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In vitro gastrointestinal digestion of cow's and sheep's dairy products: Impact of species and structure

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ABSTRACT

Sheep's milk (SM) is known to differ from cow's milk (CM) in nutritional composition and physicochemical properties, which may lead to different digestion behaviours. This work aimed to investigate the impact of the species (cow vs sheep) and the structure (milk vs yogurt) on the digestion of dairy products. Using an *in vitro* static gastrointestinal digestion model, CM, SM, cow's milk yogurt (CY) and sheep's milk yogurt (SY) were compared on particle size evolution, microscopic observations, degree of lipolysis, degree of proteolysis, specific protein degradation and calcium bioaccessibility. Species and structure affected particle size evolution during the gastric phase resulting in smaller particles for yogurts compared to milks as well as for CM products compared to SM products. Species impacted lipid composition and lipolysis, with SM products presenting higher short/medium-chain fatty acids content and higher intestinal degree of lipolysis. Proteolysis was influenced by structure, with milks showing higher intestinal degree of proteolysis compared to yogurts. Caseins were digested faster in CM, α-lactalbumin was digested faster in SM despite its higher concentration, and during gastric digestion β-lactoglobulin was more degraded in CM products compared to SM products and more in yogurts compared to milks. Lastly, SM products released more bioaccessible calcium than CM products. In conclusion, species (cow vs sheep) impacted more the digestion compared to the structure (milk vs yogurt). In fact, SM was different from CM mainly due to a denser protein network that might slow down the accessibility of the enzyme to its substrate which induce a delay of gastric disaggregation and thus lead to slower the digestion of the nutrients.

1. Introduction

The dairy industry, traditionally based on cow's milk (CM), is facing a growing trend towards diversification with the inclusion of milk from alternative sources. Although sheep's milk (SM) production remains relatively modest on a global scale in comparison to CM, SM products are receiving an increasing interest, partly due to favorable nutritional composition (Balthazar et al., 2017; Mohapatra et al., 2019; Pulina et al., 2018; Park et al., 2007). The chemical compositions of cow and sheep milks are shown in Table 1. SM contains higher levels of proteins, lipids and ashes compared to CM (Table 1), while the lactose content is similar between the two species (Nayak et al., 2020). In terms of proteins composition, both types of milk share the same caseins to whey proteins ratio (close to 80 % and 20 %, respectively). However, SM presents lower content of α-S1 casein and κ-casein, higher content of β-casein and α-S2 casein, the same amount of β-lactoglobulin and serum albumin and

Abbreviations: ANOVA, one-way analysis of variance; CLSM, confocal laser scanning microscopy; CM, cow's milk; CY, cow's milk yogurt; $D_{4,3}$, mean volume distributions; G0, gastric phase 0 min; G5, gastric phase 5 min; G60, gastric phase 60 min; G120, gastric phase 120 min; I5, intestinal phase 5 min; I60, intestinal phase 60 min; I120, intestinal phase 120 min; ICP-AES, inductively coupled plasma-atomic emission spectroscopy; MD, mean difference; OPA, o-phthaldialdehyde; SDS, sodium lauryl sulfate; SGF, simulated gastric fluid; SIF, simulated intestinal fluid; SM, sheep's milk; SPE, solid phase extraction; SY, sheep's milk yogurt; TAG, triacylglycerol.

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Table 1
Chemical composition of cow and sheep milks.

	Cow milk	Sheep milk	References
Proteins (g/100 g)	3.2	5.7	
Whey proteins WP (% total protein)	19.0	19.6	
β-lactoglobulin (% of WP)	*51*	*51*	
α-lactalbumin (% of WP)	*20*	*25*	
Serum albumin (% of WP)	*7*	*6*	Nayak et al., 2020; Alichanidis et al., 2016; Balthazar et al., 2017
Caseins CN (% total protein)	81.0	80.4	
α-S1 CN (% of CN)	*41*	*26*	
α-S2 CN (% of CN)	*11*	*14*	
β-CN (% of CN)	*33*	*42*	
κ-CN (% of CN)	*12*	*10*	
Lipids (g/100 g)	3.6	7.3	
TG (%)	98	98	Nayak et al., 2020; Alichanidis et al., 2016
SFA (%)	70	70	
SCFA and MCFA (%)	9.2	16.6	
Lactose (g/100 g)	4.6	4.6	Nayak et al., 2020
Ashes (g/100 g)	0.7	0.8	Nayak et al., 2020
Calcium (mg/100 g)	112	198	
Phosphorus (mg/100 g)	91	141	
Magnesium (mg/100 g)	11	19.5	Balthazar et al., 2017
Vitamine A (μg/100 g)	37	64	
Vitamine B3 (μg/100 g)	13	41	

WP: whey proteins
CN: caseins
TG: triglycerides
SFA: saturated fatty acids
SCFA: short-chain fatty acids
MCFA: medium-chain fatty acids

higher content of α-lactalbumin (Alichanidis et al., 2016) and differs in the amino acid sequences compared to CM (Ha et al., 2015; Maes et al., 2020). Regarding lipid composition, the milk from both species is mainly composed of triglycerides (98 %) which are constituted mostly by saturated fatty acids (70 %). However, SM contains higher proportion of short and medium-chain fatty acids compared to CM (Alichanidis et al., 2016). Concerning the micronutrients, SM contains higher amount of calcium, phosphorus, magnesium, vitamin A,) and vitamin B3 compared to CM (Balthazar et al., 2017).

To better understand the potential health benefits of SM, its digestion behaviour has been explored in comparison to CM ones. Static *in vitro* digestion models comparing milk from both species have shown different results, such as a faster release of amino acids for SM (Tagliazucchi et al., 2018), a lower gastric hydrolysis of β-lactoglobulin (Nguyen et al., 2020) and a lower final proteolysis for SM compared to CM (Shen et al., 1995). Other investigations using the same digestion model revealed a faster gastric lipolysis for SM (Teng et al., 2020) but a similar final intestinal lipolysis for both species (Santillo et al., 2018) and a lower or equivalent calcium bioaccessibility compared to CM (Bossu et al., 2020; Preedy, 2015; Shen et al., 1995). After dynamic *in vitro* digestion, gastric clot with higher firmness was observed with SM (Roy et al., 2021), while an *in vivo* study (rodent model) has reported a faster gastrointestinal transit for SM when comparing *iso*-protein skimmed milks from both species (Dalziel et al., 2018).

The composition is not the only factor influencing nutritional quality and digestion behaviour. It is well established that the food structure plays a crucial role in determining how nutrients are released during digestion, thus contributing to the differences between milks and yogurts (Fardet et al., 2019). For instance, *in vitro* digestion studies indicated higher gastric degradation of β-lactoglobulin in cow's milk yogurt (CY) compared to CM (Dupont et al., 2010; Kopf-Bolanz et al., 2014; Nguyen et al., 2020) but no differences in calcium bioaccessibility (Ünal et al., 2005). An *in vivo* study (rodent model) noted no differences in gastrointestinal transit between milk and yogurts from both species (Dalziel et al., 2018), while others authors reported a reduction in gastric emptying rate and amino acid absorption in pig for CY compared to CM (Barbé et al., 2013; Gaudichon et al., 1994; Le Feunteun et al., 2014). No differences in calcium absorption have been reported by *in vivo* studies between CY compared to CM (El-Gawad et al., 2014; Smith et al., 1985). However, comprehensive comparative analyses focusing on SM and including different structures are rare. Sheep's milk yogurt (SY) exhibits a firmer and more viscous gel compared to CY (Roy et al., 2021), due to higher protein content, presence of larger clusters of casein micelles and denser network (Ould-Eleya et al., 1998; Domagała, 2009). To our knowledge, three studies have compared the digestion of milks and yogurts from both species, in order to asses gastrointestinal transit (Dalziel et al., 2018), gut microbiota (Rettedal et al., 2019) and peptide release *in vitro* (Nguyen et al., 2020). They have shown an increased colonic transit for SM products while bacterial diversity and bioactive peptides generation were impacted by structure, which is dependent to fermentation status.

This work aimed to consider a broader approach to better understand the impact of species and structure on the digestion of cow's and sheep's milks (CM and SM) and yogurts (CY and SY) using the INFOGEST static *in vitro* gastrointestinal digestion model. CM, CY, SM and SY were compared in terms of gastric particle size evolution, microscopic observations, degree of lipolysis, degree of proteolysis, specific protein degradation and calcium bioaccessibility.

2. Materials and methods

2.1. Milks and yogurts

Cow dairy products were processed at Laiterie de Saint-Malo, Saint-Malo, France, while sheep dairy products were processed at Laiterie Le Petit Basque, Saint-Ménard-d'Eyrans, France. Both cow's milk (CM) and sheep's milk (SM) underwent pasteurization (3 min at 82 °C for CM and 20 sec at 75 °C for SM) and homogenization (200/40 bars for CM and 180/50 bars for SM). They were standardized in terms of fat, with 3.4 g/100 g for CM (whole milk) and 3.0 g/100 g for SM (semi- skimmed milk). The concentration in proteins was 3.6 g/100 g for CM and 5.9 g/100 g for SM. After a second pasteurisation for the milks (8 min at 81 °C for cow milk yogurt (CY) and 20 sec at 90 °C for sheep milk yogurt (SY)), yogurts were prepared by lactic coagulation with *Lactobacillus bulgaricus* and *Streptococcus thermophilus* at 43 °C (pH = 4.6 for CY and pH = 4.3 for SY). Caloric content of the four products were similar, with 68 ± 3 kcal/

100 g.

2.2. In vitro digestion

The static adult-stage gastrointestinal *in vitro* digestion was performed following the INFOGEST protocol (Brodkorb et al., 2019). Due to the liquid (milk) and non-chewing (yogurt) matrix structure, no oral phase was conducted. Magnetic stirring at 37 °C was maintained for 2 h during both the gastric and intestinal phases. For the gastric phase, 10 g of the sample were mixed with 8 ml of simulated gastric fluid (SGF). The pH of the gastric content was adjusted to 3 using 1 M HCl and water in order to achieve a meal-to-gastric fluid ratio of 50:50. After that, 5 µl of 0.3 M $CaCl_2$ was added. Then, rabbit gastric extract (RGE15, lot #1201, Lipolytech, France) were added to cover 60 U of gastric lipase per ml of gastric content, leading to 2344 U of pepsin per ml of gastric content according to the enzyme activities measured in RGE15 (lipase activity = 12.8 U/mg and pepsin activity = 500 U/mg).

Following gastric digestion, 8.5 ml of simulated intestinal fluid (SIF) was added. The pH was raised up to 7 using 1 M NaOH and water in order to achieve a meal-to-intestinal fluid ratio of 25:75. Additionally, 40 µl of 0.3 M $CaCl_2$ was added. Porcine pancreatin diluted in 5 ml of SIF (code P7545; lot #SLCK2311, Sigma-Aldrich) providing 100 U of trypsin per ml of intestinal content and 622 U of intestinal lipase per ml of intestinal content and 2.5 ml of bovine bile (code B3883; #SLCJ0047, Sigma-Aldrich, France) diluted in SIF to cover 10 mM, were then added.

Each product was digested in triplicate, following a randomized order. The designation "G0" corresponded to the products acidified at pH = 3, diluted in the same conditions as the gastric samples but without enzyme addition. The samples were collected at 5, 60, and 120 min of gastric (G) and intestinal (I) phases. The series of samples further used for lipid analyses were stabilized using a lipase inhibitor: 50 µl of 0.1 M 4-bromophenylboronic acid per ml of digesta. The series of sample further used for protein and calcium analysis were stabilized using protease inhibitors: 50 µl of 0.72 mM pepstatin A per ml of gastric digesta and 50 µl of 0.1 M pefabloc per ml of intestinal digesta. Other samples were collected to assess the particle size distribution. Particle size analysis (section 2.3), lipid extraction (section 2.4) and centrifugation for calcium analysis (section 2.6) were all performed the day of digestion. Samples for further analysis were stored at −18 °C.

2.3. Particle size analysis and CLSM observation

Particle size analysis was performed before and during gastric digestion using a Laser Diffraction Analyzer Mastersizer 2000 (Malvern Instrument, England). The refractive index used was 1.33 for the dispersant medium (water) and 1.46 for the particles. Each measurement was performed with and without the addition of sodium lauryl sulfate (SDS). Mean volume distributions ($D_{4,3}$) and diameter Modes (i.e., the particle diameters of the most frequent particles in the volume distribution) were determined from these measures.

The microstructure of samples was observed before and during gastric digestion using confocal laser scanning microscopy with an LSM 880 detector and a high-resolution airy scan detector (Carl Zeiss SAS, France) with a × 63 magnification. For lipid and protein staining, 200 µl of sample was mixed with 18 µl of Red Nile (0.1 %) and 6 µl of Fast Green (1 %) (Thermo Fisher Scientific, MA, USA) and incubated for 10 min at 20 °C. The mixture was added to 100 µl of 0.5 % (m/v) agarose.

2.4. Lipid analysis

2.4.1. Total fatty acid content

100 µl of undigested milks and yogurts were mixed with 500 µl of chloroform and 20 µl of Glyceryl tri-C13 (5 mg/ml) as an internal standard. The total fatty acids were *trans*-esterified with 1 ml of 0.5 % (m/v) sodium methoxide at 50 °C for 10 min and methylated with 1 ml of boron trifluoride (14 % in methanol) at 90 °C for 10 min. The fatty acid methyl esters were collected by adding 2 ml of hexane, 1 ml of potassium carbonate (10 % v/v) and centrifuged for 5 min at 300 *g*.

The analysis was performed using a Shimadzu GCMS-TableQP2010 SE gas chromatography mass spectrometer (Shimadzu Corp., Japan) equipped with a BPX70 capillary column (SGE Analytical Science, Australia). The samples were injected using a split/splitless injector at a temperature of 250 °C, with helium as carrier gas. Detection was conducted in SCAN mode. The separation was carried out under a temperature gradient ranging from 50 °C to 240 °C, with a plate maintained at 175 °C. Data acquisition was performed using GCMS solution software (Shimadzu Corp., Japan). Fatty acids were identified according to their retention time and mass spectrometry results with NIST mass spectral database library (https://www.nist.gov, 2017) and quantified using internal standards.

2.4.2. Degree of lipolysis and free fatty acid profile during digestion

Free fatty acids were extracted from 400 µl of digesta. Samples were mixed with 160 µl of hydrochloric acid and a 160 µl solution of C5:0, C11:0, C17:0 (5 mg/ml each) as an internal standard. 3 ml of a chloroform/methanol mixture (2:1, v/v) and 100 µl of sodium chloride were added. Lipids were extracted by centrifugation for 5 min at 3000 *g*. The free fatty acids were isolated using a solid-phase extraction (SPE) column (NH_2, 3 ml/500 mg, Macherey-Nagel), conditioned with 10 ml of heptane and rinsed with 10 ml of hexane/isopropanol (3:2, v/v). The fatty acids were then collected in 3.5 ml of formic acid/diethyl ether (2 % v/v). Before injection into the GC–MS, the fatty acids were methylated following the procedure described in section 2.4.1. The degree of lipolysis was calculated as followed:

$$\text{Degree of lipolysis}\,(\%) = 100 \times \frac{(\sum [FFA]_t - \sum [endoFFA]) \times F_t}{\sum [TFA]}$$

where $\sum [FFA]_t$ is the concentration of free fatty acids detected after t min of digestion (µmol/L of digesta), $\sum [endoFFA]$ is the concentration of free fatty acids coming from gastric or intestinal secretions (lipids in secretions coming mainly from bovine bile), F_t is the dilution factor due to secretions and $\sum [TFA]$ is the total fatty acids concentration (µmol/L of meal).

2.5. Protein analysis

2.5.1. Degree of proteolysis

The degree of proteolysis was calculated by measuring the primary amines (NH_2) present before and during digestion using the o-phthaldialdehyde (OPA) method (Halabi et al., 2020; Salelles et al., 2021; Nebbia et al., 2022). The quantification of primary amines was performed on the soluble fraction obtained after centrifugation of the digested samples (10,000 *g*, 20 min, 4 °C). 50 µl of the soluble fraction were diluted and mixed with 100 µl of OPA reagent (SDS: 0.5 % w/v, OPA: 0.25 mg/ml, DTT: 7 mM, sodium tetraborate: 20 mM) in a 96-well microplate (Greiner Bio-One, France). The absorbance at 340 nm was measured after 10 min using a Multiskan™ GO Microplate Spectrophotometer (Thermo Fisher Scientific). The total releasable primary amines from milk and yogurt were determined through complete hydrolysis of the matrix using 6 M HCl at 110 °C for 24 h. A standard range of methionine solution (0 to 2 mM) was used to establish the calibration curve. The degree of proteolysis was calculated as follow:

$$\text{Degree of proteolysis}\,(\%) = 100 \times \frac{\left([NH_2]_t - [endoNH_2]\right) \times F_t - [NH_2]_{t0}}{[NH_2]_{total} - [NH_2]_{t0}}$$

where $[NH_2]_t$ is the concentration of primary amine (mg/L of digesta) after t min of digestion, $[endoNH_2]$ is the concentration of NH_2 coming from gastric or intestinal secretion, F_t is the dilution factor due to secretions, $[NH_2]_{t0}$ is the concentration of initial primary amines before digestion (mg/L of meal), and $[NH_2]_{total}$ is the concentration of total

releasable primary amines.

2.5.2. Casein, α-lactalbumin and β-lactoglobulin degradation

SDS-PAGE analyses were performed using 4–12 % Bis–-Trispolyacrylamide precast gels (1.5 mm × 15 wells; NuPAGE Novex, Invitrogen). Samples were subjected to electrophoretic analysis following the procedure described by (Bouzerzour et al., 2012). 10 µg of protein was injected in each well for all samples from the gastric phase only (G0, G5, G60 and G120). The molecular marker used for the experiments was Mark 12 Unstained Standard NuPAGE 4–12 % (Invitrogen). Gel images were captured using the ChemiDoc MP Imaging System (Bio-rad). Densitometry analyses of the gel images were conducted using Image Lab Software version 6.1 (Bio-rad). Relative quantification of casein, α-lactalbumin, and β-lactoglobulin was determined by calculating the remaining amount of protein compared to the initial amount (G0 considered as 100 %).

2.6. Calcium analysis

Total calcium (Ca) has been determined by collecting ashes from milk and yogurt after mineralization 15 h at 550 °C. Ashes have been diluted in nitric acid (2 % v/v), centrifuged (1500 *g*, 15 min) and filtered (0.45 µm, Sartorius, France). Calcium was then quantified using inductively coupled plasma-atomic emission spectroscopy (ICP-AES) (iCAP 7200 Duo, Thermo Fisher Scientific, Waltham, USA).

To measure soluble Ca, milks, yogurts and digested samples were centrifuged (17,000 g,1h, 4 °C) after digestion before storage. Samples were diluted in nitric acid (2 % v/v), centrifuged (1500 *g*, 15 min) and filtered (0,45 µm, Sartorius, France) and analysed with ICP-AES as described above. The calcium bioaccessibility was calculated as follows:

$$\text{Calcium bioaccessibility}\ (\%) : 100 \times \frac{\left([solubleCa]_t - [solubleendoCa]\right) \times F_t}{[Ca]_{total}}$$

where $[solubleCa]_t$ is the concentration of soluble Ca (g/100 g of digesta) after t min of digestion, $[solubleendoCa]$ is the concentration of soluble Ca coming from gastric or intestinal secretion, F_t is the dilution factor due to secretions and $[Ca]_{total}$ is the total Ca concentration (g/100 g of meal).

2.7. Statistical analysis

Data analyses were conducted with the use of R software, version 4.1.3. A one-way analysis of variance (ANOVA) was performed for each digestion time. If significance was assessed (p-value < 0.05), pairwise multiple comparison of the means was done using Tukey's test. A multi-way ANOVA was performed to determine structure and species impact on digestion and their interactions with time. Kolmogorov-Smirnov test was used to test residues normality. Results are expressed as means ± SDs.

3. Results and discussion

The aim of this work was to determine differences between cow's milk (CM), cow's milk yogurt (CY), sheep's milk (SM) and sheep's milk yogurt (SY) during static *in vitro* gastrointestinal digestion. Impacts of species (cow vs sheep) and structure (milk vs yogurt) were investigated through particle size evolution, microscopic observations, degree of lipolysis and proteolysis, specific protein degradation and calcium bioaccessibility.

3.1. Particle size evolution during gastric phase

In the meals before digestion (Fig. 1.a,1.b and Table 2), larger fat globules were observed in SM compared to CM, and larger protein aggregates were observed in SY than in CY, which were in accordance with the literature (Ould-Eleya et al., 1998; Domagała, 2009). After acidification at pH = 3 (G0), clotting of CM and SM caseins was evidenced by both confocal observation (Fig. 1.a) as well as the increase in particle size (Fig. 1.b and Table 2) highlighting the strong effect of acidification in the disintegration of particles during gastric digestion. Following enzyme addition, particles size showed a gradual reduction, except for

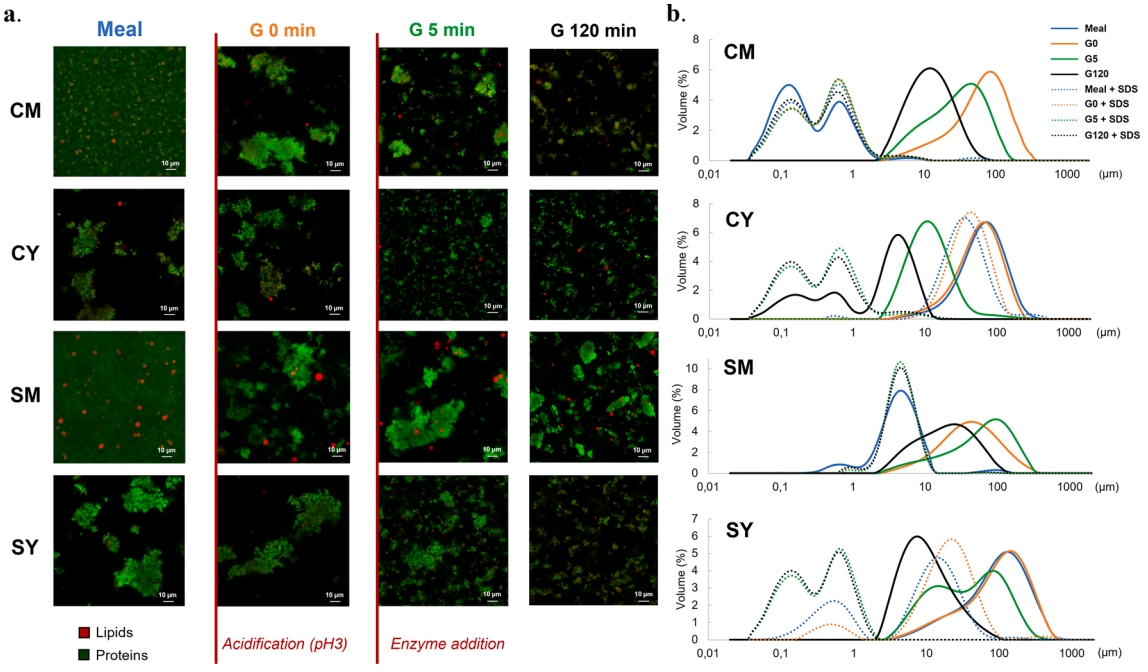

Fig. 1. Particle size evolution during gastric phase. Cow's milk (CM), cow's milk yogurt (CY), sheep's milk (SM) and sheep's milk yogurt (SY) particle size evolution have been observed with confocal laser scanning microscopy (a) and laser diffraction analyzer (b) during gastric phase of an *in vitro* digestion. Analysis was performed on meal (undigested sample), gastric phase at 0 min (G0), 5 min (G5) and 120 min (G120). The dotted lines represent the size distribution of samples with sodium lauryl sulfate (SDS) addition.

Table 2
Mean volume distributions ($D_{4,3}$) and the diameter Modes (the particle diameters of the most frequent particles in the volume distribution) of Cow's milk (CM), cow's milk yogurt (CY), sheep's milk (SM) and sheep's milk yogurt (SY) analyzed over *in vitro* digestion and performed on samples in meal (undigested sample), gastric phase at 0 min (G0), 5 min (G5) and 120 min (G120) with and without sodium lauryl sulfate (SDS) addition. D(4.3) values represent mean values ± sd (n = 2) and diameter Modes are the one presented on Fig.1b.

		Meal	G0	G5	G120	Meal + SDS	G0 + SDS	G5 + SDS	G120 + SDS
CM	D(4.3) (μm)	0.50 ± 0.02	84.64 ± 27.53	40.64 ± 4.34	15.88 ± 1.66	0.59 ± 0.01	0.67 ± 0.01	0.68 ± 0.01	0.69 ± 0.08
	Mode 1 (μm)	0.13	79.62	44.77	12.62	0.16	0.16	0.16	0.16
	Mode 2 (μm)	0.80				0.71	0.71	0.71	0.56
CY	D(4.3) (μm)	79.13 ± 8.59	71.50 ± 4.62	17.17 ± 3.74	3.36 ± 0.68	50.21 ± 2.79	50.21 ± 2.24	0.81 ± 0.02	0.92 ± 0.08
	Mode 1 (μm)	63.24	56.37	12.62	0.16	35.57	50.24	0.16	0.14
	Mode 2 (μm)				0.56			0.63	0.63
	Mode 3 (μm)				5.02			5.64	5.64
SM	D(4.3) (μm)	5.10 ± 0.03	45.57 ± 3.04	90.26 ± 14.08	27.89 ± 7.01	5.10 ± 0.03	5.14 ± 0.05	5.18 ± 0.09	5.24 ± 0.04
	Mode 1 (μm)	0.71	44.78	112.47	28.25	4.47	4.47	4.47	4.47
	Mode 2 (μm)	5.02							
SY	D(4.3) (μm)	135.60 ± 25.52	138.04 ± 20.51	63.57 ± 10.99	14.45 ± 2.04	26.16 ± 13.75	41.84 ± 15.26	3.51 ± 3.54	0.45 ± 0.01
	Mode 1 (μm)	126.19	158.87	15.89	7.96	0.50	0.56	0.16	0.16
	Mode 2 (μm)			79.62		14.16	25.18	0.63	0.71

SM showing the biggest particles even at the end of gastric phase (Fig. 1.a, 1.b and Table 2). At the end of gastric digestion (G120), the impact of species and structure on mean volume distributions ($D_{4,3}$) was significant (p-value < 0.05 for species impact and structure impact). Indeed, when comparing milk and yogurt from the same species at G120, yogurt showed significantly smaller particles (p-value < 0.05) than its respective milk: D(4.3) = 15.88 ± 1.66 μm for CM and D(4.3) = 3.36 ± 0.68 μm for CY; D(4.3) = 27.89 ± 7.01 μm for SM and D(4.3) = 14.45 ± 2.04 μm for SY. Similarly, when comparing the different species for the same structure at G120, CM products showed significantly smaller particles (p-value < 0.05) than SM products. The diameter Mode values at G120 illustrated as well these differences with the following results (Table 2): Mode 1 = 12.62 μm for CM and Modes 1, 2 and 3 = 0.16, 0.56 and 5.02 μm for CY; Mode 1 = 28.25 μm for SM and Mode 1 = 7.96 μm for SY. The observations from confocal microscopy (Fig. 1.a) illustrated this structural evolution coherently: a progressive disaggregation of particles with larger particles for SM products compared to CM products, as well as larger particles for milks compared to yogurts throughout gastric digestion.

After the addition of SDS, the particle size profiles (Fig. 1.b, dotted lines and Table 2) of digested samples returned to the undigested milk's profile, indicating the formation of non-covalent aggregation during gastric digestion. For digested yogurt samples, it was noted that SDS addition caused a return to undigested milk's profile only after the enzymes addition (G5), suggesting stronger particle aggregation formed before digestion.

The delay of disaggregation observed for SM products may be explained by the firmer clot formed during gastric digestion (Roy et al., 2021). *In vivo* studies have shown that higher viscosity can slow down gastric emptying (Mackie et al., 2013; Zhu et al., 2013), similar results were observed with a higher protein content (Anderson and Moore, 2004; Anderson et al., 2011). Therefore, SM products showed firmer clot in the stomach due to a higher protein content compared to CM products (concentration in proteins was 3.6 g/100 g for CM and 5.9 g/100 g for SM; section 2.1) leading to a denser protein network which could slow down the accessibility of the enzyme to its substrate and probably lead to a slower gastric disaggregation. It may be expected for instance that SM products, with a naturally firmer texture and a higher protein content, could be a good option for populations looking for high satiety food (Dougkas and Östman, 2016).. In fact, in study on mice, it has been demonstrated that the consumption of SM after a period of caloric restriction has led to a higher blood levels of leptin compared to CM (Gauffin Cano et al., 2009), a satiety-inducing hormone (Mendoza-Herrera et al., 2021).

3.2. Degree of lipolysis and fatty acid profiles

Lipid content was 3.4 g/100 g for CM products and 3.0 g/100 g for SM products, as described in section 2.1. The fatty acid composition of the four meals has been measured and are the following (Supplementary Material Table S4): the fatty acid composition was globally similar between the four meals, with 73.00 ± 0.79 % of saturated fatty acids,

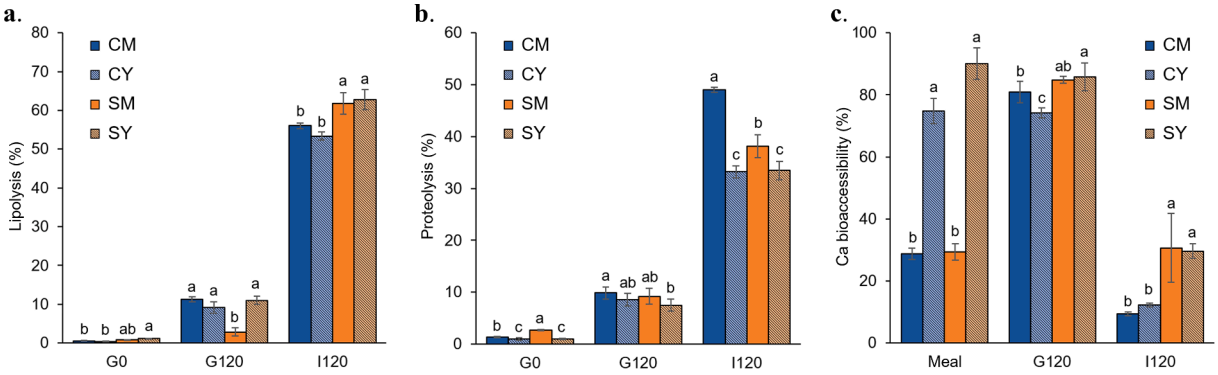

Fig. 2. Degree of lipolysis (a), proteolysis (b) and calcium bioaccessibility (c) during digestion time. Cow's milk (CM), cow's milk yogurt (CY), sheep's milk (SM) and sheep's milk yogurt (SY) has been characterized through a gastrointestinal *in vitro* digestion. Analysis was performed on undigested sample (meal) or gastric phase at 0 min (G0), 120 min (G120) and intestinal phase at 120 min (I120). a. Degree of Lipolysis has been measured with gas chromatography mass spectrometry. b. Degree of Proteolysis has been measured with o-phthaldialdehyde (OPA) method. c. Calcium (Ca) bioaccessibility has been measured with inductively coupled plasma-atomic emission spectroscopy. All values represent mean values ± sd (n = 3) and are expressed as percentage (%) over the total amount. Letters represent significative difference between groups (p-value < 0.05) with a Tuckey test.

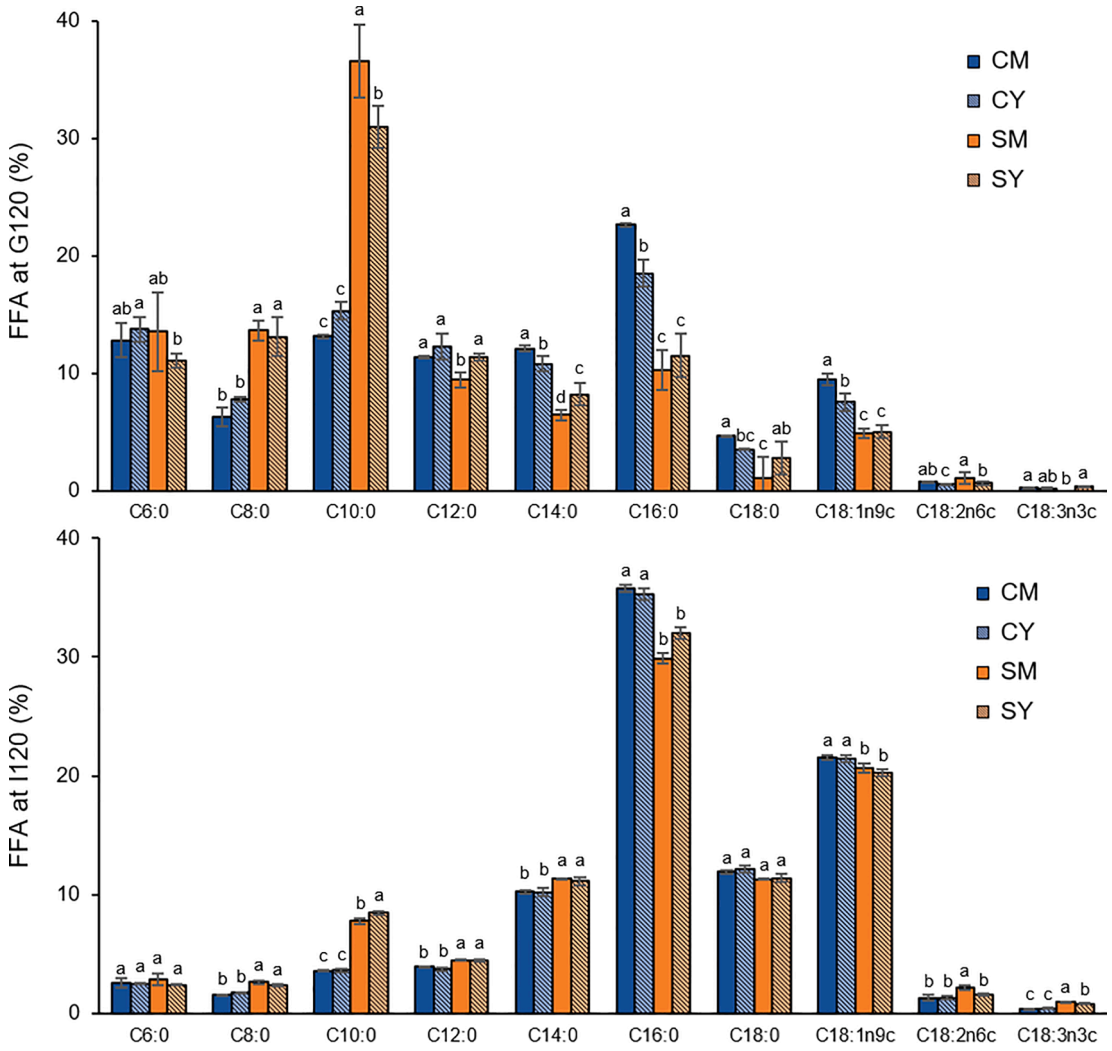

Fig. 3. Free fatty acid (FFA) profile of gastric (a) and intestinal (b) digestas. Cow's milk (CM), cow's milk yogurt (CY), sheep's milk (SM) and sheep's milk yogurt (SY) free fatty acid (FFA) composition at the end of gastric phase (G120) and intestinal phase (I120) has been measured with gas chromatography mass spectrometry. Mean values ± sd (n = 3) are expressed as the percentage (%) of free fatty acids over total free fatty acids. Letters represent significative difference between groups (p-value < 0.05) with a Tuckey test.

24.00 ± 1.10 % of mono-unsaturated fatty acids and 2.80 ± 0.48 % of poly-unsaturated fatty acids. Palmitic acid (C16:0) was the predominant fatty acid for all meals, with a slightly lower concentration found in SM products compared to CM ones (29 ± 0.50 % against 36 ± 0.18 %, p-value < 0.05). Proportions of short and medium chain fatty acids, from C:6 to C12:0, was higher in SM products than in CM ones (14.6 ± 0.16 against 9.2 ± 0.18 %, p-value < 0.05). This last difference was mainly due to the high concentration in capric acid (C:10) in SM. The ω6/ω3 ratio was lower in SM products than in CM ones (2.8 ± 0.2 vs 5.0 ± 0.5, p-value < 0.05).

As shown in Fig. 2.a and in Supplementary material Table 1, there was no difference in lipolysis during gastric phase between CM, CY and SY, only SM stood out with a reduced extent of lipolysis. This lower lipolysis level for SM observed at the end of gastric phase could be linked to its lower structure destabilization observed in Fig. 1. Indeed, an aggregated structure is a limiting factor for lipolysis (Nguyen et al., 2018), due to more restricted access for the gastric lipase to its substrate. At the end of digestion (I120), a significant species impact was observed (p-value < 0.05), SM products showing higher lipolysis compared to CM products with a mean difference (MD) of 7 %. However, no structure impact was found.

As observed in Fig. 3 and in Supplementary material Table S5, short and medium chain fatty acids were released in majority in gastric phase and in higher extent in SM products. Teng et al. (2020) found similar results and suggested that, in addition to a higher short and medium-chain fatty acids content, this could also be attributed to the presence of higher levels of short and medium chain fatty acids at the *sn*-1 or *sn*-3 location within the triacylglycerols (TAG) structure in SM compared to CM. Indeed TAG in *sn*-2 location are not hydrolyzed by digestive lipases (Nagata et al., 2018). Furthermore, the stereo-preference of gastric lipase for short and medium-chain fatty acid due to their location in *sn*-1 and *sn*-3 within the TAG could explain the differences in lipolysis observed between CM and SM (Rogalska et al., 1990).

In terms of lipidic nutritional quality, SM products seem to exhibit a more favorable composition compared to CM products. Limiting palmitic acid intake in favor of short and medium chain fatty acids is suggested to be preferable for cardiometabolic health (Dulloo et al., 1996; Hill et al., 1989; Huang et al., 2021; Panth et al., 2018). Moreover, a balanced ω6/ω3 ratio, between 1 and 4, could limit chronic inflammation (Simopoulos, 2002), although the total amount of poly-unsaturated fatty acids in these products were relatively low. A clinical study suggested that SM products consumption, in comparison to CM products, resulted in a small reduction of total cholesterol (Skeaff et al., 2004). Other authors showed a similar postprandial triglycerides response and cholesterol levels between CM and SM products despite a higher absolute quantity of lipids in SM products, and higher blood level

of short and medium chain fatty acids (Olmedilla-Alonso et al., 2017; Teng et al., 2022).

3.3. Degree of proteolysis and gastric degradation of specific proteins

Protein content was 3.6 g/100 g for CM products and 5.9 g/100 g for SM products, as described in section 2.1. At G120 (in Fig. 2.b. and Supplementary material Table S2), no major difference in gastric proteolysis degree was found between the four products with 8.8 ± 1.2 % proteolysis, despite the higher absolute quantity of proteins in SM products. These results are consistent with the literature (Roy et al., 2021).

There was a significant structure impact on proteolysis results (p-value < 0.05). CM exhibited the highest degree of proteolysis at the end of digestion (I120) with 49.00 ± 0.47 % (Fig. 2.b.). The milks from both species demonstrated higher degree of proteolysis than their respective yogurts with a mean difference (MD) = 16 % for CM vs CY (p-value < 0.05) and 5 % for SM vs SY (p-value < 0.05). CM presented higher degree of proteolysis than SM, MD = 11 % (p-value < 0.05), while no significant differences in proteolysis between yogurts from both species were observed with 33.0 ± 1.2 %, being the least hydrolyzed. These differences in proteolysis between SM and CM could be explained by their difference in terms of structure and protein content. Indeed, as described in section 3.1, SM products showed firmer clot in the stomach due to a higher protein content and a denser protein network, that could slow down the accessibility of the enzyme to its substrate and thus lead to a slower protein hydrolysis. Similarly, the differences in proteolysis observed between milk and yogurt for both species could be explained also by their difference in structure. Indeed, yogurts presented an aggregated structure due to the coagulation by lactic bacteria, which slow down the protein hydrolysis.

Caseins, α-lactalbumin and β-lactoglobulin were considered separately during gastric phase in Fig. 4 and supplementary material Table S6 and supplementary material Fig. 1. Caseins were degraded faster in CM products than in SM ones, likely due to its higher absolute quantities in SM products. Interestingly, α-lactalbumin were degraded faster in SM products despite its higher concentration compared to CM products, which has never been shown in literature before, with our knowledge. No intact caseins and α-lactalbumin remained at the end of gastric phase. As for β-lactoglobulin, significative structure and species impacts were observed (interaction Time*Species p-value < 0.05, interaction Time*Structure p-value < 0.05). Indeed, β-lactoglobulin from yogurts were digested faster than their respective milks as well as β-lactoglobulin from CM products compared to SM products, for a same structure. SM β-lactoglobulin was not degraded at all contrary to CY, from which no intact β-lactoglobulin remained. These findings are in accordance with the literature (Dupont et al., 2010; Kopf-Bolanz et al., 2014; Nguyen et al., 2020).

It should be noted that CM products and SM products, as commercially available products, have been pasteurized at different temperatures (as described in section 2.1) following routine industrial manufacturing processes. It has been demonstrated that pasteurization temperature could influence protein digestion and thus could also explain the differences observed in protein hydrolysis between CM and SM.

3.4. Calcium bioaccessibility

The total calcium content measured was 163 ± 13 mg/100 g for CM products and 214 ± 11 mg/100 g for SM products, as described in section 2.1. As exposed in Fig. 2.c. and in Supplementary material Table S3, calcium was predominantly found in soluble form in yogurts, which is due to the acidic pH of these meals, that is why yogurts showed the highest calcium bioaccessibility before digestion compared to milks. Similarly, the solubilization of most of the calcium of the four products was caused by the acidification during gastric phase, that is why the calcium bioaccessibility is high for all the four products in gastric samples (G120). Finally, calcium bioaccessibility at the end of the digestion (I120), presented a significant specie impact (p-value < 0.05) but no structure impact. Indeed, both SM and SY showed higher levels of bioaccessible calcium compared to CM products with a mean difference (MD) of 18.77 %. Thus, SM products had the advantage of containing more calcium and exhibiting greater calcium bioaccessibility during the intestinal phase. It was observed that the amount of bioaccessible calcium was correlated with the ratio [total Ca] / [C16:0 released at I120] (correlation coefficient $r^2 = 0.81$). This could be explained by a saponification phenomenon between calcium and palmitic acid (C16:0), making the calcium insoluble and therefore non-bioaccessible (Mulet-Cabero & Wilde, 2023). The amount of palmitic acid was higher in CM products than in SM ones (36.11 ± 0.18 % in CM and 28.97 ± 0.26 % in SM, p-value < 0.05; Supplementary material Table S4), which could explain the lower calcium bioaccessibility found in CM products compared to SM products. However, while no structure impact was shown in literature with various models (El-Gawad et al., 2014; Smith et al., 1985; Ünal et al., 2005), only slight differences were found for the calcium bioaccessibility between SM and CM (Bossu et al., 2020; Preedy, 2015), with or without milk skimming (i.e. the removing of C16:0) (Shen et al., 1995). The contradiction with *in vitro* studies described above and our results may be due to the use of different digestion models, in fact dialysis model being used in cited studies compared to an *in vitro* static model in the current study. However, considering the calcium bioaccessibility measured in the present study, SM products remain a better source of calcium than CM products. An adequate calcium intake supports bone development in youth, slows down age-related bone decline and lowers the risk of osteoporotic fractures, with dairy products as effective as calcium supplements in achieving these benefits (Heaney, 2000; Khan et al., 2015).

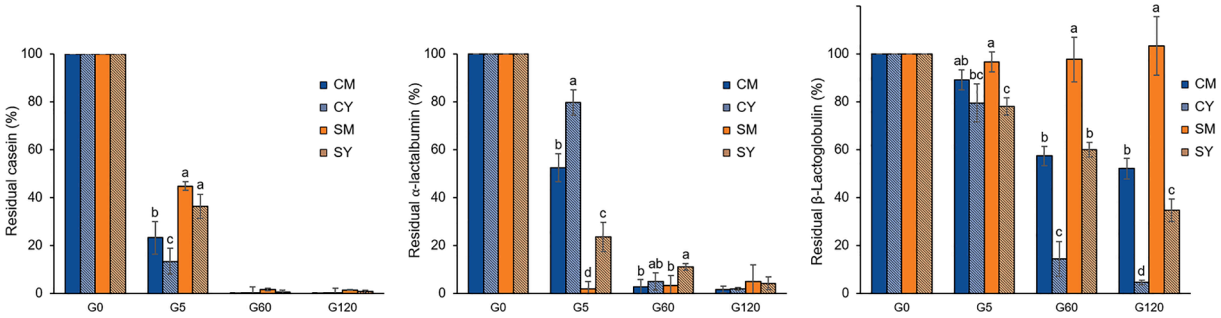

Fig. 4. Evolution of Caseins, α-lactalbumin and β-lactoglobulins during gastric digestion. Cow's milk (CM), cow's milk yogurt (CY), sheep's milk (SM) and sheep's milk yogurt (SY) relative proteins quantification has been performed with SDS-PAGE electrophoretic analysis and densitometry during gastric phase at 0 min (G0), 5 min (G5), 60 min (G60) and 120 min (G120). Mean values ± sd (n = 3) are expressed as the percentage (%) of the remaining protein in respect of the initial value (before digestion). Letters represent significative difference between groups (p-value < 0.05) with a Tuckey test.

4. Conclusion

Considering the results from this study, cow's and sheep's milks and yogurts revealed a higher impact of the species (cow vs sheep) than the structure (milk vs yogurt) under *in vitro* static digestion. In these conditions, the structure and the species influenced the microstructure evolution during the gastric phase: yogurts exhibited smaller particles after gastric phase compared to milks, as well as CM products compared to SM ones. Furthermore, the species appeared to impact the lipid composition and the lipolysis: SM products displayed higher levels of short and medium-chain fatty acids and a greater intestinal degree of lipolysis than their CM counterparts. Concerning proteins, caseins were degraded more rapidly in CM likely due to its lower concentration in this product, and α-lactalbumin more rapidly in SM despite its higher concentration in this product. β-lactoglobulin gastric digestion exhibited significant impacts of species and structure, being more degraded in yogurts compared to milks and in CM products compared to SM products. Moreover, the structure influenced proteolysis: milks led to a higher degree of proteolysis compared to yogurts, as did CM compared to SM, but no difference was found between yogurts. Lastly, the species appeared to affect calcium bioaccessibility, with SM products releasing more soluble calcium at the end of digestion compared to CM products.

Accordingly, it was shown that SM products seemed to be degraded slower than CM products mainly due to a higher protein content and a denser protein network in SM, that could slow down the accessibility of the enzyme to its substrate and thus lead to slower the digestion of the nutrients. This attribute of SM could be interesting in the research of satiety food effect to regulate appetite in adults, and thus could be a good option for population focusing on appetite management. Moreover, SM products displayed a more balanced lipid profile as well as higher protein content and more bioaccessible calcium than CM products, which could be beneficial for population suffering from cholesterol, sarcopenia, and osteoporosis like elderly.

To conclude, this study highlights the role of composition and structural attributes in the digestion of SM and CM products. This deeper understanding contributes to the knowledge of dairy product digestion but also offers insights for nutritional outcomes. Further research could be completed with products manufactured within the same technological conditions and also with *in vivo* studies in order to come closer to more physiological conditions, and to confirm these promising results.

CRediT authorship contribution statement

Tanguy Saviard: Writing – original draft, Investigation, Formal analysis. **Olivia Menard:** Validation, Investigation, Conceptualization. **Stefano Nebbia:** Validation, Supervision. **Jordane Ossemond:** Validation, Investigation. **Gwénaële Henry:** Validation, Investigation, Conceptualization. **Raphaël Chacon:** Validation, Project administration, Conceptualization. **Steven Le Feunteun:** Validation, Conceptualization. **Didier Dupont:** Validation, Conceptualization. **Linda Le Roux:** Writing – review & editing, Validation, Supervision, Investigation, Conceptualization.

Declaration of competing interest

Tanguy Saviard, Linda Le Roux, and Raphaël Chacon are employees of Sill Entreprises. Other 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

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2024.114604.

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