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Human milk *vs*. Infant formula digestive fate: *In vitro* dynamic digestion and *in vivo* mini-piglet models lead to similar conclusions

Elise Charton [a,b], Olivia Menard [a], Marie-Françoise Cochet [a], Yann Le Gouar [a], Julien Jardin [a], Gwénaële Henry [a], Jordane Ossemond [a], Amandine Bellanger [e,f,g], Carlos A. Montoya [c,d], Paul J. Moughan [c], Didier Dupont [a], Isabelle Le Huërou-Luron [b,1], Amélie Deglaire [a,1,*]

[a] *STLO, L'Institut Agro, INRAE, 35042 Rennes, France*
[b] *Institut NuMeCan, INRAE, INSERM, Univ Rennes, 35590 Saint Gilles, France*
[c] *Riddet Institute, Massey University, Palmerston North, New Zealand*
[d] *Smart Foods Innovation and Bioproducts Group, AgResearch Limited, Palmerston North, New Zealand*
[e] *CHU Rennes, Pediatrics Department, 35000 Rennes, France*
[f] *University of Rennes, Faculty of Medicine, 35000 Rennes, France*
[g] *CHU Rennes, CIC-Inserm 1414, 35000 Rennes, France*

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A B S T R A C T

Infant formula (IF), the only nutritionally adequate substitute for human milk (HM), still needs to be improved to be more biomimetic with HM, including in terms of digestive fate. The latter can be explored using different digestion models. The present study aimed to compare IF and HM digestion using *in vivo* (mini-piglet) and *in vitro* (dynamic system, DIDGI®) models. Fresh mature HM was collected and compared with a standard bovine IF. *In vivo*, 18 Yucatan mini-piglets (24-day-old) received HM or IF and were euthanized 30 min after the last meal. The entire digestive content was collected from the stomach to the colon. *In vitro*, the same meals were fed to an *in vitro* dynamic digestion model simulating the term infant at four weeks of age. Digesta were sampled regularly in the gastric and intestinal compartments. Structure (confocal microscopy and laser light scattering) and proteolysis (SDS-PAGE for residual intact proteins, OPA for hydrolysis degree, LC-MS/MS for peptides) were investigated along digestion. The digesta microstructure differed between HM and IF in a similar way between *in vitro* and *in vivo* digestion. *In vitro* gastric proteolysis of caseins and α-lactalbumin was significantly slower for HM than for IF, such as for the early intestinal proteolysis degree. *In vitro* bioaccessibility of free AAs explained only 30 % of the true ileal digestibility of AAs. Peptide mapping of caseins differed between HM and IF along their digestion. The relative peptide mapping data over six proteins from HM and IF were highly correlated between *in vitro* and *in vivo* digestion, particularly at 80 and 120 min of *in vitro* gastric digestion *vs*. *in vivo* stomach data and at 20 and 40 min of *in vitro* intestinal digestion *vs*. *in vivo* proximal jejunum data ($r = 0.7–0.9$, $p < 0.0001$, $n = 1604$). 40 to 50 % of the bioactive peptides identified *in vivo* were also found *in vitro*, with a good correlation of their abundances ($r = 0.5$, $p < 0.0001$, $n = 61$). Overall, *in vitro* and *in vivo* digestion were in good agreement, both indicating a different digestive fate for HM and IF.

Abbreviations: AA, amino acid; ACE, angiotensin converting enzyme; CLSM, confocal laser light microscopy; EAA, essential amino acid; G, gastric; HM, human milk; I, intestinal; IF, infant formula; LC-MS-MS, liquid chromatography-mass spectrometry-mass spectrometry; MFA, multi-factorial analysis; NEAA, non-essential amino acid; OPA, *ortho*-phtalaldehyde; SDS-PAGE, sodium dodecyl-sulfate polyacrylamide gel electrophoresis; TAA, total amino acid.

* Corresponding author.
E-mail addresses: elise.charton@agrocampus-ouest.fr (E. Charton), olivia.menard@inrae.fr (O. Menard), marie-francoise.cochet@institut-agro.fr (M.-F. Cochet), yann.le-gouar@inrae.fr (Y. Le Gouar), julien.jardin@inrae.fr (J. Jardin), gwenaele.henry@inrae.fr (G. Henry), jordane.ossemond@inrae.fr (J. Ossemond), Carlos.Montoya@agresearch.co.nz (C.A. Montoya), P.J.Moughan@massey.ac.nz (P.J. Moughan), didier.dupon@inrae.fr (D. Dupont), isabelle.luron@inrae.fr (I. Le Huërou-Luron), amelie.deglaire@institut-agro.fr (A. Deglaire).
[1] Authors contributed equally.

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1. Introduction

Despite breastfeeding recommendations (WHO, 2011), a high proportion of infants are still fed with infant formula (IF), justifying the importance of IF mimicking as closely as possible human milk (HM) to get closer to HM nutritional and health benefits. IFs, mainly based on bovine milk, have been widely optimized over the years to reduce differences between HM and IF (Bourlieu et al., 2017; Deglaire et al., 2023; Totzauer et al., 2018). However, some differences persist notably regarding the fine composition and the structure (before and during digestion), including that of the protein fraction. For example, the true protein content is lower in mature HM than in IF (0.8–1.0 g/100 mL HM *vs*. 1.1–1.7 g/100 mL IF) (Boudry et al., 2021) with a different protein profile, particularly regarding the whey proteins. Indeed, in HM, whey proteins are predominantly in the form of α-lactalbumin and lactoferrin whereas β-lactoglobulin is the major whey protein in IF but absent in HM (Chatterton et al., 2004). In addition, HM and IF caseins are differently organized and mineralized, with smaller micelles in HM than in bovine milk (Calapaj, 1968). Besides, IF manufacturing leads to structural differences mainly owing to the homogenization process, which reduces the size of fat droplets into submicronic particles in IF (Bourlieu et al., 2015; Michalski et al., 2005), and to successive heat treatments resulting in protein denaturation/aggregation and favoring the Maillard reaction (Halabi et al., 2022; Hendricks & Guo, 2014; Deglaire et al., 2023).

Many studies have investigated the digestive behaviour of HM proteins, using *in vivo* (Darragh & Moughan, 1998; de Oliveira et al., 2017; Wada et al., 2017) or *in vitro* (de Oliveira et al., 2016; Deglaire et al., 2016) digestion models, or that of IF proteins using *in vivo* (Bouzerzour et al., 2012; Rutherfurd et al., 2006) or *in vitro* (Chatterton et al., 2004; De Figueiredo Furtado et al., 2021; Halabi et al., 2022) digestion models. A few studies have directly compared protein digestion between these two types of food, and this has only been performed *in vitro*, using semi-dynamic (Abrahamse et al., 2022; Chatterton et al., 2004; He et al., 2022) or dynamic models (Maathuis et al., 2017). A different digestion behavior between HM and conventional bovine IF has been reported. In the gastric phase, HM formed larger aggregates and presented a different protein distribution, with a higher protein content in the serum phase for HM than for bovine IF (He et al., 2022). In the intestinal phase, opposite results of proteolysis kinetics have been reported, with either a slower protein hydrolysis for HM (Abrahamse et al., 2022) or on the contrary a faster bioaccessible N release for HM in comparison with a conventional bovine IF (Maathuis et al., 2017). *In vivo*, to date, no study has compared HM and IF in the same experiment, except in our previous mini-piglet study in which we reported a similar true ileal digestibility for most AAs between HM and IF (Charton et al., 2023), but with no information on the digestive kinetics.

Because digestion studies in infants are difficult to conduct for ethical reasons, suitable animal models such as rat pups or piglets are needed to better understand the mechanisms of infant digestion (Deglaire & Moughan, 2012; Moughan et al., 1992). Particularly, the three-week-old piglet has been previously reported to mimic HM protein digestion of the 3-month-old human infant (Darragh & Moughan, 1995). The 3- to 4-week-old piglet has thus been used to investigate the protein digestion of IFs (Abrahamse et al., 2015; Bouzerzour et al., 2012; Charton et al., 2023; Chauvet et al., 2024) and less frequently of HM (Charton et al., 2023; Darragh et al., 1998). However, such animal experiments are gradually being replaced by *in vitro* digestion models due to ethical and societal concerns. These latter models have been developed and improved over the past decades and are useful to compare the digestive fate of IF in regards to HM in order to design more biomimetic IF. Such *in vitro* models are assumed to represent what occurs during human infant digestion, although few *in vivo* and *in vitro* comparisons have been undertaken particularly at the infant stage. Chatterton et al. (2004) have reported that HM protein digestion in the gastric phase was comparable between human newborns and an *in vitro* static model using human neonate gastric juice. The comparison was limited due to the very low number of human neonates (n = 2) and as this was solely based on visual observations on SDS-PAGE. A dynamic *in vitro* model has been developed within our team and validated against the piglet model for the digestion of a porcine IF (Ménard et al., 2014). A high correlation coefficient (r = 0.99) was observed between *in vitro* and *in vivo* for gastro-intestinal proteolysis, as determined by ELISA, in addition to similar *in vitro* and *in vivo* gastric volumes. This dynamic *in vitro* model, parametered for a 1-month-old human infant, has been since used to investigate separately the protein digestion of HM (De Oliveira et al., 2016; Nebbia et al., 2020) or of IF (Chauvet et al., 2023; Le Roux et al., 2020), but not yet concomitantly.

The aim of the present study was thus two fold: (1) to compare the digestion fate of HM and IF, in terms of structure and proteolysis and (2) to compare two existing models of the human infant digestion, i.e. an *in vitro* dynamic gastrointestinal system *vs*. an *in vivo* model that is the milk-fed piglet. We assumed that HM and IF presented a different digestive behaviour, particularly regarding proteins and that the *in vitro* dynamic digestion model could predict the *in vivo* response, particularly in the context of infant digestion. The same infant foods (HM and IF) were fed to mini-piglets and to the *in vitro* dynamic system (DIDGI®). Piglets were fed hourly over 6 times before euthanasia 30 min after the last meal. *In vivo* digesta were sampled along the entire digestive tract, while *in vitro* digesta were sampled at regular intervals after meal ingestion. The microstructure of the meal and of the digesta was followed (confocal microscopy and laser light scattering) as well as the proteolysis kinetics using a variety of complementary techniques (SDS-PAGE, OPA, LC-MS-MS).

2. Materials and Methods

2.1. Diets

Two types of HM pools, pasteurized HM for the piglet dietary adaptation and fresh HM for the *in vitro* & *in vivo* digestion study, were used. Pasteurized HM, arising from 22 donating mothers, was obtained from the donor milk bank of Rennes, as previously described (Charton et al., 2022). Fresh HM was collected for each of the three experimental blocks of the animal experiment and originated in total from 50 volunteer mothers of full-term infants. For each block, fifteen to twenty samples of fresh mature HM (1.8–2 months post-delivery) were collected in sterile infant-bottles. HM was expressed in the morning from a complete expression of a single breast. Fresh HM was then pooled in sterile bottles and stored at 4 °C until distribution to the piglets the day after collection. An aliquot of each fresh HM pool was kept frozen at −80 °C until *in vitro* digestion. Ethical approval for HM collection was granted by the Institutional Review Board of South Mediterranean V (n°19.12.12.65653).

The infant formula (IF) used in the present study was produced at a semi-industrial scale within our laboratory, as described by Yu et al. (2021). IF proteins, composed at 41 % of caseins and 59 % of whey proteins, were derived from skimmed bovine milk and bovine whey obtained by microfiltration. IF lipids originated from a commercial oil blend intended for infant nutrition. The present IF received the highest level of heat treatment, as presented in Yu et al. (2021), in order to mimic the protein denaturation level of commercial IFs. Lipid and protein content of the present fresh HM and IF has been detailed in Charton et al. (2023).

HM and IF diets, when fed to piglets, were supplemented with liquid vanilla (3 g/L of HM or IF) to encourage intake and with undigestible markers (0.3 % dry matter of cobalt-EDTA and Ytterbium).

2.2. In vivo digestion

The present study procedure, designed in agreement with the current ethical standards of the European and French guidelines and approved by the ethics committees of CREEA (Rennes Committee of Ethics in

Animal Experimentation) and of the French Ministry of Higher Education and Research (authorization #2020020610329770), was detailed previously (Charton et al., 2022; 2023). Briefly, eighteen healthy Yucatan piglets (10 ± 1 days) were assigned to the experimental diet (n = 9 per group) according to their body weight, litter origin and sex. They were first adapted to the experimental conditions during 8 ± 2 days during which they received a full fat bovine milk supplemented with vitamins and minerals (Charton et al., 2023). Piglets then received an IF for 6 days or a pool of pasteurized HM during 5 days, followed by a pool of fresh HM for the last day of the experiment. During the entire experiment, piglets were fed via a drinking trough ten times (from 7:30 to 22:00) except on the last day of the experiment, where they were hourly-fed over six times (Charton et al., 2022; 2023) and received 468 ± 134 mL of HM or IF.

The animal experiment was conducted over three independent blocks, with each block having half of the piglets fed with HM and the other half with IF, as detailed in Charton et al. (2023). On the last day of the experiment, animals were euthanized 30 min after the last meal by electrical stunning immediately followed by exsanguination as described elsewhere (Charton et al., 2022). As described previously (Charton et al., 2023), the entire digestive content was collected along the digestive tract [stomach, proximal (first 2.5 m of the small intestine) and median jejunum, ileum (60 cm before ileocecal junction) and proximal colon (first-third of colon)], and mixed with 50 μL of protease inhibitor/mL digesta (gastric digesta: pepstatin A solution at 0.73 μM; intestinal digesta: Pefabloc solution at 0.1 M, purchased from Sigma-Aldrich, St Quentin Fallavier, France). Structural analysis (laser light scattering and confocal microscopy) were performed on fresh digesta mixed with the appropriate protease inhibitor solution. The remaining samples were homogenized using an ultra-thurax homogenizer and then stored at −20 °C until SDS-PAGE, OPA, amino acid and peptide analyses or until freeze-drying prior cobalt-EDTA analysis, as described in Charton et al. (2023). Ytterbium was not considered due to inconsistent results (Charton et al., 2023).

2.3. In vitro digestion

The three fresh HM pools, stored frozen, were thawed and pooled by mixing equal volume of each pool. The same IF as fed *in vivo* was used. These meals were supplemented with a solution of 0.2 g/L of norleucine as a marker. A volume of 100 mL of each meal was digested during 180 min with the *in vitro* dynamic gastrointestinal digestion model using the bi-compartmental system DIDGI® set up to simulate the term infant digestion conditions at the postnatal age of four weeks, as described previously (de Oliveira et al., 2016), but with some adaptations. The gastric emptying followed the Elashoff equation with $t_{1/2} = 47$ min and $\beta = 0.9$ for HM and $t_{1/2} = 78$ min and $\beta = 1.2$ for IF. The Elashoff intestinal emptying equation was identical for both diets with $t_{1/2} = 200$ min and $\beta = 2.2$. The gastric pH acidification was similar to that presented in Nebbia et al. (2020) (Eq. (1)) with t the time after ingestion in min.

$$pH\_acidification = 8 \times 10^{-5} \times t^2 - 0.031 \times t + pH_{Diet} \quad (1)$$

The intestinal pH was maintained constant at 6.6. A rabbit gastric extract solution (Lipolytech®, Marseille) was used to provide pepsin and lipase (268 U/mL and 19.2 U/mL of gastric content, respectively) and was added at 1 mL/min during the first 10 min and at 0.5 mL/min until the end of digestion. The intestinal enzymes were provided by porcine pancreatin solution (Sigma-Aldrich, Saint-Quentin Fallavier, France; trypsin and lipase: 16 U/mL and 200 U/mL of intestinal content, respectively) that was added at a rate of 0.25 mL/min from 10 min after ingestion until the end of digestion. A solution of bovine bile salts (Sigma-Aldrich, Saint-Quentin Fallavier, France), such as indicated by the INFOGEST protocol (Brodkorb et al., 2019) was added at a level of 3.1 mmol/L of intestinal content at 0.5 mL/min during all the digestion duration.

Digestion was performed in triplicate for each meal. Gastric contents were sampled at 20, 40, 80,120 and 180 min of digestion for both diets except at 180 min for HM due to the faster gastric emptying. Intestinal digesta were sampled at the same times, from 20 to 180 min. At each time, 1.25 mL of the digestive contents were sampled; they appeared to be visually homogeneous and thus considered as representative.

In order to inhibit proteolysis, 10 μL of a pepstatin solution (0.73 μM, Sigma-Aldrich, St Quentin Fallavier, France) was added per mL of gastric content, while 50 μL of a Pefabloc solution (0.1 M; Sigma-Aldrich, St Quentin Fallavier, France) was added per mL of intestinal digesta. Structural analyses (laser light scattering and confocal microscopy) were performed on fresh digesta mixed with protease inhibitor. The remaining samples were stored at −20 °C until SDS-PAGE, OPA, amino acid and peptide analysis.

2.4. Samples characterization

2.4.1. Structure

2.4.1.1. Confocal microscopy. Microstructure was analyzed in meals, in the *in vivo* digesta samples collected post-mortem from the stomach, proximal and median jejunum and ileum, and in the *in vitro* gastric digesta by confocal laser scanning microscopy (CSLM) using a ZEISS LSM880 inverted confocal microscope (Carl Zeiss AG, Oberkochen, Germany) at X 20 magnification. Each sample (200 μL) was mixed with 12 μL LipidTOX (apolar lipid staining), 3 μL Rhodamine PE (polar lipid staining) and 6 μL of Fast Green (protein staining), kept at room temperature during 10 min before being mixed (1:1) with low melting point agarose at 0.5 %. A volume of 20 μL of this sample was deposited on a glass slice spacer of 25 μL with a cover slip on the top. Three lasers were used to excite the fluorescent dyes (LipidTox: λ_{ex} 488 / λ_{em} 488–530; Fast Green: λ_{ex} 633 / λ_{em} 635–735; Rhodamine PE: λ_{ex} 561 / λ_{em} 565–610). The detection was performed using photomultiplier tube and gallium arsenide phosphide photomultiplier tube detectors. Images were processed using confocal acquisition software ZenBlack and recorded in panorama mode of 2x2 images at a resolution of 1944x1944 pixel each. CSLM analysis was performed for two replicates of digestion of each meal, for *in vivo* and *in vitro* conditions.

2.4.1.2. Particle size distribution. The particle size distribution was measured by laser light scattering (Malvern Mastersizer 2000, Malvern Instrument Ltd., Worcestershire, UK) with two laser sources (633 and 466 nm). The refractive indices were 1.33 for the water (dispersant solution), 1.458 for lipids in HM and 1.462 for lipids in IF. The average of the mode diameter, the volume- (D[4,3]) and surface- D[3,2]) weighted average diameter of the particles were calculated from triplicate particle size measurements performed on *in vivo* samples collected in four out of the nine piglets from each diet group and on *in vitro* samples collected in two out of the three *in vitro* digestion replicates for each meal.

2.4.2. Proteolysis

2.4.2.1. Semi quantification of individual protein hydrolysis (SDS-PAGE) and gastric residual meal. The meals and the *in vivo* and *in vitro* gastric digesta were analyzed by SDS-PAGE under reducing conditions. Samples were first diluted 2-fold with NuPAGE® LDS buffer before being diluted with distilled water at the same fold as that used for the meal to load 5 μg of protein per well. The diluted samples were mixed with 0.5 M DTT and heated at 80 °C for 10 min.

A molecular weight marker ranging from 10 to 250 kDa (Precision Plus Protein™ Kaleidoscope™ Standards, Bio-Rad, California, USA) was used to identify the protein bands. Samples (15 μL) and the molecular weight marker (15 μL) were loaded in the wells of a precast 4–20 %

polyacrylamide gel (Mini-PROTEAN® TGX™ Precast gels, Bio-Rad, California, USA). The gels were placed on the Mini-PROTEAN Tetra Vertical Electrophoresis Cell system (Bio-Rad, California, USA) containing the 10x Tris/glycine/SDS running buffer (Bio-Rad Laboratories). The electrophoresis migration was carried out until total migration at 150 V (≈45 min).

After migration, the gels were immersed in aqueous solution of 40 % (v/v) ethanol and 10 % (v/v) acetic acid for 30 min prior to overnight coloration in Bio-Safe Coomassie staining solution (Bio-Rad Laboratories). The gels were rinsed in ethanol 20 % (v/v) solution until complete discoloration of gel background and scanned using an Image Scanner III LabScan 6.0 (GE Heal hcare Europe GbmH, Velizy-Villacoublay, France).

Bands on the gels were quantified by densitometry using software Image Quant TL™ (GE Healthcare Europe183 GbmH, Velizy-Villacoublay, France) to determine the percentage of residual intact proteins of α-lactalbumin and caseins in HM and IF, IF-β-lactoglobulin and HM-lactoferrin during digestion. Caseins were considered as a single band. Proportions of intact proteins remaining in the gastric digesta were determined following the equation Eq. (2) after considering the meal dilution by secretions using the marker dilution, *i.e.* the cobalt-EDTA concentration (*in vivo*) or the norleucine concentration (*in vitro*) (Eq. (3)):

$$\%residual\ intact\ protein = \frac{Protein\ peak\ optic\ density_{digesta}}{\%meal\ in\ the\ digesta\ x\ Protein\ peak\ optic\ density_{meal}} \times 100 \quad (2)$$

$$\%meal\ in\ the\ digesta = \frac{[Co-EDTA]\ or\ [NorLeu]\ digesta\ in\ g/100mL}{[Co-EDTA]\ or\ [NorLeu]\ meal\ in\ g/100mL} \times 100 \quad (3)$$

The proportion of the ingested meal volume remaining in the stomach at a given time was determined using the following equation (Eq. (4)):

$$\%ingested\ meal\ in\ the\ gastric\ phase_t = \frac{Total\ stomach\ volume_t \times \%meal\ in\ the\ digesta}{Volume\ of\ ingested\ meal_{t_0}} \times 100 \quad (4)$$

with t, the time t and t_0 the time before digestion.

2.4.2.2. Protein hydrolysis degree. The primary amino group quantification in the undigested meals, *in vivo* gastric digesta, *in vitro* gastric and intestinal digesta, and in the pancreatin solution was performed using the OPA (*o*-phtalaldehyde) method (Church et al., 1983; Nielsen et al., 2001). Supernatant of centrifuged samples (10 000 g, 20 min, 4 °C) were firstly diluted in 50 mM sodium tetraborate buffer before being diluted in OPA reagent (SDS 0.5 % [w/v]; 7 mM DTT; 1.9 mM OPA solution in ethanol; 47.15 mM sodium tetraborate; pH 9.5) and then incubated at 37 °C during 10 min in a 96-well plate. The absorbance was measured at 340 nm using MultiskanTM GO Microplate spectrophotometer (Thermo Scientific, Waltham, MA, USA). NH_2 concentration (mg/L) was determined using a calibration curve based on a methionine solution (0 to 2 mM). Analysis were conducted in triplicate for each sample. NH_2 concentration in the digesta was corrected by the meal dilution, as calculated in Eq. (3). Degree of protein hydrolysis (DH) was then determined at each sampling time t according to the following equation (Eq. (5)):

$$DH_t(\%) = \frac{[NH_2]_{\ digesta\ t} - [NH_2]_{\ undigested\ meal\ t}}{[NH_2]_{\ total\ meal} - [NH_2]_{\ undigested\ meal}} \times 100 \quad (5)$$

with $NH_{2\ digesta\_t}$ the primary amino group concentration in the gastric or intestinal digesta sampled at 20, 40, 80, 120 and 180 min, $NH_{2\ undigested\ meal\_t}$ the primary amino group concentration in the undigested meal adjusted at the pH of the corresponding sample (*i.e.*, for HM: pH_{G20} = 6.16, pH_{G40} = 5.65, pH_{G80} = 4.67, pH_{G80} = 4.07 and for IF: pH_{G20} = 6.20, pH_{G40} = 5.60, pH_{G80} = 4.81, pH_{G120} = 4.30, pH_{G180} = 3.85 and at pH 6.6 for intestinal samples for the *in vitro* dynamic digestion of both diets) and $NH_{2\ total\ meal}$ the total content of primary amino group after meal acid hydrolysis (6 N HCl at 110 °C during 24 h). All concentrations were expressed in g/100 g of meal. The intestinal *in vitro* DH was additionally corrected for the NH_2 content in the pancreatin solution.

2.4.2.3. Free amino acid bioaccessibility. Free amino acids (AAs) were determined in the *in vitro* intestinal digesta at 20, 40, 80, 120 and 180 min and in the intestinal secretions (pancreatin + bile). The total AAs including sulfuric AAs (Cys + Met) were quantified in both meals as described previously (Charton et al., 2023). Total tyrosine content was determined after acid hydrolysis containing 0.1 % phenol. AA concentrations were expressed in g/kg of meal. Tryptophan was not determined. The overall free AA bioaccessibility was calculated using the following equation (Eq. (6):

$$Overall\ free\ AA\ bioaccessibility(\%) = \frac{[Free\ AA]_{digesta} - [Free\ AA]_{secretions}}{[Total\ AA]_{meal} - [Free\ AA]_{meal}} \times 100 \quad (6)$$

with $[Free\ AA]_{digesta\_t}$ the free AA concentration in the intestinal digesta at time t (20, 40, 80, 120 and 180 min), $[Free\ AA]_{secretions}$ the free AA concentration in the pancreatin and bile secretions, $[Free\ AA]_{meal}$ and $[Total\ AA\ ]_{meal}$ the free and total AA concentration in the undigested meal, respectively. All concentrations were expressed in g/100 g of meal.

The net free AA bioaccessibility was determined after correction for the free AAs present in HM or IF before digestion.

2.4.2.4. Peptidomic profile by LC/MS-MS. Peptidomic analyses were performed on undigested HM and IF meals, on *in vivo* digesta (gastric, jejunal, and ileal digesta), *in vitro* gastric digesta (at 80 and 120 min) and *in vitro* intestinal digesta (at 20, 40, 80 and 180 min). Gastric digesta obtained *in vivo* and *in vitro* were diluted at 1/200 and 1/100, respectively, and intestinal digesta samples from both digestion models were diluted at 1/500 in the acid buffer (water + 0.1 % TFA+2 % ACN) of the chromatography system before being centrifuged and filtered on 0.45 µm filters (Millex HV, Merck KGaA, Darmstadt, Germany). Filtered samples were injected and identified as previously described (Halabi et al., 2022). Peptides were identified from the MS/MS spectra using the X!Tandem Pipeline software (version 0.4.34) against a HM-protein database (Molinari et al., 2012) and an internal bovine milk protein database to which was added the common Repository of Adventitious Protein (http://thegpm.org/crap). For *in vivo* digesta, the pig proteome "*Sus scrofa*" from UniprotKB (accessed in 2021–02-09) was also used to correctly identify endogenous peptides. Peptide abundance corresponded to the area under the curve of the eluted peak (ion intensity).

Peptidomic analyses were performed as three technical replicates of each *in vitro* digesta (n = 9 analyses by digesta time point), while no technical replicate was performed for *in vivo* digesta with each piglet considered as a replicate (n = 9 analyses).

2.4.2.5. Bioactive peptide identification. Bioactive peptides were identified by comparison against the BIOPEP database (accessed in 2023–03-24; 4668 sequences; (Minkiewicz et al., 2019)) and the Milk Bioactive Peptide Database [accessed in 2023–04-11; 804 sequences; (Nielsen et al., 2017)]. Only exact matches between sequences were considered.

2.5. Statistical analysis

All statistical analyses were performed using the R software, version 4.0.2 (R Core Team, 2020).

A linear model was used to test the statistical significance of the dietary treatment, block and sex on the *in vivo* measurement of particle size distribution, intact residual protein proportion, and DH. When the block or sex effects were not significant ($p > 0.05$), they were removed from the linear model. Normality distribution and homoscedasticity of the residuals were tested using Shapiro-Wilk and Levene's Test, respectively. When one of the previous conditions was not fulfilled, linear models were tested on transformed data (natural logarithmic transformation). If normality and homoscedasticity were still not fulfilled, a non-parametric data was used to test the diet effect (Kruskal-Wallis test). A mixed linear model ('lmer' package) was used to test the impact of dietary treatment and time and their 2-by-2 interactions on *in vitro* measurements of intact residual protein proportion, DH and free AA bioaccessibility. Digestion replicates were considered as a random effect, and other parameters were fixed effects. Normality distribution and homoscedasticity of residuals were tested using Shapiro-Wilk and Levene's Test, respectively and considered as acceptable when $P>0.01$. When differences were significant ($P<0.05$), post hoc tests were performed using a Tukey correction ('lsmeans' package). Unless otherwise stated, results are expressed in mean ± standard error of the mean (SEM).

Regarding peptidomic data analysis, only peptides detected in at least 5 out of 9 piglets or *in vitro* digesta replicates for HM or IF group were kept for statistical analysis. Peptides having the same AA sequence but different post-translational modifications were considered as different. Missing abundances were set to 0. Peptide abundances were summed per protein to obtain an overall protein abundance and the maximum abundance for each protein was set to 1. Hierarchical clustering was then performed, based on the minimum within-cluster variance Ward's agglomeration ('hclust' function; 'stats' package) applied to protein–protein Euclidian distance matrix. The number of clusters was determined with the bar heights at one of the most marked jumps. The heatmap and its dendrogram were displayed using the heatmap.2 function ('ggplot' package). A peptide mapping along the parent protein sequence was built using an in-house program. For each time or site of digestion, the peptide abundances were summed at each AA position over the different peptides covering this position and the different replicates, and then log10-transformed [log10(abundance + 1)]. A visual overview was then provided by a heatmap representation of the cumulative abundances along the parent protein sequence (heatmap.2 function; stats package).

In order to evaluate the proximity of the peptidomic data between *in vivo* and *in vitro* digestion sites and times, a multi-factor analysis (MFA) was conducted on the AA cumulative abundances using the mfa function [factominer package (Lê et al., 2008)] after setting these abundances at a maximum of 1 within each protein. These data were grouped according to the protein origin. Pearson correlation coefficients were determined on these AA cumulative abundances between *in vivo* and *in vitro* digestion sites and times. MFA and Pearson correlation were also conducted on the average abundances of the bioactive peptides common to *in vivo* and *in vitro* digestion models.

3. Results & Discussion

3.1. Structure of the meal before and during digestion

The microstructure of meals differed between undigested HM and IF as observed by CLSM (Fig. 1A and B) and by laser light scattering (Fig. 1C). The modal diameter was statistically significantly higher in HM (4.7–5.1 μm) than in IF (0.2–0.6 μm) (Fig. 1C), due to the presence of native milk fat globules in HM *vs*. submicronic droplets in IF, resulting from the lipid homogenization step during IF manufacturing (Bourlieu et al., 2015; Michalski et al., 2005).

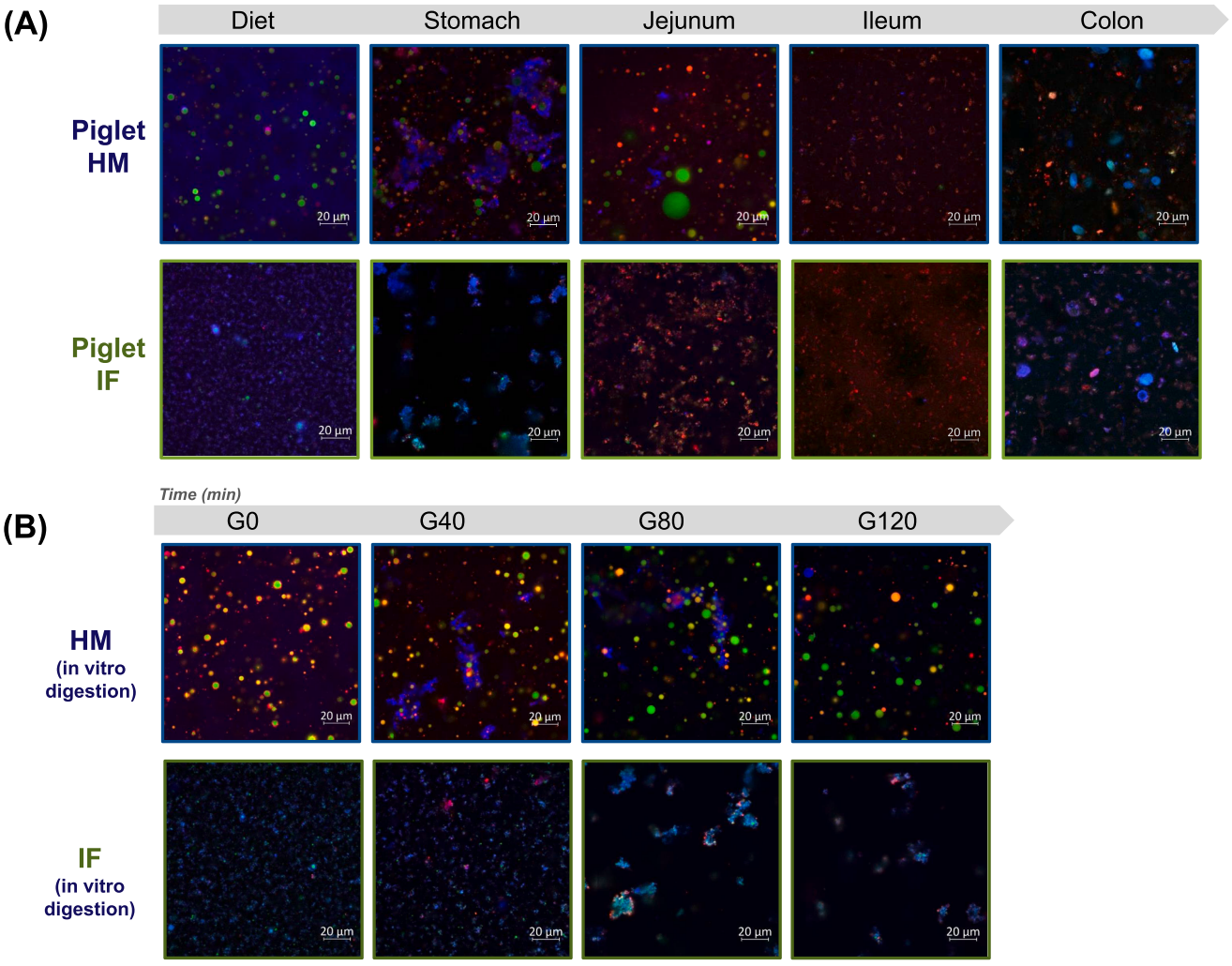
Different types of aggregates were observed between HM and IF in the gastric phase, with a larger particle size (Fig. 1C) and a potentially more open structure (Fig. 1A and B) for HM aggregates than for IF ones, the latter appearing smaller (Fig. 1C) and denser. This was observed both *in vivo* and *in vitro*, with a significantly higher particle size for HM than for IF *in vivo* and *in vitro* at G40 and similar CLSM images between *in vivo* and *in vitro* since G40 for HM and since G80 for IF. The different aggregates between HM and IF can be partially attributed to the different heat treatment received by HM (raw) and IF (highly heat treated), inducing in a different pattern of casein coagulation, as previously reported (Ahlborn et al., 2023; de Oliveira et al., 2016; Halabi et al., 2022; Nebbia et al., 2020). For the raw HM, the pepsinolysis of κ-caseins at the surface of the native micelles must have induced the aggregation of *para*-casein micelles (Halabi et al., 2022), while for IF, the caseins, coated with the denatured whey proteins (58 %, Yu et al. 2021), hindering the action of pepsin on κ-caseins, must have coagulated due to the acidic condition (Halabi et al., 2022). This was confirmed by acidification (pH=4.8) of the meal without enzyme, which induced aggregation in IF but not in HM (data not shown). The different pattern of protein coagulation observed for HM and IF can also be due to the different structure and mineralization pattern between human and bovine casein micelles (He et al., 2022; Sood et al., 1997; Yang et al., 2024), as well as to the different fat droplet size between HM and IF.

During the *in vitro* gastric digestion, the difference of particle size between HM and IF disappeared after 80 min of gastric digestion, likely due to the combined action of acid coagulation and pepsinolysis (Fig. 1C), however the particle nature largely differed (Fig. 1B). The modal diameter of the particles observed during *in vivo* and *in vitro* gastric digestions differed somewhat, with values being higher *in vivo* than *in vitro*. This could be partly due to the different mechanical constraints, being gentler *in vivo* than *in vitro*, or due to a faster acidification *in vivo*. Nevertheless, overall, both *in vivo* and *in vitro* digestion led to similar conclusions regarding the comparative disintegration of IF *vs*. HM during their gastric digestion, although the pH at which it occurred differed.

During *in vivo* digestion, the microstructural differences between HM and IF, as observed by CLSM (Fig. 1A), were maintained until the jejunum, particularly with the persistence of native HM milk fat globules. In the ileum and colon, there was no more structural differences between HM and IF (Fig. 1A). In the ileum, a low signal was observed for proteins and lipids likely due to their digestion and absorption, whereas in the colon, proteins re-appeared probably due to the metabolic activity of the gut microbiota.

3.2. Gastric meal dilution and emptying

The meal dilution by the gastric secretions could be compared *in vitro* and *in vivo* solely at 30 min of digestion due to the present experimental design. This was determined thanks to the indigestible marker dilution (Eq. (3)) and was found to be in a similar range for HM and IF and between *in vitro and in vivo* at this time point (Fig. 2A), with about 18 % of secretions in the total gastric volume. This value is consistent with previous observations in the preterm infant, where values of 10–20 % of



(C)

HM
IF

		In vivo		*In vitro*			
		Diets	**Stomach**	**Diets**	**G40**	**G80**	**G120**
			pH = 4.83 ± 0.15 pH = 4.74 ± 0.19	pH = 6.74 pH = 6.84	pH = 5.61 pH = 5.70	pH = 4.78 pH = 4.85	pH = 4.20 pH = 4.27
Mode (µm)	HM	4.74 ± 0.21	95.40 ± 15.01	5.14 ± 0.08	25.18 ± 9.38	23.34 ± 7.69	21.95 ± 7.34
	IF	0.58 ± 0.005	45.50 ± 0.93	0.16 ± 0.002	5.87 ± 0.76	22.21 ± 3.57	24.71 ± 3.67
	p-value	<0.001	0.009	<0.001	0.007	0.760	0.457
D [3, 2] - Surface weighted mean (µm)	HM	1.84 ± 0.97	26.07 ± 3.87	2.84 ± 2.59	9.36 ± 2.17	7.30 ± 0.56	8.39 ± 2.26
	IF	0.27 ± 0.001	29.70 ± 0.72	0.24 ± 0.02	0.51 ± 0.15	19.15 ± 2.59	21.21 ± 2.84
	p-value	0.181	0.384	0.070	0.001	<0.001	0.001
D [4, 3] - Volume weighted mean (µm)	HM	5.07 ± 0.14	134.47 ± 21.62	5.61 ± 0.18	22.47 ± 6.96	20.91 ± 3.62	20.21 ± 3.88
	IF	6.41 ± 1.33	62.06 ± 4.59	4.44 ± 2.46	5.60 ± 0.24	23.46 ± 3.95	26.71 ± 4.28
	p-value	0.233	0.004	0.309	0.004	0.309	0.051

Fig. 1. Structural evolution of Human milk (HM, blue) and Infant formula (IF, green) during *in vivo* and *in vitro* digestion. Confocal laser scanning microscopy images of HM and IF digesta (A) through the gastrointestinal tract of piglet and (B) during *in vitro* dynamic gastric digestion. Proteins are colored in blue (Fast Green), polar lipids are colored in red (Rhodamine) and apolar lipids are colored in green (LipidTOX). Scale bar: 20 µm. (C) Particle size distribution and characteristics (mode, diameters) of the diets and of their gastric *in vivo* (30 min of digestion) and *in vitro* digesta at 40, 80 and 120 min of *in vitro* digestion. *In vivo* data represents means of two independent diets (n = 2 piglets) and gastric samples (n piglets = 2), with each measurement performed in duplicate. *In vitro* data represents means of two independent digestions (n = 2), with each measurement performed in triplicate. All particle size data (*in vivo* and *in vitro*) represents means ± standard error of the mean.

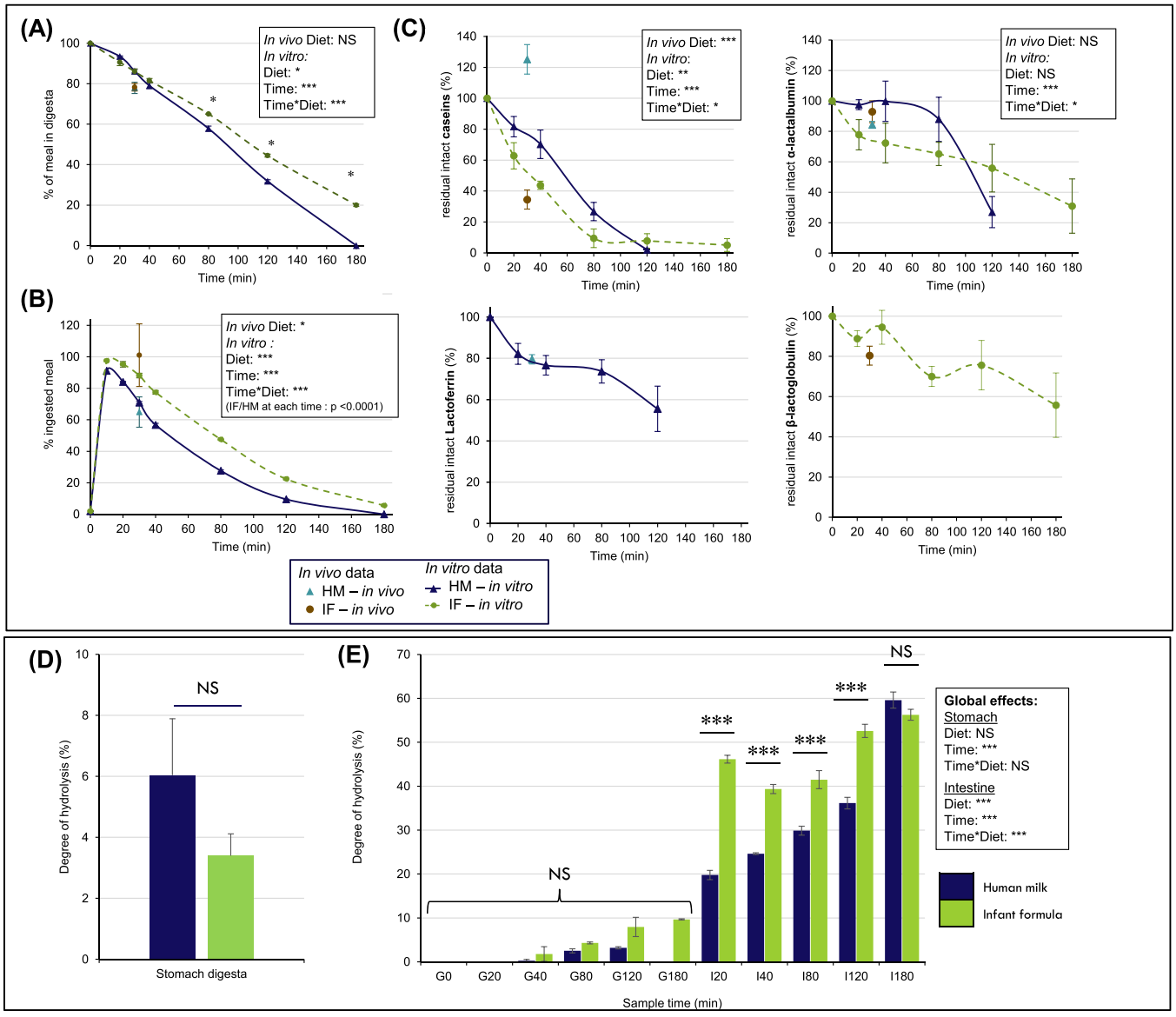


Fig. 2. (A) Proportion of meal in the gastric digesta, after gastric secretion dilution, as determined *in vitro* or *in vivo*, (B) proportion of the ingested meal remaining in the gastric phase as determined *in vitro* or *in vivo* and (C) residual proportions of intact caseins, α-lactalbumin (α-LA), lactoferrin (LF) and β-lactoglobulin (β-LG) in the gastric phase as observed by SDS-PAGE followed by densitometry and as corrected for the meal dilution, (D) degree of protein hydrolysis in the stomach of HM- and IF-fed piglets and (E) degree of protein hydrolysis during the *in vitro* digestion of HM and IF. In panels A, B, C and D, *in vivo* data represents means ± standard error (n_{HM}=8, n_{IF}=8). In panels A, B, C and E, *in vitro* data represents means ± standard error of the mean (n_{HM}=3, n_{IF}=3). Statistically significant factors were referenced with P<0.001 (***), P<0.01 (**), P<0.05 (*) and P>0.05 (NS, non-significant). *HM: Human milk; IF: Infant formula: G: Gastric; I: Intestinal. Statistics on in vitro data did not take into account the value at 0* min *nor at 10* min *that are theoretical values (% ingested meal).*

secretions were observed during the 90 min following meal ingestion (De Oliveira et al., 2016). *In vitro*, the dilution level increased linearly with postprandial time, in a greater manner for HM than for IF, due to a faster gastric emptying for HM (Fig. 2B) and a constant fluid secretion over time. Whether this high dilution level reflects the *in vivo* situation remains to be investigated. The proportion of ingested meal remaining in the piglet stomach 30 min postprandially (Fig. 2B) was significantly higher for IF (100 %) than for HM (65 %), as determined based on the marker dilution and the total gastric volume [Co-EDTA, Eq. (4)]. Whether the piglet frequent feeding had an impact on the gastric emptying kinetics remains unknown. Nevertheless, this result suggests a slower emptying of IF *vs* HM, such as parametered in the present *in vitro* system based on the literature review of Bourlieu et al. (2014) and as recently reported (Camps et al., 2021). This different emptying between HM and IF could be partly linked to the different coagulate formed in the stomach, as discussed in section 3.1. In the *in vitro* digestion system, a proportion of 88 % and 71 % of the ingested IF and HM, respectively, was remaining in the gastric compartment at 30 min, which was in the same range as *in vivo*, although the difference between meals was lower.

3.3. Residual intact proteins in the gastric phase

The *in vivo* and *in vitro* gastric proteolysis of lactoferrin, caseins, α-lactalbumin and β-lactoglobulin, as observed by SDS-PAGE is presented in Fig. 2C and in the Fig. S1. Regarding proteins common between HM and IF (caseins and α-lactalbumin), a similar impact of the diet was observed *in vivo* and *in vitro* with similar proportions of residual intact proteins *in vivo* and *in vitro* at 30–40 min of digestion, except for HM caseins being more concentrated *in vivo* than *in vitro*. Regarding α-lactalbumin, the rate of proteolysis did not differ between diets and

was low, likely due to its globular conformation stabilized by four intramolecular disulphide bridges, but destabilized by the pH decrease. Regarding caseins, a higher level of residual intact proteins was observed for HM than for IF in the piglet stomach and *in vitro* along the gastric digestion. This can be due to a lower level of proteolysis for HM caseins than for IF caseins and/or to a more heterogeneous emptying of HM proteins, with a slower emptying for HM caseins, such as suggested by the high proportion (>100 %) of intact caseins in the HM piglet stomach. This can be potentially linked to the different aggregates formed between HM and IF, as discussed in section 3.1.

Regarding the digestion of proteins specific to each matrix, i.e. HM-lactoferrin and IF-β-lactoglobulin, a good agreement between *in vitro* and *in vivo* values was observed at 30–40 min of gastric digestion. However, it should be noted that *in vivo* digesta were collected at a single time (30 min after the last meal). The final proteolysis level of these specific proteins was lower than that observed *in vitro* for caseins due to their pepsinolysis resistance, as previously reported (Abrahamse et al., 2022; Bouzerzour et al., 2012; de Oliveira et al., 2016; Halabi et al., 2022; Nebbia et al., 2020), and mainly due to their globular structure (Dalgalarrondo et al., 1995; Nebbia et al., 2020).

Overall, the *in vitro* gastric hydrolysis of individual proteins followed the same trend as that occurring *in vivo* at the same digestion time

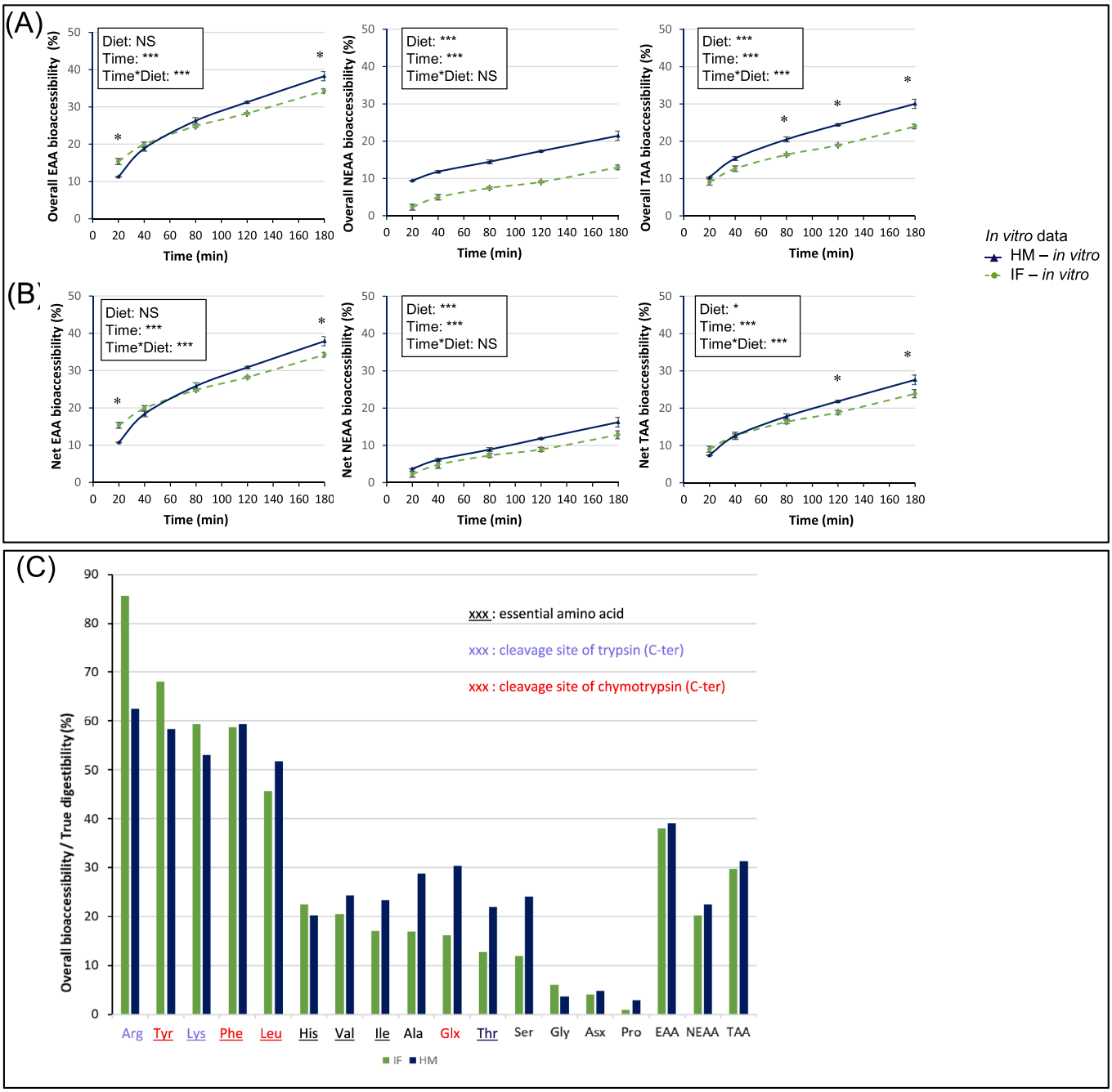


Fig. 3. Overall (A) and net (B) bioaccessibility of essential amino acids (EAA), of non-essential amino acids (NEAA) and of total amino acids (TAA). (C) Ratio (%) between the overall AA bioaccessibility and the corresponding true ileal digestibility as published in Charton et al. (2023) (%).The in vitro digesta at 180 min cumulates the amino acid measured in the last intestinal digesta samples and in the cumulated emptied fraction of the intestinal compartment. Statistically significant factors were referenced with P<0.001 (***), P<0.05 (*) and P>0.05 (NS, non-significant). HM: Human milk; IF: Infant formula: G: Gastric; I: Intestinal. Time in min; Glx: Glutamate + Glutamine; Asx: Asparagine + Aspartate.

(30–40 min), although greater differences occurred for caseins *in vivo*.

3.4. Degree of protein hydrolysis

The degree of protein hydrolysis is shown in Fig. 1D. There was no significant difference in the degree of protein hydrolysis in the gastric phase between HM and IF, both *in vitro* and *in vivo*, with values remaining below 10 %, such as previously reported (Abrahamse et al., 2022; Halabi et al., 2022; Huang et al., 2022; Le Roux et al., 2020). The limited gastric protein hydrolysis can be explained by the lower pepsin activity [18 % of the adult level (Henderson et al., 2001)] and the lower level of acidification than that observed in adults (Mason, 1962). In addition, the rate of gastric acidification being slower *in vitro* than *in vivo* (4.8 at 30 min *in vivo vs*. 5.7 at 40 min *in vitro*, Fig. 1C) could partly explain the lower values for DH observed *in vitro* than *in vivo* at the same digestion time (30–40 min). The *in vitro* DH at the intestinal level was significantly lower for HM than for IF up to 120 min of digestion ($P<0.001$) with no more significant difference between diets at 180 min of digestion (Fig. 2E), which agrees with that observed by Abrahamse et al. (2022). The lower hydrolysis rate determined during *in vitro* intestinal digestion of HM could be due to the presence of antiproteases in HM such as α1-anti-trypsin and anti-chymotrypsin, as described by Chowanadisai and Lönnerdal (2002), thus allowing a reduced intestinal proteolysis of bioactive proteins, which can subsequently exert their biological properties (Lönnerdal, 2003, 2016). The lower hydrolysis rate for HM could also be linked to the different kinetics of gastric emptying, resulting in a faster HM delivery in the intestine, combined with a limited level of proteases, probably not present in excess and thus having a limited capacity of proteolysis. This, however, should be explored further.

3.5. Bioaccessibility vs. Digestibility of amino acids

The overall intestinal AA bioaccessibility was followed over time in the *in vitro* model, as presented in Fig. 3.A, and was based on the free AA analysis of the digesta. The overall intestinal AA bioaccessibility was significantly higher for HM than for IF throughout digestion for the non-essential AAs and mostly at the end of intestinal digestion for total and essential AAs. However, when considering the net bioaccessibility (*i.e.* after correction for the free AAs already present before digestion) the magnitude of the difference was very small (Fig. 3B). This was due to the presence of free glutamic acid and to a lesser extent of glutamine in HM. While the present overall AA bioaccessibility values were similar to those reported by Abrahamse et al. (2022), this was not true for the net bioaccesssibility values. The present bioaccessibility values are not in line with the degree of protein hydrolysis of HM *vs*. IF (Fig. 2D) for which an opposite relationship was found, indicating that intestinal digesta of IF contained smaller peptides than HM, such as observed by mass spectrometry (Fig S2B and C) during the first 80 min of intestinal digestion.

The ratio between the present overall bioaccessibility and the true ileal digestibility of each AA, as measured in the same experiment and published previously (Charton et al., 2023), was determined and is presented in Fig. 3C. On average, the overall bioaccessibility of the total AAs explained 30 % of their true ileal digestibility (Fig. 3C, TAA). This low contribution of the *in vitro* to the *in vivo* value is due to (1) the fact that only free AAs and not di- or tri-peptides were considered for bioaccessibility and (2) the absence of brush border peptidases in our *in vitro* model (Picariello et al., 2015). Such a ratio hides large discrepancies among individual AAs. The highest ratio between bioaccessibility and digestibility (>50 %) was observed for Arg, Tyr, Lys, Phe and Leu, which are the preferential cleavage sites for trypsin (Arg, Lys) (Keil, 1992) and chymotrypsin (Tyr, Phe, Leu) (Appel, 1986), when located on the C-ter position of the peptidic chain and then further released as free AA by carboxypeptidases. These values were higher for IF than for HM for Arg, Tyr and Lys, while the opposite was true for Leu, Ile, Ala, Glx, Thr and Ser. These differences between meals is likely due to the different AA sequences between HM and IF proteins. Regarding Glx (Glutamic acid + Glutamine), the difference observed was solely due to the presence of free Glx, at 80 % under the form of glutamic acid in HM.

The differences between HM and IF for overall bioaccessibility or true ileal digestibility of each AA were compared over all the AAs. The extent of the HM *vs.* IF differences between *in vitro* and *in vivo* was not significantly correlated, although close to the statistically significant level (Pearson correlation coefficient: −0.51, p: 0.054, n = 15). However, the correlation coefficient was negative, indicating an opposite relationship between *in vitro* and *in vivo* values. Thus, the measurement of free AA bioaccessibility is not a predictive parameter of true ileal AA digestibility. A further step of fractionation of the digesta into absorbable and non– absorbable fractions through physical (e.g. filtration) or chemical (e.g. acidic or methanol precipitation), followed by analysis of the total AAs after acidic hydrolysis of the absorbable fraction should improve the relationship.

3.6. Peptides

3.6.1. Peptide identification

The number of different peptides identified *in vivo* and specific to the stomach was higher (62 % of all the *in vivo* HM-peptides, n = 1064; 56 % of all the *in vivo* IF-peptides, n = 1065) than those specific to the proximal jejunum (11 % for HM and 21 % for IF), with, in addition, 25 % and 19 % for HM and IF, respectively, of peptides common between these two digestion sites (data not shown). A high proportion of peptides specific to the gastric phase was also found *in vitro*, with 66 % or 75 % of all the *in vitro* HM-deriving peptides (n = 1798) or IF-deriving peptides (n = 1976), respectively, while only 15 % or 14 % of HM or IF-peptides, respectively, were solely present in the intestinal phase, in addition to 19 % and 11 % of HM and IF-peptides common between these two compartments (data not shown). Among all the gastric peptides, 46 and 28 % of the HM- and IF-peptides were common between the *in vivo* chyme (30 min of digestion) and the *in vitro* chyme at 80 or 120 min of digestion (Fig S2A). Only 8 to 10 % of HM and IF gastric peptides were specific to the *in vivo* digestion. In the intestinal phase, only 11 % of all the intestinal peptides were common between the proximal jejunum (*in vivo)* and the *in vitro* intestinal phase (all sampling times combined) for HM and IF, respectively. A low number of peptides was identified in the median jejunum and in the ileum in the piglet (n = 12 to 41, Fig. S2B), with a very low number of common peptides between *in vitro* and *in vivo* digestion (Fig S2A). Overall, the distribution of the peptides being either specific or common to the different digestion times or sites were similar *in vitro* and *in vivo*. The proportion of peptides common between *in vitro* and *in vivo* was much lower in the intestinal phase than in the gastric phase, likely due to the lack of the brush border peptidases in the *in vitro* model. The latter can also explain the much lower diminution of the peptide number in the *in vitro* intestinal phase than along the piglet intestine (Fig S2B and C.). Regarding the median molecular weights of the peptides (Fig. S2B & C), the *in vivo* and *in vitro* values were in the same range in the gastric and intestinal phases. Along the intestinal tract, the *in vivo* peptides had a slightly higher molecular weight for HM than for IF, such as observed *in vitro* during the early intestinal digestion times but to a lesser extent.

A high proportion of the peptides identified during *in vivo* digestion of HM originated from caseins (84 %) and to a minor extent from whey proteins, with lactoferrin being the most contributing whey protein (9 % of the peptides, Fig. 4A). In a similar manner for IF, a large proportion of the *in vivo* peptides was from caseins (72 %) and from β-lactoglobulin (17 %). *In vitro*, a similar parent protein distribution was observed with 66 % and 78 % of the peptides arising from caseins for HM and IF, respectively. However, a greater number of minor parent proteins was observed, particularly for HM (Fig. 4B). The low number of peptides originating from whey proteins except for β-lactoglobulin can be attributed to the presence of disulfide bonds within these peptides, such

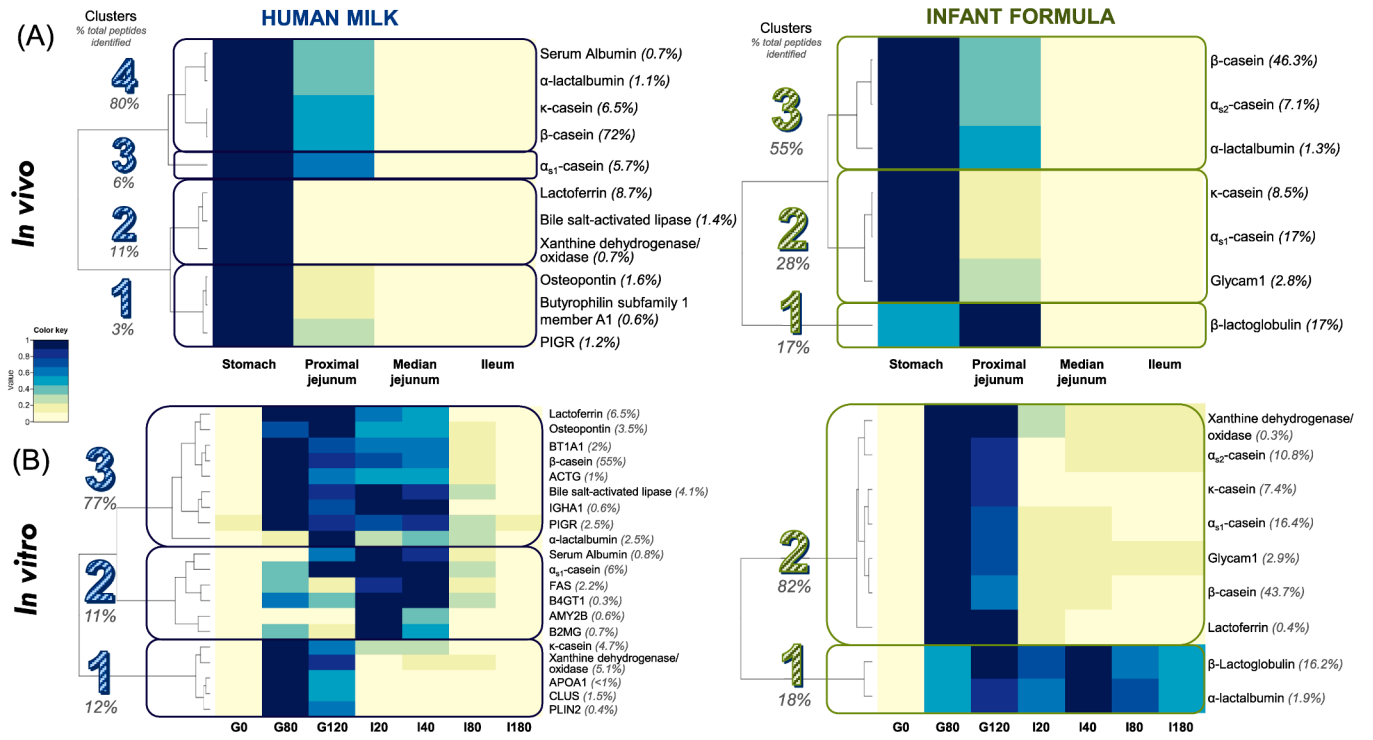


Fig. 4. Heatmap of the peptide abundances summed by parent protein for human milk (Blue) and infant formula (Green) along the piglet digestive tract (A) and across digestion time in gastric and intestinal phases (B). Clusters were identified by hierarchical classification and are surrounded. Abundance was gradually colored from low abundance (light yellow) to high abundance (dark blue). G0: undigested diet, G80: 80 min of gastric phase, G120: 120 min of gastric phase, I20: 20 min of intestinal phase, I40: 40 min of intestinal phase, I80: 80 min of intestinal phase, I180: 180 min of intestinal phase.

as for α-lactalbumin, which prevents their detection by LC-MS/MS, but could also be caused by their hydrolysis resistance particularly in the gastric phase (Fig. 2C). Overall, the peptide identifications were in line with previous data reported for HM (Deglaire et al., 2016; Wada et al., 2017) and IF (Halabi et al., 2022; Hodgkinson et al., 2019; Wada et al., 2017).

3.6.2. *Peptide clustering*

The clustering analysis, performed after summing the peptide abundances by protein nature, grouped the parent proteins according to the kinetics of peptide release (Fig. 4). The latter differed between HM and IF due to their different protein origins. *In vivo*, while the maximal abundances were reached in the stomach for all proteins in HM, this was not true in IF for β-lactoglobulin, accounting for 17 % of the peptides. *In vitro*, greater differences between HM and IF were observed, as higher abundances were observed in the early intestinal digestion times (I20, I40) for peptides from β-casein and α_{s1}-casein from HM than those from the same proteins in IF.

When compared within each food, the parent protein clusters differed *in vitro* and *in vivo,* likely due to the different digestion times and/or due to the lack of brush border enzymes in the *in vitro* model. Nevertheless, some similarities can be drawn between *in vivo* and *in vitro* digestion. For most proteins, the maximal peptide abundance was reached in the stomach or at 80 min of *in vitro* gastric digestion. In the intestinal phase, the proximal jejunum was closer to that observed at 40 min of *in vitro* intestinal digestion, except for HM-lactoferrin being closer to that observed at 80 min of *in vitro* digestion. The median jejunum and ileum were closer to the observations made at 180 min of *in vitro* intestinal digestion.

3.6.3. *Peptide mapping along the protein sequence*

The peptide abundances summed at each AA position along the parent protein sequence is given for *in vivo* and *in vitro* digestion in Fig. 5A and B. Before digestion, HM-peptides from β-casein were identified while no or very few peptides deriving from κ-casein and lactoferrin were identified (Fig. 5A), such as previously demonstrated (Dallas et al., 2013; Deglaire et al., 2016; Ferranti et al., 2004). IF-peptides deriving from β-casein, κ-casein and β-lactoglobulin were also identified before digestion but in lower abundance than in HM (Fig. 5B). The greater abundances for β-casein-peptides in HM than in IF may be due to the action of endogenous milk proteases (Deglaire et al., 2016; Nielsen et al., 2017). In bovine milk, endogenous proteases are also present particularly in milk somatic cells that were removed by the milk microfiltration used for the present IF production (Wang et al., 2019; Yu et al., 2021).

When comparing the peptide mapping of β-casein between HM and IF, a greater abundance over a longer sequence part was observed in HM than in IF on the C-terminal side of the protein, both *in vitro* and *in vivo*. Regarding K-casein, an opposite sequence coverage was observed between HM and IF, with the central area of the HM-K-casein sequence being covered by peptides, unlike that in IF, where peptides covered almost all the sequence except in the central area. This different peptide mapping is likely due to their different AA sequences between HM and IF (based on bovine milk), with only 55 and 58 % of sequence homology for K-casein and β-casein between human and bovine origin (The UniProt Consortium, 2023).

The peptide mapping onto the β-casein sequence was visually very close between *in vivo* and *in vitro* digestions, especially when comparing the *in vivo* stomach data *vs.* the first *in vitro* gastric samples (80 and 120 min of digestion) and the proximal jejunum data *vs.* the early intestinal phase of digestion (Fig. 5A and B). More precisely, peptides released from β-casein covered almost all of the sequence *in vivo*, in the stomach and the proximal jejunum, and *in vitro* for both gastric and intestinal phases. These data corroborated previous observations (Halabi et al., 2022; Hodgkinson et al., 2019; Wada et al., 2017; Giribaldi et al., 2022). The same peptide mapping was also observed *in vitro* and *in vivo* for K-casein from HM and IF (Fig. 4A and B), with no peptide in the C-terminal region (105–160) of HM-κ-casein and no peptide only in the region

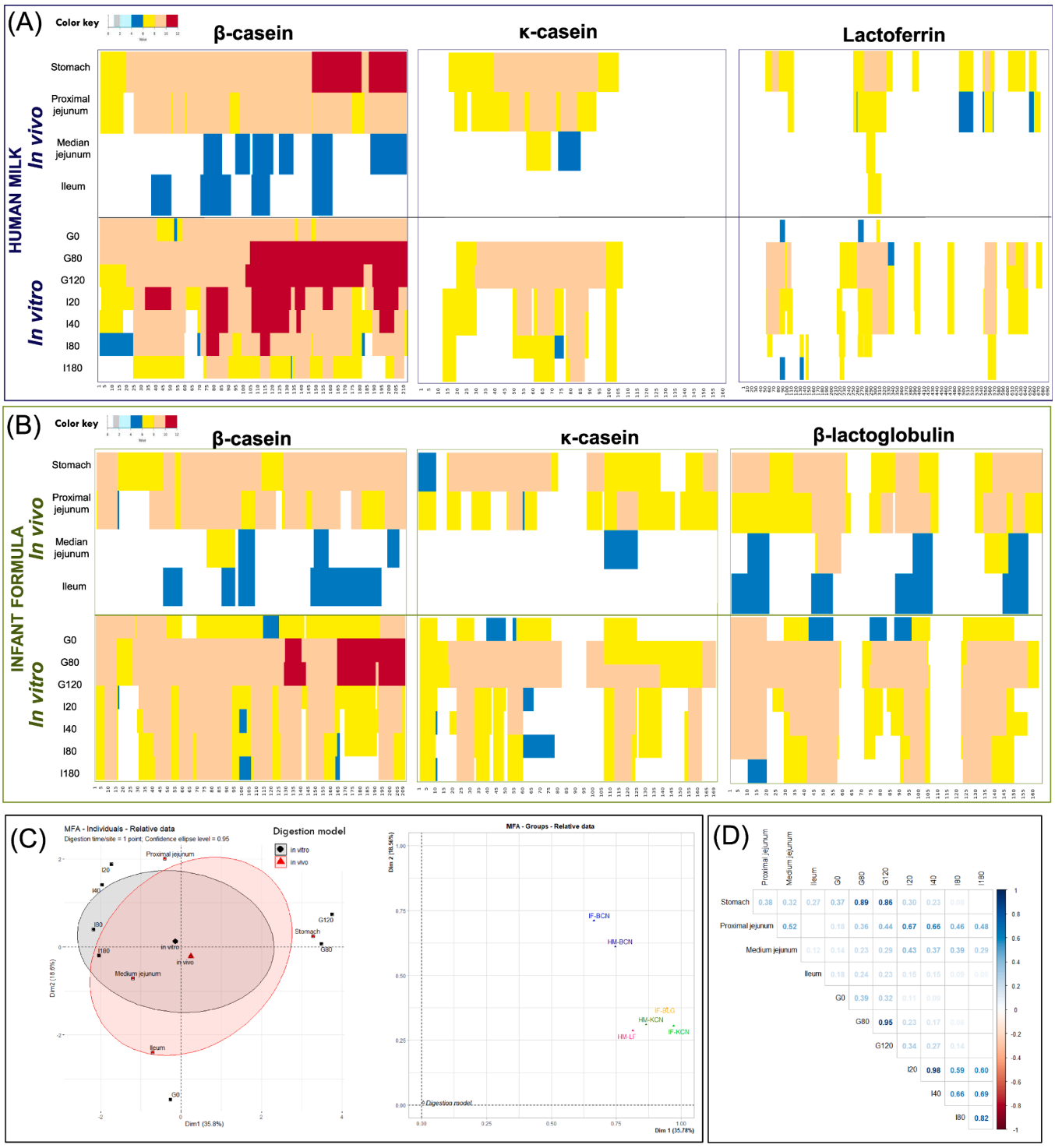


Fig. 5. Peptide color mapping along the sequence of the major parent proteins of (A) Human milk and (B) Infant formula over the piglet digestive tract after 30 min of digestion and during the *in vitro* dynamic digestion. Multi-factorial analysis (C) and Pearson correlation coefficient matrix (D) of the relative peptide mapping data. (A and B) The x-axis represents the amino acid sequence of parent protein. The y-axis represents the digestive tract compartment for *in vivo* digestion and the digestion time during *in vitro* digestion. Abundance of amino acid identification within the protein sequence was gradually colored from white for low abundance to red for high abundance. G0: undigested diet, G80: 80 min of gastric phase, G120: 120 min of gastric phase, I20: 20 min of intestinal phase, I40: 40 min of intestinal phase, I80: 80 min of intestinal phase, I180: 180 min of intestinal phase.

82–78 of IF-κ-casein, such as reported previously (Giribaldi et al., 2022; Hodgkinson et al., 2019). Regarding lactoferrin and β-lactoglobulin, the same peptide mapping was observed *in vivo* and *in vitro* highlighting that three regions were mainly covered by the identified peptides for HM-lactoferrin (50–100, 260–360 and 550–570), such as reported previously (Deglaire et al., 2019) while almost the entire IF-β-lactoglobulin sequence was covered except in two regions (60–72, 106–124) that contain bridged cysteins at the positions 66–160, 106–119 and 106–121.

To evaluate the proximity of the peptidomic data between *in vivo* and *in vitro* digestion sites and times, a multi-dimensional comparison of the relative data from the peptide mapping (*i.e.* after setting the amino acid maximal value to 1 within each of the six proteins) was conducted across

the different digestion sites and times (*in vivo* and *in vitro*) and is presented in Fig. 5C. The first two dimensions of the map represented the data well as it gathered a high level of variance (55 %). The relative peptide mapping data from the stomach and from the *in vitro* gastric digestion at 80 and 120 min were close in the individual map, such as supported by the very high Pearson correlation coefficient ($r = 0.88$, $p < 0.0001$, $n = 1604$ AAs; Fig. 5D). They were highly correlated to the first dimension, mainly explained by peptide mapping data of κ-casein, lactoferrin, β-lactoglobulin and partly from β-casein. On the second dimension, the proximal jejunum and the *in vitro* intestinal digestion at 20 and 40 min were close, such as supported by the high Pearson correlation coefficient ($r = 0.67$, $p < 0.0001$, $n = 1604$ AAs; Fig. 5D), and

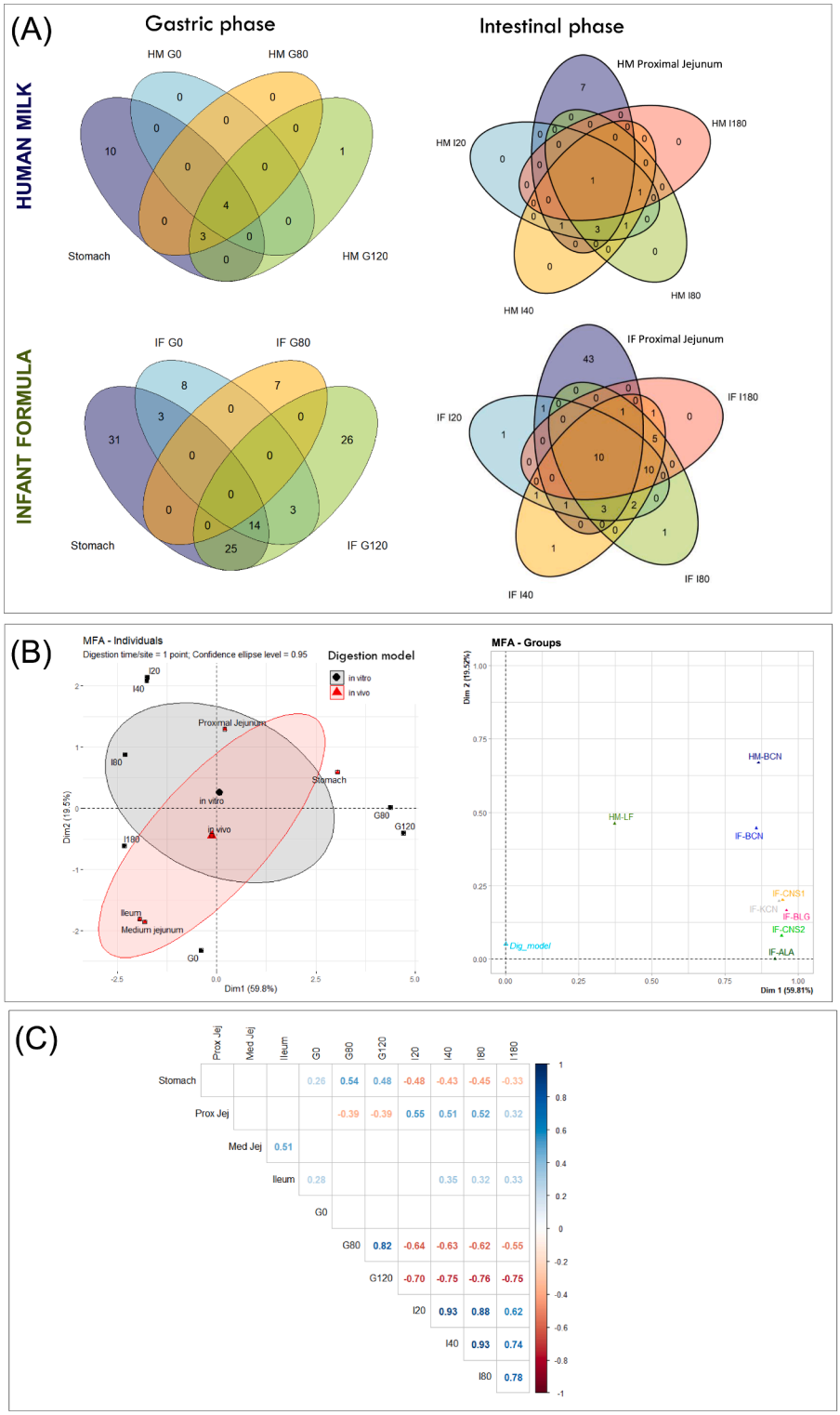


Fig. 6. (A) Venn diagram of the number of bioactive peptides identified in the gastric and intestinal phases for human milk (HM) and infant formula (IF) during *in vivo* and *in vitro* digestions. Multi-factorial analysis (B) and Pearson correlation coefficient matrix (C) performed on the bioactive peptide abundances common between *in vitro* and *in vivo* digestion ($n = 61$).

were mainly associated to data from β-casein. When considering the peptide mapping data from all the digestion sites and times, the *in vitro* and *in vivo* data were not statistically significantly different over the first two dimensions, such as represented by the confidence ellipses presented in Fig. 5C. The present results are in good agreement with previous Spearman correlation coefficients reported for peptidomic data obtained from adult *in vitro* digestion (static or dynamic) and pig digestion of bovine milk proteins [r = 0.7–0.8 for the gastric phase

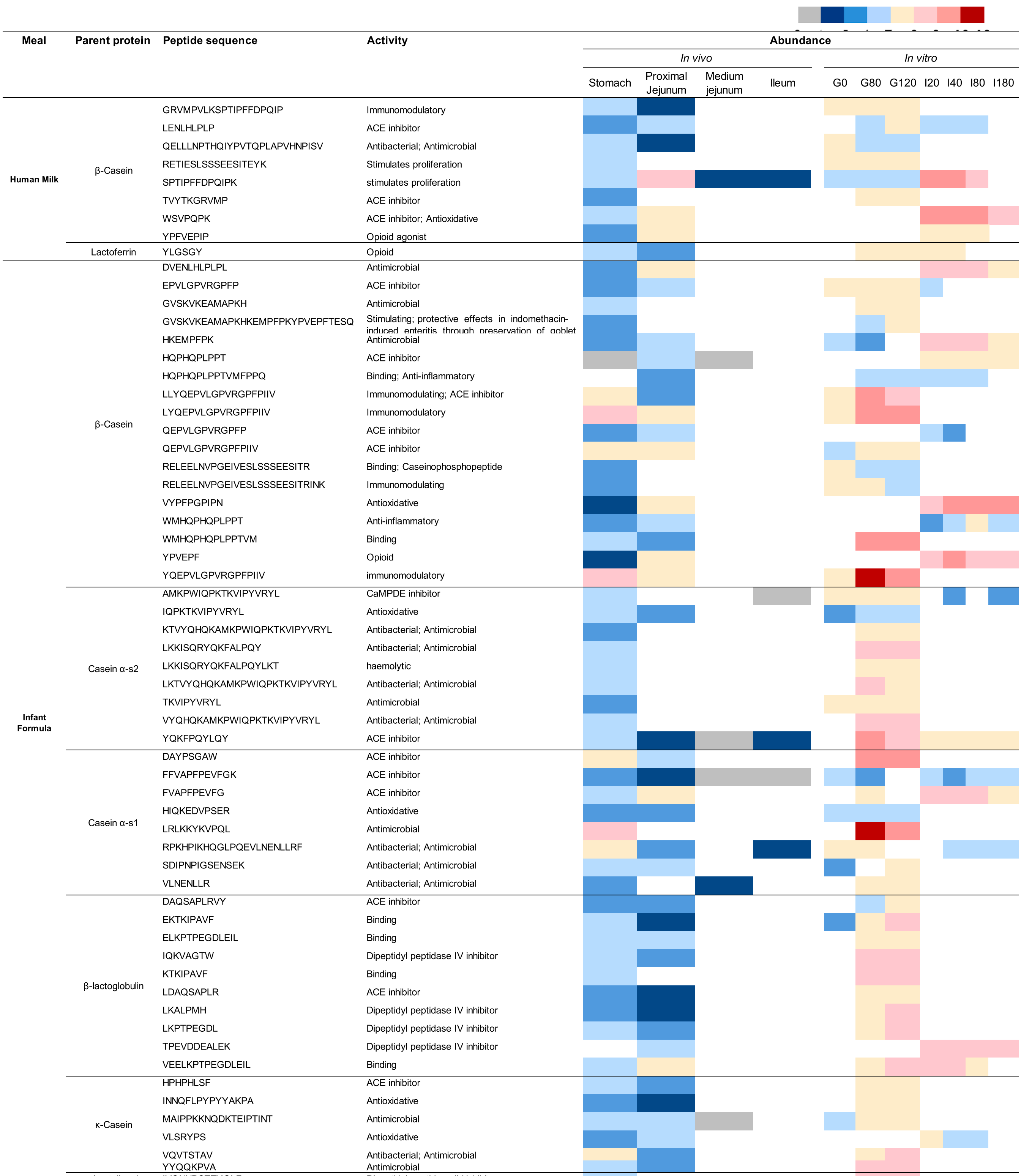


Fig. 7. Bioactive peptide abundances that are common between *in vivo* and *in vitro dynamic* digestion of human milk or infant formula. Color is function of $\log_{10}$ transformed abundance value.

(Egger et al., 2019; Miralles et al., 2021) and r = 0.5 for the intestinal phase, Egger et al., (2019)].

3.6.4. Bioactive peptides

The number of bioactive peptides, determined using BIOPEP and MBPDB databases, was higher for IF than for HM digesta (127 *vs.* 19). During HM digestion, a low proportion of all the peptides was identified as bioactive (<2%), corresponding to 28 or 44 bioactive peptides during *in vivo* or *in vitro* digestion, respectively. The majority of these bioactive peptides originated from β-casein, as previously observed (Deglaire et al., 2019; Wada & Lönnerdal, 2015). During IF digestion, a higher proportion of peptides was identified as bioactive (7–10 %), corresponding to 129 or 304 bioactive peptides during *in vivo* or *in vitro* digestion, respectively. These bioactive peptides mostly originated from caseins (α_{s1}-, α_{s2}-, κ- and β-caseins, representing 75–80 % of bioactive peptides identified) and β-lactoglobulin (19–25 % of bioactive peptides identified). The low diversity of parent proteins of bioactive peptides is partly related to the low number of such peptides yet identified in the bioactive peptide databases. The majority of bioactive peptides identified during HM and IF *in vivo* and *in vitro* digestion had antimicrobial, antibacterial, antioxidant and angiotensin converting enzyme (ACE) inhibitor properties, or proliferation stimulation (Fig. 7), as observed previously (Halabi et al., 2022; Hodgkinson et al., 2019).

As shown in Fig. 6A, the proportion of the *in vivo* bioactive peptides also found *in vitro* amounted to 41 % for the gastric digestion of HM (7 out of 17 bioactive peptides identified *in vivo*) and 49 % for the gastric digestion of IF (43 out of 73 bioactive peptides identified *in vivo*). Bioactive peptides detected in the gastric phase may not be fully physiologically relevant if not persistent in the intestinal phase. In the latter phase, 50 % of the *in vivo* HM-derived bioactive peptides identified in the proximal jejunum were also identified *in vitro* (6/12 bioactive peptides identified *in vivo,* Fig. 6A), while the sole HM bioactive peptide still present in the lower part of the piglet intestine was also identified *in vitro* until 80 min of intestinal digestion (Fig. 7). Regarding IF intestinal digestion, despite the high number of bioactive peptides identified in the proximal jejunum of IF-fed piglets (59), only 23 % of them were also identified *in vitro* (Fig. 6A). A lower number of bioactive peptides was identified in the lower intestine (8), but 63 % of them were also found *in vitro*. When comparing the abundances of the bioactive peptides common to *in vivo* and *in vitro* digestion (n = 61, Fig. 6B, C and Fig. 7), similarities were highlighted through the multifactorial analysis (Fig. 6B), particularly for the bioactive peptides identified in the stomach *vs.* 80 and 120 min of the *in vitro* gastric digestion and for the bioactive peptides identified in the proximal jejunum *vs.* 20, 40 and 80 min of *in vitro* intestinal digestion, as shown on the first two dimensions (80 % of the data variance) of the individual map (Fig. 6B). This was confirmed by the Pearson correlation coefficients (Fig. 6C), with an average value of 0.5 ($p < 0.0001$, n = 61) between *in vitro* and *in vivo* at the digestion site and times listed above.

A substantial amount of bioactive peptides remained specific to each digestion model, which can be explained by the different digestion sampling times and sites and by the different experimental conditions between *in vivo* and *in vitro*, particularly with the absence of brush border enzymes in the *in vitro* model likely contributing to the longer persistence of some peptides, particularly those containing the proline residue (Picariello et al., 2015). In addition, other bioactive peptides may have been released but not identified, either due to their absence in the bioactive peptide database or due to a size lower than the identification level (5 AAs).

3.7. Limitations of the study

The present study aimed to compare two existing models of the human infant digestion that were the *in vitro* dynamic model developed for a 4-week-old infant (De Oliveira et al., 2016; Halabi et al., 2022; Le Roux et al., 2020) and the 3-week-old piglet (Darragh & Moughan, 1995; Moughan et al., 1992) using HM collected at 2 months of lactation. HM is mature at three to four weeks of lactation, thus 2-month HM was assumed to be similar to a 1-month HM, which corresponds to the digestion stage of the *in vitro* model. Regarding the *in vivo* model, it has been demonstrated that a 3-week-old piglet would be equivalent to a 3-month infant in terms of protein digestion (Darragh & Moughan, 1995), in line with the comparative digestive tract evolution between human and pig reported by Sangild (2006). Ideally, the present *in vitro* model mimicking a 1-month old human infant should have been compared to a 7-day old piglet. However, it was not possible to include such a young piglet, which is too fragile to support a HM-feeding due to the different nutritional content between HM and sow milk, which contains two times more energy and lipids, three times more proteins but equal amount of lactose than in HM.

The digesta sampling differed between the *in vitro* and *in vivo* digestion, being along the time after a single meal ingestion for the *in vitro* digestion and along the digestive tract after a frequent feeding for the *in vivo* digestion. The frequent feeding regimen (6 meals every hour with digesta collection 30-min after the last meal) is assumed to ensure a more continuous flow of digesta along the digestive tract, and to allow a more representative sample collection (James et al., 2002). Nevertheless, these different experimental conditions between *in vitro* and *in vivo* may have impaired the comparison between the two models.

4. Conclusion

The present study confirmed that differences in protein nature and structure of HM and IF impacted their digestion, with major differences mainly observed in the gastric and early intestinal digestion. These results were observed both *in vitro* and *in vivo,* thus demonstrating that the *in vitro* dynamic digestion model at the infant stage allowed to a satisfactory prediction of the *in vivo* digestion of infant milks. This study highlighted that *in vitro* gastric digesta sampled between 40 and 80 min corresponded to gastric digesta of frequently-fed piglets after 30 min of *in vivo* gastric digestion and that the early *in vitro* intestinal phase (20, 40 min) was associated with the proximal jejunum of piglets after 30 min of digestion. This was particularly true for gastric digesta microstructure, gastric residual intact proteins and gastro-intestinal peptide release, while the*in vivo* – *in vitro* correspondence was less true for bioactive peptides and for *in vivo* AA digestibility *vs. in vitro* free AA bioaccessibility. Overall, the present study demonstrates that the dynamic digestion model used in the present study leads to similar conclusions as the *in vivo* model of human infant digestion that is the mini-piglet and can be thus useful for designing and testing more biomimetic IF. Further research is needed to better predict the bioactive peptide release and the AA bioavailability by adding brush border peptidases to the *in vitro* model and/or by performing further digesta fractionation and acid hydrolysis.

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CRediT authorship contribution statement

Elise Charton: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Olivia Menard:** Writing – review & editing, Investigation. **Marie-Françoise Cochet:** Writing – original draft, Investigation. **Yann Le Gouar:** Writing – review & editing, Investigation. **Julien Jardin:** Writing – review & editing, Visualization, Investigation, Formal analysis. **Gwénaële Henry:** Writing – review &

editing, Investigation. **Jordane Ossemond:** Writing – review & editing, Investigation. **Amandine Bellanger:** Writing – review & editing, Resources. **Carlos A. Montoya:** Writing – review & editing, Methodology, Funding acquisition. **Paul J. Moughan:** Writing – review & editing, Methodology, Funding acquisition. **Didier Dupont:** Writing – review & editing, Conceptualization. **Isabelle Le Huërou-Luron:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization. **Amélie Deglaire:** Writing – review & editing, Visualization, Supervision, Methodology, Funding acquisition, 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

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2024.115070.

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