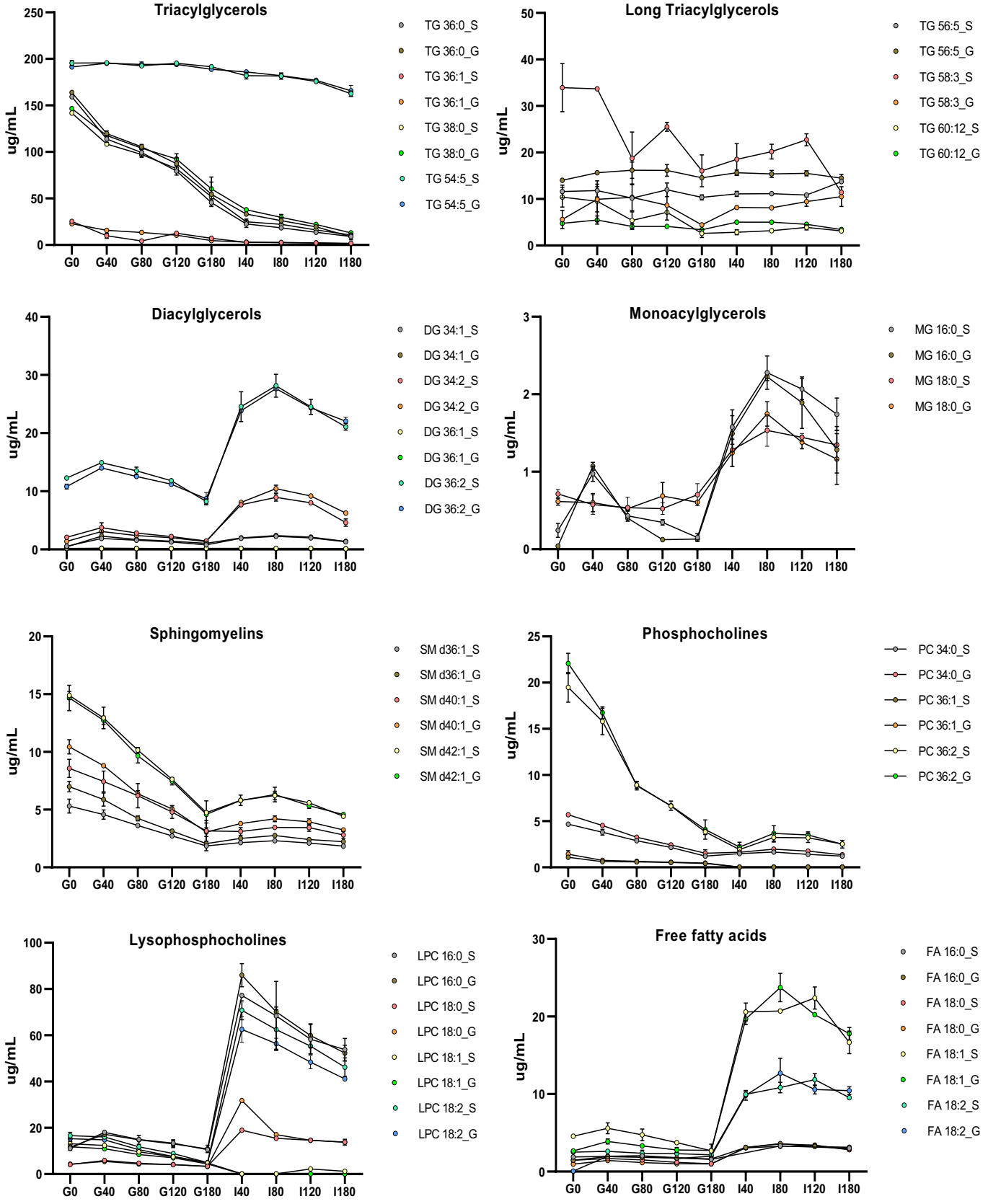

Fig. 4. Levels of complex lipids (expressed as μg/mL of digested samples) detected in sheep and goat IF samples during *in vitro* dynamic digestion (n = 54; 2 groups, 9 time points, 3 sample replicates).

Table 3
TGs percent digestion (mean % ± SD) of sheep and goat IFs. The following TGs: TG(38:0), TG(44:0), TG(44:2) and TG(46:0) resulted a mix of isomers.

Molecular specie	Sheep	Goat	*p*
TG 36:0 (12:0/12:0/12:0)	94 ± 0.3	93 ± 0.8	
TG 36:1 (12:0/12:0/12:1)	93 ± 0.3	96 ± 3.1	
TG 38:0	92 ± 0.4	91 ± 1.7	
TG 38:1 (18:1/8:0/12:0)	92 ± 0.4	91 ± 1.7	
TG 40:2 (12:0/12:1/16:1)	94 ± 0.1	94 ± 0.1	
TG 42:1 (12:0/12:0/18:1)	89 ± 1.7	87 ± 1.8	
TG 42:2 (12:0/12:1/18:1)	87 ± 1.1	86 ± 0.9	
TG 44:0	73 ± 2.1	64 ± 3.0	**
TG 44:1 (12:0/14:0/18:1)	74 ± 4.0	72 ± 2.5	
TG 44:2	70 ± 3.0	62 ± 3.0	**
TG 46:0	57 ± 0.5	52 ± 5.1	
TG 46:1 (12:0/16:0/18:1)	53 ± 1.6	47 ± 5.3	
TG 47:0 (15:0/16:0/16:0)	17 ± 1.1	11 ± 1.8	
TG 48:4 (12:0/18:2/18:2)	43 ± 0.2	34 ± 3.0	**
TG 50:0 (16:0/16:0/18:0)	24 ± 2.7	15 ± 4.8	**
TG 50:2 (16:0/16:1/18:1)	26 ± 2.5	19 ± 4.5	*
TG 54:5 (18:1/18:2/18:2)	17 ± 2.7	13 ± 2.7	
TG 54:6 (18:2/18:2/18:2)	33 ± 3.0	29 ± 3.1	

* $p < 0.05$.
** $p < 0.01$.

these two fatty acids in ruminant milk and in our IF. Considering the reported high variability of *trans*-vaccenic acid levels in ruminant's milk (Scano et al., 2019), we shifted our focus to the less abundant caproleic acid (Conte et al., 2022; Mele et al., 2016; Rapetti et al., 2021). Referring our data to those reported in literature we estimated that the IF lipid fraction contains a 6–10% of milk fat. Furthermore, because AA and DHA are added to the IFs for their beneficial effects to newborns (Bakshi et al., 2023; Green Corkins & Shurley, 2016), we were not able to distinguish whether these originated from milk fat or if they were added as ingredients to the formula.

The IFs studied in the present work are composed by goat or sheep whole milk, thus retaining the milk fat globule membrane (MFGM) components that are lacking in IF not containing milk fat (Gallier et al., 2020; Hageman et al., 2019; Prosser, 2021). The MFGM is a rich source of bioactive polar lipids and membrane specific proteins, including glycosylated proteins and enzymes (Gallier et al., 2020). It has been demonstrated that supplementation of IF with bovine MFGM decreases the infectious morbidity in formula-fed infants (Timby et al., 2015). During the digestion process MFGM becomes less stable at low pH, pepsin digests proteins of the membrane, and gastric lipases gain access to TGs (He et al., 2020). However, it should be considered that in manufacturing powdered IF, the technological treatments can induce changes in morphology of fat globules increasing the crystallization and melting temperatures of the TGs in the MFG (Ren et al., 2019).

Upon the action of lipases on complex lipids, release of breakdown

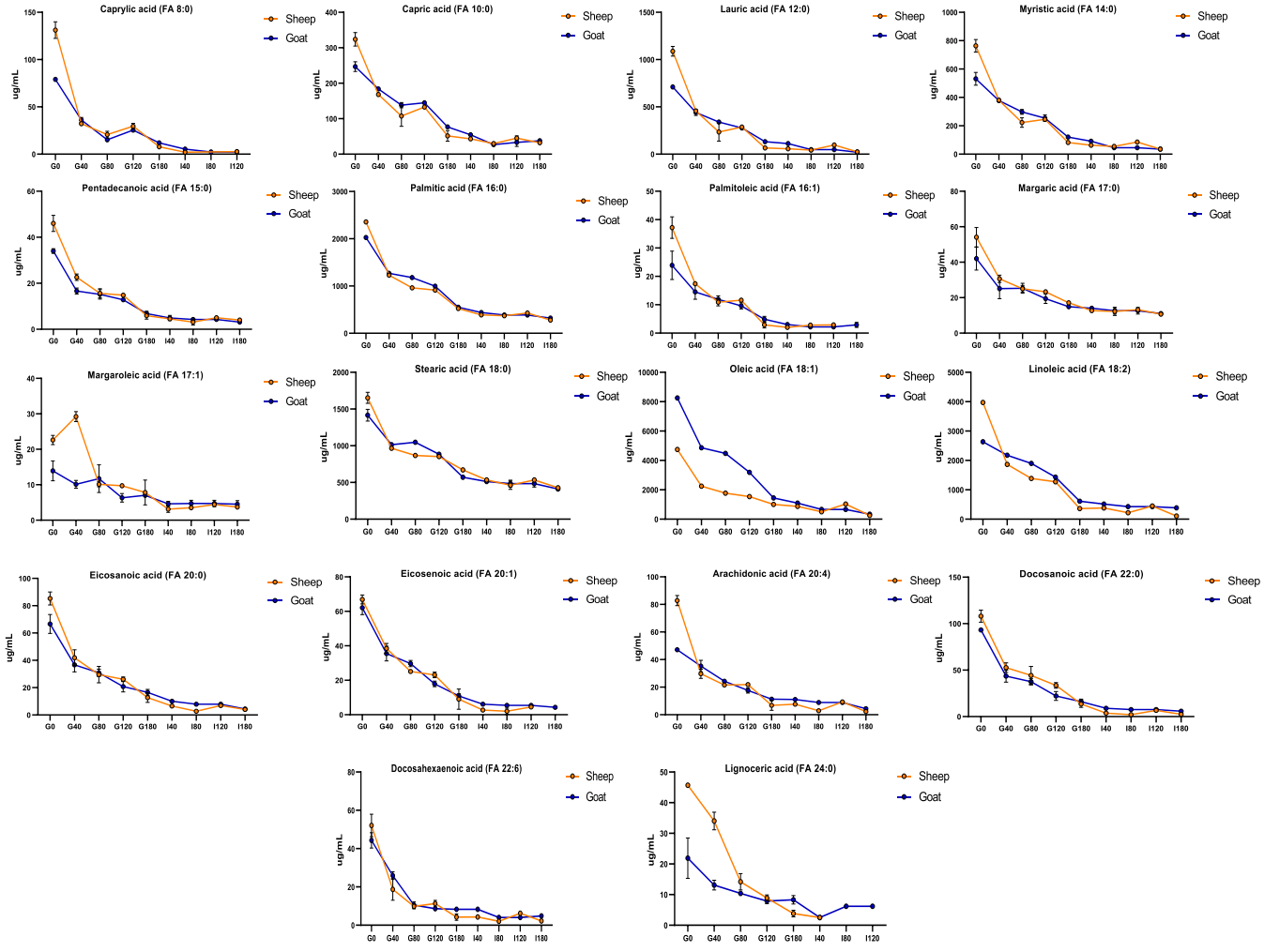

Fig. 5. Levels of FAMEs (expressed as μg/mL of digested samples) detected in sheep and goat IF samples during *in vitro* dynamic digestion (n = 54; 2 groups, 9 time points, 3 sample replicates).

products, such as LPCs, DGs, FFAs and *sn*-2 MGs is expected. Secretory pancreatic phospholipases (*i.e.* A2 IB, sPLA2 and sPLA2X) hydrolyze phosphatidylcholine at the *sn*-2 position to yield FFA and LPC (Borgström et al., 1957). In accordance with this physiological process, our results showed that in the intestine there are higher levels of LPCs, and free fatty acids compared the gastric tract, while the progenitors PCs, are more present in the stomach tract, suggesting their efficient hydrolysis in the gastric phase.

We also observed that levels of the sphingomyelins SM(d36:1), SM (d40:1), and SM(d42:1) decreased from the stomach to the intestine, and their hydrolysis showed the same trend of PC. This hydrolysis trend is consistent with data proposed by Armand (2008) who observed approximatively 25% of SM hydrolysis activity of gastric human lipase. On the other hand, we observed that levels of these SMs were not relevantly changed in the intestinal tract, despite Nilsson (1968, and 1969) and Schmelz et al. (1994) reported that in rats and mice sphingomyelins undergo little cleavage in the stomach but are hydrolyzed in all subsequent regions of the small intestine and colon. Free fatty acids were found upregulated in the intestinal compartment. The dramatic increase of oleic acid and linoleic acid with respect to palmitic acid and stearic acid can be explained by the higher abundance of the former in IFs samples. In this regard is worth to note that Teng et al. (Teng et al., 2019) studying the *in vitro* digestion of ruminant milk, reported that the in the intestinal tract the most abundant free fatty acids were palmitic acid and oleic acid, mirroring milk fat FA composition.

In the IFs and in the digested samples we annotated phytosterols as glycosylated campesterols and glycosylated stigmasterols that can be originated from the vegetable oil blend as well as from the goat and sheep diet (**Tables 1** and **2**). Sterols were detected at higher levels in goat IF digested samples, in both gastric and intestinal compartments, compared to sheep IF. The negative effects of exposure of the infant to phytosterols have been matter of debate (Claumarchirant et al., 2015; Prosser, 2021). However, to study the bio accessibility of sterols during the *in vitro* digestion systems, the addition of sterol esterases must be considered (Makran et al., 2022).

The *in vitro* dynamic method used in this study has several limitations. These include the lack of precise digestive parameters that accurately mimic the *in vivo* model, and the potential for inadequate mixing of the substrate and lipolytic enzymes. Additionally, this analytical approach may not fully detect or cover complex lipids, such as cholesterol.

5. Conclusion and prospective

Using an untargeted UHPLC-QTOF/MS lipidomics method, we analyzed the hydrolysis of complex lipids during the *in vitro* digestion of sheep and goat milk-based IFs. We specifically tracked the hydrolysis of complex lipids such as triacylglycerols, phosphatidylcholines, and sphingomyelins by monitoring their concentrations throughout digestion. Our findings revealed higher levels of monoacylglycerols, diacylglycerols, and lysophosphatidylcholines during the intestinal phase. Additionally, sphingomyelins were less affected by hydrolysis during digestion. Interestingly, the sheep milk-based IF exhibited slightly higher triacylglycerol digestibility. Furthermore, we evaluated the complex lipid building blocks, specifically fatty acids as methyl esters, and found that short- and medium-chain fatty acids were present at higher levels in the sheep IF compared to the goat. These results suggest that sheep milk is comparable to goat milk as an ingredient for IF manufacturing.

Future research should focus on the analysis of sheep IFs low molecular weight metabolites by a metabolomics and proteomics platforms after *in vitro* digestion. This approach would provide a comprehensive understanding of the digestion of sheep IFs, crucial for accurately assessing the nutritional value and physiological function. Such insights would enable the customization of IFs in industrial production.

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CRediT authorship contribution statement

Mattia Casula: Methodology, Formal analysis, Data curation. **Cristina Manis:** Writing – original draft, Supervision, Formal analysis, Data curation, Conceptualization. **Olivia Menard:** Writing – original draft, Software, Resources, Methodology, Data curation. **Giulia Tolle:** Formal analysis. **Marie-Francoise Cochet:** Formal analysis. **Didier Dupont:** Writing – original draft, Investigation, Data curation, Conceptualization. **Paola Scano:** Writing – original draft, Data curation, Conceptualization. **Viviana Garau:** Investigation, Data curation, Conceptualization. **Pierluigi Caboni:** Writing – original draft, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary Data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2024.140850.

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