British Journal of Nutrition (2024), 132, 351–358 doi:10.1017/S0007114524001260

Comparison of protein digestibility of human milk and infant formula using the INFOGEST method under infant digestion conditions

Yosuke Komatsu[1]*, Yasuaki Wada[2]*, Takuya Shibasaki[1], Yohei Kitamura[1], Tatsuya Ehara[1], Hirohiko Nakamura[1] and Kazuhiro Miyaji[1]

[1]*Health Care & Nutritional Science Institute, Morinaga Milk Industry Co., Ltd., 1-83, 5-Chome, Higashihara, Zama-City 252-8583, Kanagawa-Pref., Japan*
[2]*Innovative Research Institute, Morinaga Milk Industry Co., Ltd., 1-83, 5-Chome, Higashihara, Zama-City 252-8583, Kanagawa-Pref., Japan*

(Submitted 11 January 2024 – Final revision received 21 April 2024 – Accepted 23 May 2024 – First published online 3 June 2024)

Abstract

Many improvements have been made to bring infant formula (IF) closer to human milk (HM) regarding its nutritional and biological properties. Nevertheless, the protein components of HM and IF are still different, which may affect their digestibility. This study aimed to evaluate and compare the protein digestibility of HM and IF using the infant INFOGEST digestion method. Pooled HM and a commercial IF were subjected to the infant INFOGEST method, which simulates the physiological digestion conditions of infants, with multiple directions, i.e. the curd state, gel images of SDS-PAGE, molecular weight distribution, free amino acid concentrations and *in vitro* protein digestion rate. HM underwent proteolysis before digestion and tended to have a higher protein digestion rate with finer curds during gastric digestion, than the IF. However, multifaceted analyses showed that the protein digestibility of HM and IF was not significantly different after gastrointestinal digestion. In conclusion, the infant INFOGEST method showed that the digestibility of HM and IF proteins differed to some extent before digestion and after gastric digestion, but not at the end of gastrointestinal digestion. The findings of this study will contribute to the refinement of IF with better protein digestibility in infant stomach.

Keywords: **Human milk: infant formula: protein digestibility: infant INFOGEST digestion**

Human milk (HM) is the ideal food for infants and should be continuously provided in combination with complementary foods thereafter[1]. When HM is inadequate for an infant or breastfeeding is not possible, a bovine milk protein-based infant formula (IF) is used as an alternative formulated to have a high nutritional value[2]. However, an increased risk of obesity at 6 years of age has been reported in infants who consumed IF with high-protein content[3]. Compared with HM, proteins are included in higher amounts in IF due to their inferior digestibility and amino acid (AA) balance[4,5]. Protein intake in the early postnatal period may influence metabolic activity at 2 years and older[3,6]; therefore, a difference in protein compositions between HM and IF should be one of the focuses of attention. Milk proteins are classified into two fractions, that is, casein (CN) and whey proteins[7,8]. The CN:whey protein ratio changes from 10:90 in colostrum to 40:60 in mature HM during lactation, whereas that of commercial bovine milk is normally 80:20[9]. Therefore, commercial bovine milk protein-based IF are generally formulated with the CN:whey protein ratio adjusted from 80:20 to 40:60[9]. However, the characteristics of proteins in HM and bovine milk are still different. The dominant CN in HM is β-CN, whereas α_{s1}-CN is dominant in bovine milk. β-lactoglobulin is the most abundant protein in whey proteins of bovine milk, but the counterpart is absent in HM[10]. Furthermore, AA sequences of HM proteins and bovine milk counterparts are homologous but different to some extents[11]. These differences between HM and bovine milk are considered to affect the digestive trajectories of proteins, as manifested by curd formation of CN in the stomach proteolysis kinetics, and the resulting protein digestibility *in vitro* and *in vivo* experiments[12–16]. This has greatly hampered the 'humanisation' of proteins in IF.

In vivo assessment of protein digestibility in animals, including pigs and rats, has widely been used to date[17,18]. However, these *in vivo* models are not only expensive, time-consuming and entail ethical issues but also make the digestive trajectory difficult to monitor over time. Therefore, there has

Abbreviations: AA, amino acid; CN, casein; HM, human milk; IF, infant formula; MW, molecular weight; TCA, trichloroacetic acid.

* **Corresponding authors:** Yosuke Komatsu, email yo-komatsu@morinagamilk.co.jp; Yasuaki Wada, email ya-wada@morinagamilk.co.jp

been a need for digestion models that closely mimic the physiological processes of human gastrointestinal digestion, which has led to the development of *in vitro* digestion models as alternatives to *in vivo* models[19]. In recent years, a series of *in vitro* digestion models with different digestion conditions for adults has been internationally consolidated to the harmonised INFOGEST method[20,21]. Although no protocols are currently authorised for infants, Menard *et al.* proposed an *in vitro* infant digestion model that mimics the physiological digestion conditions of infants[22]. Recently, the digestibility of protein ingredients with different degrees of hydrolysis was compared using the infant digestion model[23]. Moreover, the digestibility of milk from different species such as camels, as well as colostrum and mature milk from lactating Chinese women, has been previously studied[24,25]. However, HM and commercially available IF are both complex food matrixes consumed by infants, and they have not sufficiently been compared in terms of protein digestibility using an infant digestion model. IFs have been improved by bringing their macronutrient content closer to that of HM. In particular, its protein content has been continuously reduced step by step with caution[26]. However, there is still room for improvement in the quantity and quality of milk proteins in IFs.

This study aimed to evaluate and compare the protein digestibility of HM and IF over time using the infant INFOGEST digestion method by simulating the physiological digestion conditions of infants. The digesta were compared based on the curd state, gel images of SDS-PAGE, molecular weight (MW) distribution and free AA concentrations. Moreover, *in vitro* digestion rates of HM and IF proteins were calculated after fractionating them into digestible and non-digestible components.

Methods

Samples and chemicals

HM samples were collected from fourteen healthy volunteers (lactation period: 1–3 months post-delivery). Prior to collection, nipples were wiped with sterile cotton, and milk was collected in γ-sterilised 50 ml centrifuge tubes to the extent that breast-feeding was not affected. When a breast pump was used, subjects were instructed to disassemble and sanitise it and maintain its cleanliness until use. Some subjects did not use a breast pump, and the milk was directly collected into the centrifuge tubes. The timing of milk collection during the day was not specified. Samples were transferred to our laboratory and temporarily stored −80°C. The samples were then defrosted, pooled for each volunteer and aliquoted and stored again at −80°C until analysis. The Institutional Review Board of the Japan Clinical Research Conference approved this study (approval number: BONYU-01), which was conducted in accordance with the Declaration of Helsinki of 2013. The participants provided written informed consent for all the procedures related to this study. A commercially available standard IF (Morinaga Milk Industry Co., Ltd.) was used for comparison with HM. The IF is a standard formula (ingredients: lactose, vegetable oil, bovine milk protein, starch, etc.) with typical nutritional components and can be viewed as being rationally representing the characteristics of HM substitutes. The energy, fat and carbohydrate contents of HM were analysed using the human milk analyzer (MIRIS AB), while those of the IF were analysed by our in-house quality control department (Table 1). The nitrogen (N) content of both HM and IF was determined with the Dumas method using SUMIGRAPH NC-220F (Sumika Chemical Analysis Service), which was then multiplied by 6·25 to estimate the crude protein content (Table 1). Porcine pepsin, pancreatin, gastric lipase and bile extract were purchased from Sigma-Aldrich (Cat. P7012, P7545, BCCF2430 and B8631, respectively). Rabbit gastric lipase was replaced with *Rhizopus oryzae* lipase and porcine pepsin[21,27]. Pepsin, pancreatin, gastric lipase, and bile acid activities were determined according to the protocol described by Minekus *et al.*[20]. Bile activity was measured using the bile acid assay kit (DiaSys Diagnostic Systems, Cat. 122129990313).

Table 1. Macronutrients in human milk and infant formula

	Human milk*	Infant formula†
Energy (kcal/100 ml)	56	67
Protein (g/100 ml)‡	1·3	1·3
Fat (g/100 ml)	2·2	3·4
Carbohydrate (g/100 ml)	6·8	7·6

* Energy, fat and carbohydrate contents in human milk are the values measured by the human milk analyser.
† Energy, fat and carbohydrate contents in infant formula were analysed by our in-house quality control department.
‡ Protein in human milk and infant formula was measured using the DUMAS method for nitrogen content and converted using a conversion factor of 6·25.

In vitro-simulated gastrointestinal digestion

HM and IF were digested using the INFOGEST method under infant gastrointestinal digestion conditions at 1 month of age (Table 2)[22]. As permitted by the INFOGEST protocol[21], oral digestion by α-amylase was skipped due to the short residence time of HM and IF in the infant's oral cavity. Simulated gastric fluid was formulated to include 94 mM sodium chloride and 13 mM potassium chloride at pH 5·3. HM and IF were mixed with simulated gastric fluid containing enzymes at a ratio of 63:37, and the pH was adjusted to 5·3. Gastric enzyme activity was set at 19 U/ml for gastric lipase and 268 U/ml for pepsin in the final gastric fluid mixture. The mixture was shaken continuously at 160 rpm for 60 min in a water bath equipped with an incubation shaker (Yamato Scientific). After gastric digestion, gastric chyme and simulated intestinal fluid were mixed with the enzymes at a ratio of 62:38, and the pH was adjusted to 6·6. Simulated intestinal fluid was composed of 164 mM sodium chloride, 10 mM potassium chloride and 85 mM sodium bicarbonate and was adjusted to pH 7. Calcium chloride (3 mM) was added to the final intestinal fluid mixture. Intestinal enzyme activities were set to 90 U/ml for intestinal lipase and 16 U/ml for trypsin in the final intestinal fluid mixture. The bovine bile extract was added to the final intestinal fluid mixture containing 3·1 mM bile salts. The mixture was shaken continuously at 160 rpm for 60 min in a water bath equipped with an incubation shaker. Enzymes in each sample were inactivated in a water bath at 90°C for 5 min. The digesta were freeze-dried using a lyophiliser (FreeZone 4·5, LABCONCO) and then ground into a fine powder using a grinder

Table 2. Digestion condition of infant and adult INFOGEST models

Digestion condition	Model	
	Infant*	Adult†
Gastric phase		
Gastric lipase activity (U ml^{-1})	19	21
Pepsin activity (U ml^{-1})	268	2000
Reaction pH	5·3	3·0
Reaction time (min)	60	120
Intestinal phase		
Bile acid activity (mmol l^{-1})	3·1	10
Pancreatic lipase activity (U ml^{-1})	90	2000
Trypsin activity (U ml^{-1})	16	100
Reaction pH	6·6	7·0
Reaction time (min)	60	120

* With reference to the physiological digestion conditions of infants, the *in vitro* infant digestion model proposed by Menard *et al.*[22].
† The adult INFOGEST model condition is presented as a reference for the infant model[21].

(Multi-beads shocker, YASUI KIKAI). The freeze-dried powders were stored at −25°C until further analysis. A 'blank' sample containing neither HM nor IF was prepared in the same manner as above.

SDS-PAGE

HM, IF and their digesta were analysed by SDS-PAGE (Bio-Rad Laboratories) under reducing conditions. Electrophoresis was conducted at 120 V for 50 min after loading 50 µg of protein onto a Mini-PROTEAN TGX Any KD gel (Bio-Rad Laboratories). The gels were stained with Bio-Safe Coomassie Stain (Bio-Rad Laboratories) for 60 min and then destained overnight in Milli-Q water.

Size-exclusion chromatography

The MW distributions of HM, IF and their digesta were determined by size-exclusion chromatography using an HPLC U3000 system (Thermo Fisher Scientific). HM, IF and their digesta were diluted with a buffer containing 30 % acetonitrile and 0·1 % formic acid to 1 mg/ml of protein (before digestion) and centrifuged at 3000 × ***g*** for 30 min to remove the lipid layer. Then, 20 µg of protein was loaded onto an XBridge BEH125 SEC column (3·5 µm, 7·8 × 300 mm; Waters, Milford, CT, USA). Size-exclusion chromatography involved isocratic elution at 40 °C using 30 % acetonitrile and 0·1 % formic acid as the mobile phase at a flow rate of 0·8 ml/min. Spectrophotometric detection was performed at 214 nm. To determine the MW, the following standards were used: L(−)-phenylalanine (MW 165; FUJIFILM Wako Pure Chemical Corporation), enkephalin (MW 588; Bachem Americas), oxytocin (MW 1007; Bachem Americas), bacitracin (MW 1427; Sigma-Aldrich), insulin (MW 5740; FUJIFILM Wako Pure Chemical Corporation), chymotrypsinogen A (MW 25,000; Pharmacia), ovalbumin (MW 43,000; Sigma-Aldrich), lactoperoxidase (MW 93,000; Sigma-Aldrich) and immunoglobulin G (MW 160,000; Sigma-Aldrich). A calibration curve was constructed by plotting the logarithmic MW of the standards against their respective elution times. Each total peak area was integrated and separated into five ranges (–300, 301–1000, 1001–2000, 2001–3000 and 3001–) and expressed as a percentage of the total area.

Free aminoacid analysis

HM, IF and their digesta were diluted with Milli-Q water to 2 mg/ml protein (before digestion), and the non-protein fraction was separated using 12 % trichloroacetic acid (TCA). The supernatants were passed through a 0·22 µm filter and analysed using an AA analyzer L-8900 (Hitachi-Hitech) equipped with an ion-exchange column (2622SC-PF; 4·6 mm × 60 mm; Hitachi-Hitech). L-8900 buffer solutions (PF-1, 2, 3, 4; Kanto Chemical Co., Inc. and RG; FUJIFILM Wako Pure Chemical Corporation) were used as the mobile phase, and about 20 µl of each sample was injected into the HPLC column. The 148 min mode, outlined in the Hitachi LC110012 manual, was employed.

In vitro *protein digestion rate*

Supernatants obtained from the digesta after precipitation with TCA were defined here as digestible fractions. The freeze-dried digesta were reconstituted with Milli-Q water and subjected to 12 % TCA precipitation. The supernatant was obtained by centrifugation at 4°C, 2150 × ***g*** for 10 min and at 4°C, 12 000 × ***g*** for 5 min. The *in vitro* protein digestion rate was defined as the ratio of the *N* content in the digestible fraction to the *N* content in the entire lyophilised digesta. Thus, the *N* content was corrected for the *N* content of the blank sample

$$\textit{In vitro protein digestion rate}(\%) = \frac{N\ \textit{digestible fraction}_{samples} - N\ \textit{digestible fraction}_{blank}}{N\ \textit{whole digesta}_{samples} - N\ \textit{whole digesta}_{blank}} \times 100$$

Statistical analysis

The JMP software (SAS Institute) was used for statistical analyses. Data for free AA and *in vitro* protein digestion rate are expressed as the mean (standard deviation). Differences between groups were analysed by Welch's *t* test, and significance was set at $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$ and $^{***}P < 0{\cdot}001$.

Results

Photographic images

HM and IF were digested using the infant INFOGEST digestion method, and the changes over time are shown in the photographic images (Fig. 1). Curd aggregates were only observed during the gastric digestion phase of IF. No aggregates were observed in HM and IF during the intestinal digestion phase.

SDS-PAGE

Protein band patterns during the infant INFOGEST digestion were compared (Fig. 2). Bands derived from CN and whey proteins were observed in the HM and IF before digestion, with different patterns. The intensities of CN bands for both HM and IF were lower in the gastric digestion phase, but no significant changes

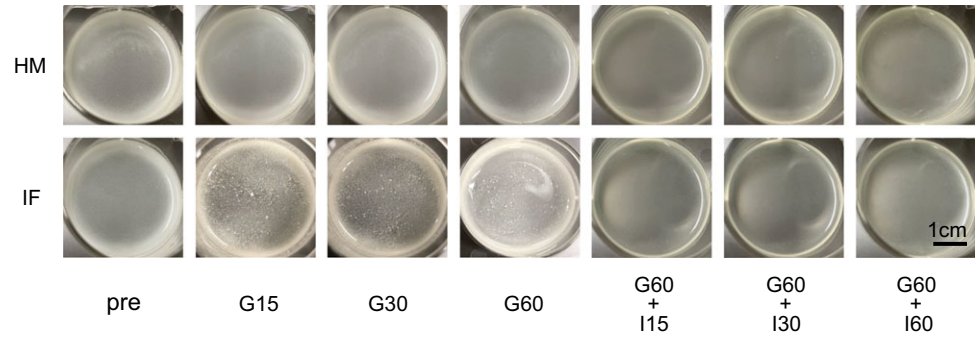

Fig. 1. Photographic images of human milk (HM) and infant formula (IF) during the infant INFOGEST digestion. The infant INFOGEST digestion assays were independently conducted three times for HM and IF. The images are representatives of the three experiments. G, gastric digestion; I, intestinal digestion.

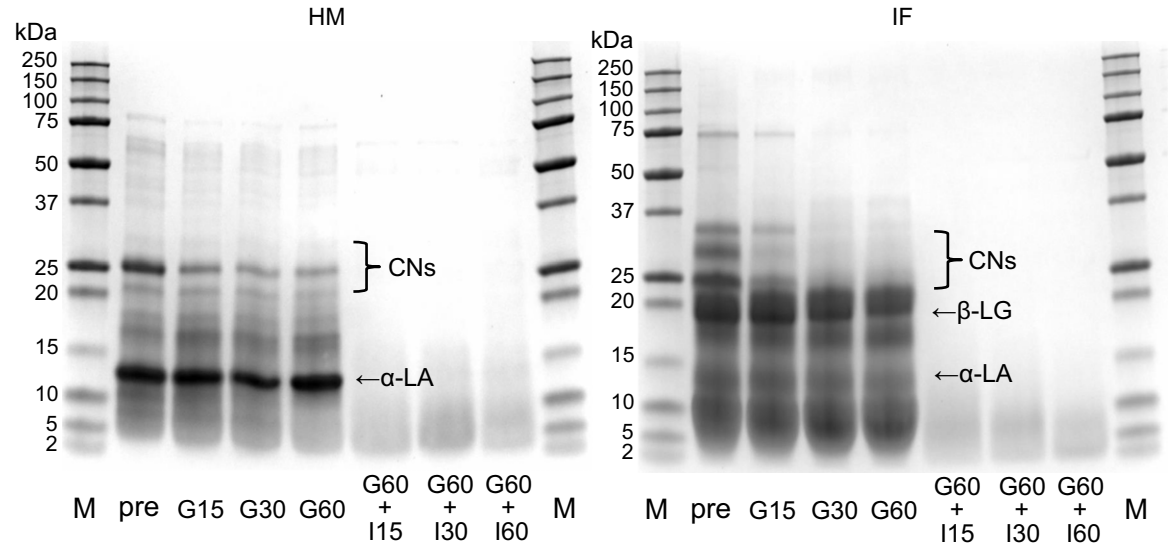

Fig. 2. SDS-PAGE of human milk (HM) and infant formula (IF) during the infant INFOGEST digestion. The infant INFOGEST digestion assays were independently conducted three times for HM and IF, where 50 μg of protein was resolved per lane. Samples were analysed under reducing conditions. The images are representatives of the three experiments. CN, casein; G, gastric digestion; I, intestinal digestion; α-LA, α-lactalbumin; β-LG, β-lactoglobulin; M, molecular weight marker.

were observed in the other bands. In the intestinal digestion phase, all bands derived from the high-MW (above approximately 10 kDa) region disappeared, and smear-like bands were observed in the low-MW (below approximately 10 kDa) region.

Molecular weight distributions

MW distributions were examined for HM, IF and their gastric and intestinal digesta, by pooling the digesta of independent digestion experiments and then subjecting them to the size-exclusion chromatography (Fig. 3). Before digestion and during the gastric digestion phase, fractions above 3000 Da were dominant in both HM and IF. No remarkable differences were observed in the proportions of molecules above 3000 Da between the HM and IF. Before and after digestion, the proportion of molecules ranging from 1001 to 3000 Da in IF was higher than that in HM. HM had a higher proportion of molecules below 300 Da before digestion compared with IF, and the difference was maintained throughout the digestion phases.

Free aminoacid concentrations

The free AA concentrations during the infant INFOGEST digestion are shown in Fig. 4. In the gastric digestion phase, the free total and indispensable AA concentrations in HM were significantly higher than those in IF. In contrast, no significant differences were observed between HM and IF during the intestinal digestion phase.

In vitro *protein digestion rates*

In vitro protein digestion rates during infant INFOGEST digestion are shown in Fig. 5. Before digestion, HM had a significantly higher *in vitro* protein digestion rate than IF. The *in vitro* protein digestion rate during the gastric digestion phase tended to be higher for HM than for IF, although the difference was not significant. In contrast, the *in vitro* protein digestion rate did not significantly change during the intestinal digestion phase.

British Journal of Nutrition

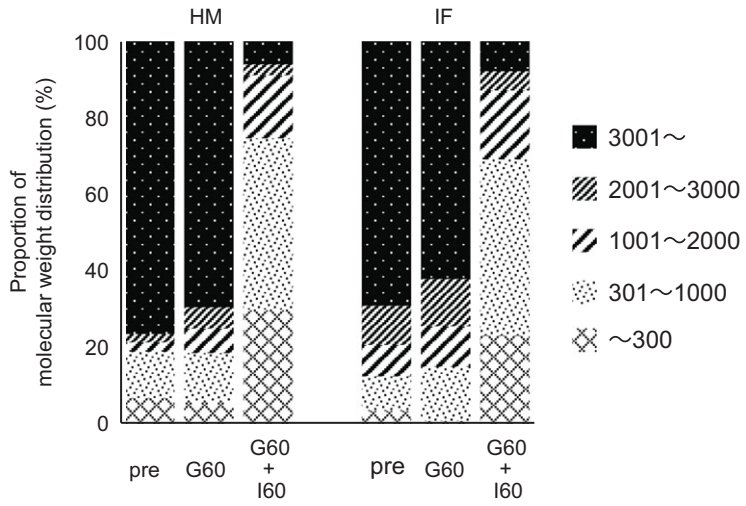

Fig. 3. Molecular weight distributions of human milk (HM) and infant formula (IF) during the infant INFOGEST digestion. The infant INFOGEST digestion assays were independently conducted three times for HM and IF. The obtained digesta were pooled and subjected to size-exclusion chromatography. G, gastric digestion; I, intestinal digestion; figures following G or I indicate digestion time (min).

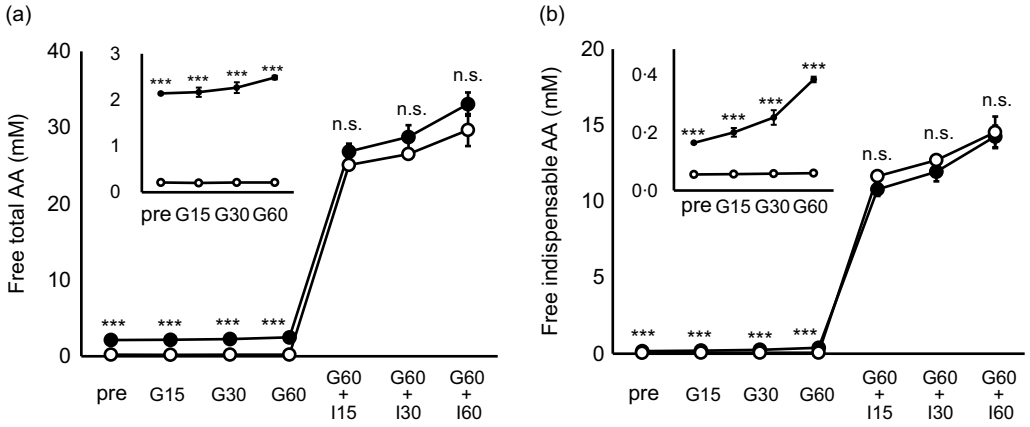

Fig. 4. Changes in the amounts of (a) total amino acids (AA) and (b) indispensable AA in human milk (HM) and infant formula (IF) during the infant INFOGEST digestion. The infant INFOGEST digestion assays were independently conducted three times for HM and IF. Data are shown as the means (standard deviations) (n 3). Differences between groups were analysed using Welch's t tests. ***$P < 0{\cdot}001$. G, gastric digestion; I, intestinal digestion; figures following G or I indicate digestion time (min). –●–, HM; –○–, IF.

Discussion

Differences in the protein components of HM and IF are considered to affect protein digestibility. To clarify differences in protein digestibility between HM and IF, an appropriate evaluation method is required. Recently, an *in vitro* infant digestion model that mimics the physiological digestion conditions of infants was proposed by Menard *et al.*[22]. This study aimed to evaluate and compare the nutritional qualities of HM and IF by subjecting HM and a commercial IF to the infant INFOGEST digestion method, followed by comparison of the protein digestibility using multiple directions.

Images of the gastric and intestinal digestion fluids derived from HM and the IF at different time points were compared. During the gastric digestion phase, no curd aggregates were observed in HM, whereas aggregates were visible in IF. The lower CN to whey protein ratio and higher β-CN to α_{s1}-CN ratio in HM reduce the size of its CN micelles which promote the formation of soft and very fragile curds under acidic conditions[15,28]. In addition, our study confirmed that IF forms a harder curd than HM in the stomach of infants, which was also found in previous studies[29]. The SDS-PAGE results indicated that HM and IF showed limited protein degradation in the gastric digestion phase. In contrast, protein degradation proceeded rapidly in the subsequent intestinal digestion phase, and high MW protein bands were rarely observed. These results are similar to those previously observed during *in vitro* dynamic simulations of HM digestion in full-term infants[30] and IF digestion in piglets[31]. The MW distribution results before digestion showed that the proportion of molecules below 1000 Da in HM was higher than in IF. This was probably due to the higher amount of non-protein N-containing molecules in HM, such as AA, short peptides and urea[32–34]. In the intestinal digestion phase, the proportion of molecules below 1000 Da increased while that of molecules above 3000 Da rapidly decreased for both HM and IF. This result was consistent with the

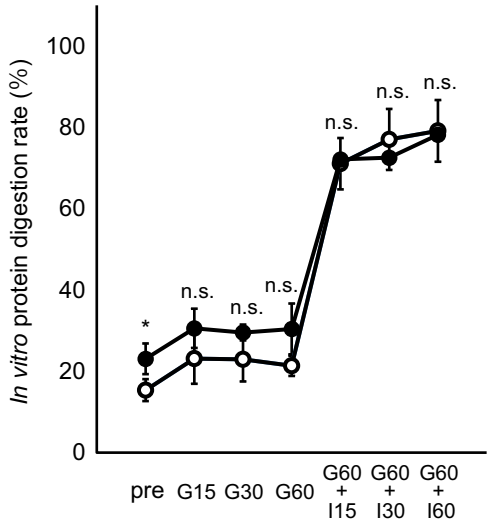

Fig. 5. Changes in *in vitro* protein digestion rates of human milk (HM) and infant formula (IF) during the infant INFOGEST digestion. The infant INFOGEST digestion assays were independently conducted three times for HM and IF. Data are shown as the means (standard deviations) (*n* 3). Differences between groups were analysed using Welch's *t* tests. $^{*}P < 0{\cdot}05$. G, gastric digestion; I, intestinal digestion; figures following G or I indicate digestion time (min). –●–, HM; –○–, IF.

rapid degradation of proteins with a larger MW in the intestinal digestion phase, as determined by SDS-PAGE in the present study. Free AA analysis showed that HM had significantly higher concentrations of free total and indispensable AA before digestion and in the gastric digestion phases compared with IF. In the intestinal digestion phase, the concentrations of free AA increased dramatically compared with the concentrations in the gastric digestion phase for both HM and IF, with no significant difference between HM and IF. This observation suggests that both HM and IF proteins would be degraded to the same extent into readily absorbed free AA during intestinal digestion in infants.

The infant INFOGEST model utilised in this study was designed to mimic *in vivo* infant digestive conditions as closely as possible. Digestive parameters, including pH of the reaction system, the amount of digestive enzymes and the digestion reaction time, were determined based on a comprehensive review summarising previous physiological digestive conditions in infants[35]. Specifically, in the infant model, the pH of the intestinal phase (6·6) was almost the same as in the adult model (7·0), whereas the pH of the gastric phase in the infant model (5·3) was set much higher than in the adult model (3·0). The pH of the gastric phase in the infant model was calculated based on the gastric emptying half-time (78 min), which was measured from an *in vivo* experiment of infants[35,36]. Pepsin activity, which is known to vary greatly depending on pH, is maximal at pH 2·0, whereas only approximately 10 % of the maximal activity is obtained at around pH 5·3 in the gastric digestion phase of the infant model[37]. Moreover, the pepsin concentration is only about one-seventh of that in the adult digestion model. This was calculated based on the enzyme activities in infant gastric aspirates and the infant's body weight[38,39]. Trypsin, the primary proteolytic enzyme in the intestinal phase, was added at only about one-sixth of the amount in the adult model. This concentration was set based on the enzyme level in the digestive fluid collected from infants[40]. The reaction time was set at 60 min for both the gastric and intestinal phases. The reaction time for the gastric phase was based on the gastric emptying time in full-term infants[35,36], whereas for the intestinal phase, it was set in consideration of the contraction amplitude, propagation speed of food passage and frequency of intestinal peristalsis[41,42], along with conducting *in vitro* experiments to verify the length of the digestion time. Thus, in the infant model, digestive capacity was set considerably weaker in both gastric and intestinal phases compared with those in the adult model.

Notably, the difference in curd formation observed during the gastric digestion phase, even though not significant, may have led to the higher digestibility of proteins in HM than those in IF. This may be because curd formation partially prevents the access of pepsin to the substrate, as previously demonstrated by a dynamic digestion model[43]. Finally, no difference in protein digestibility was observed between HM and IF at the end of gastrointestinal digestion in the multifaceted analyses. Using the infant INFOGEST digestion method, the results of this study suggest that HM and IF proteins would eventually be equally digestible after gastrointestinal digestion. A study using mini-piglet as a model demonstrated that the digestibility of individual AA in HM and IF was similar at the ileal terminal, except for threonine[44]. However, studies evaluating the protein digestibility of HM and IF *in vivo* are scarce. Several studies have investigated the *in vivo* digestion dynamics by aspirating digestive fluids from the infant's digestive tract[45,46]. In the future, such techniques will likely elucidate a more detailed understanding of the *in vivo* digestive dynamics of HM and IF in infants.

One limitation of this study was that only one type of commercially distributed IF was tested. Still, this IF is a standard formula with typical nutritional components (ingredients: lactose, vegetable oil, bovine milk protein, starch. etc.) and can be viewed as being rationally representing the characteristics of HM substitutes. Future comparative studies between HM and different types of IF are required. Another limitation was that the digestive ability of infants increases with growth. Therefore, the infant INFOGEST digestion method should be modified to accommodate such changes, aiming to more accurately simulate infant digestion. Recently, *in vitro* digestion models based on gastric digestion in infants aged 1, 3 and 6 months were proposed, and the digestion kinetics of skim milk were reported[47]. Further studies that aim to improve the current infant *in vitro* digestion method are required. It is also to be noted that external factors might affect protein digestibility. Specifically, the collection of HM was not sterile, potentially leading to microbial proteolysis[48], and protein degradation may also occur during defrosting. These factors cannot be completely eliminated. Furthermore, considering that approximately 25 % of HM is comprised of NPN[49], the application of a conversion factor 6·25 to the amount of N obtained by elemental analysis could potentially lead to an overestimation of protein digestibility. Thus, it is crucial to employ a multifaceted approach to further compare protein digestibility. In this study, we used a combination of analyses such as SDS-PAGE, MW distribution and free AA analysis to ensure the certainty of protein digestibility.

In conclusion, the infant INFOGEST method used in this study confirmed that the protein digestibility of HM and IF differed to some extent before digestion as well as after gastric digestion, although their digestibility was similar after the intestinal digestion phase. These differences between HM and the IF may be critical, especially for infants with an immature digestive capacity. Previous studies have shown that protein dephosphorylation improves gastric clotting property and gastrointestinal digestibility in infant gastric models[50]. Applying such modification to IF would make their protein digestibility significantly similar to that of HM.

Acknowledgements

We thank Vu Tuan Bui (Health Care & Nutritional Science Institute, Morinaga Milk Industry Co., Ltd.) for assistance with the AA and SDS-PAGE analyses.

Conceptualisation and methodology, Yosuke Komatsu, Y. W., Yohei Kitamura, T. E., H. N. and K. M.; data curation, Yosuke Komatsu and T. S.; formal analysis and writing-original draft, Yosuke Komatsu; writing-review & editing, Y. W., Y. K. and Y. W. were primarily responsible for the final manuscript. All the authors have read and approved the final version of the manuscript.

All the authors are employees of Morinaga Milk Industry Co., Ltd. This research was also funded by the Morinaga Milk Industry Co., Ltd.

References

1. Horta BL & Victora CG (2013) *Short-Term Effects of Breastfeeding: A Systematic Review of the Benefits of Breastfeeding on Diarrhoea and Pneumonia Mortality*. Geneva: World Health Organization.
2. Kim SY & Yi DY (2020) Components of human breast milk: from macronutrient to microbiome and microRNA. *Clin Exp Pediatr* **63**, 301.
3. Koletzko B, Demmelmair H, Grote V, *et al.* (2016) High protein intake in young children and increased weight gain and obesity risk. *Am J Clin Nutr* **103**, 303–304.
4. Maathuis A, Havenaar R, He T, *et al.* (2017) Protein digestion and quality of goat and cow milk infant formula and human milk under simulated infant conditions. *J Pediatr Gastroenterol Nutr* **65**, 661–666.
5. Purkiewicz A, Stasiewicz M, Nowakowski JJ, *et al.* (2023) The influence of the lactation period and the type of milk on the content of amino acids and minerals in human milk and infant formulas. *Foods* **12**, 3674.
6. Socha P, Grote V, Gruszfeld D, *et al.* (2011) Milk protein intake, the metabolic-endocrine response, and growth in infancy: data from a randomized clinical trial. *Am J Clin Nutr* **94**, 1776S–1784S.
7. Carter BG, Cheng N, Kapoor R, *et al.* (2021) Invited review: microfiltration-derived casein and whey proteins from milk. *J Dairy Sci* **104**, 2465–2479.
8. Liao Y, Weber D, Xu W, *et al.* (2017) Absolute quantification of human milk caseins and the whey/casein ratio during the first year of lactation. *J Proteome Res* **16**, 4113–4121.
9. Fenelon MA, Hickey RM, Buggy A, *et al.* (2019) Whey proteins in infant formula. In Whey Proteins: From Milk to Medicine, pp. 439–494 [HC Deeth & N Bansal, editors]. Cambridge, Massachusetts, the United States: Academic Press.
10. Ballard O & Morrow AL (2013) Human milk composition: nutrients and bioactive factors. *Pediatr Clin* **60**, 49–74.
11. Lajnaf R, Feki S, Ameur SB, *et al.* (2023) Recent advances in selective allergies to mammalian milk proteins not associated with Cow's Milk Proteins Allergy. *Food Chem Toxicol* **178**, 113929.
12. Yang T, Liu D, Tang J, *et al.* (2024) Formation of casein micelles simulating human milk casein composition from bovine caseins: Micellar structure and *in vitro* infant gastrointestinal digestion. *Food Hydrocoll* **149**, 109610.
13. Xiao, T., et al. Comparative Analysis of Protein Digestion Characteristics in Human, Cow, Goat, Sheep, Mare, and Camel Milk under Simulated Infant Condition. *J. Agric. Food Chem.* **71**, 15035–15047 (2023).
14. Meng F, Uniacke-Lowe T, Ryan AC, *et al.* (2021) The composition and physico-chemical properties of human milk: a review. *Trends Food Sci Technol* **112**, 608–621.
15. Roy D, Ye A, Moughan PJ, *et al.* (2020) Composition, structure, and digestive dynamics of milk from different species—a review. *Front Nutr* **7**, 577759.
16. Nguyen TT, Bhandari B, Cichero J, *et al.* (2015) Gastrointestinal digestion of dairy and soy proteins in infant formulas: an *in vitro* study. *Food Res Int* **76**, 348–358.
17. Mathai JK, Liu Y & Stein HH (2017) Values for Digestible Indispensable Amino Acid Scores (DIAAS) for some dairy and plant proteins may better describe protein quality than values calculated using the concept for Protein Digestibility-Corrected Amino Acid Scores (PDCAAS). *Br J Nutr* **117**, 490–499.
18. Reynaud Y, Buffière C & Cohade B (2021) True ileal amino acid digestibility and Digestible Indispensable Amino Acid Scores (DIAASs) of plant-based protein foods. *Food Chem* **338**, 128020.
19. Hur SJ, Lim BO, Decker EA, *et al.* (2011) *In vitro* human digestion models for food applications. *Food Chem* **125**, 1–12.
20. Minekus M, Alminger M, Alvito P, *et al.* (2014) A standardised static *in vitro* digestion method suitable for food–an international consensus. *Food Funct* **5**, 1113–1124.
21. Brodkorb A, Egger L, Alminger M, *et al.* (2019) INFOGEST static *in vitro* simulation of gastrointestinal food digestion. *Nat Protoc* **14**, 991–1014.
22. Ménard O, Bourlieu C, De Oliveira SC, *et al.* (2018) A first step towards a consensus static *in vitro* model for simulating full-term infant digestion. *Food Chem* **240**, 338–345.
23. Corrigan B & Brodkorb A (2020) The effect of pre-treatment of protein ingredients for infant formula on their *in vitro* gastro-intestinal behaviour. *Int Dairy J* **110**, 104810.
24. Elwakiel M, Boeren S, Wang W, *et al.* (2020) Degradation of proteins from colostrum and mature milk from Chinese mothers using an *in vitro* infant digestion model. *Front Nutr* **7**, 162.
25. Zou Z, Duley JA, Cowley DM, *et al.* (2022) Digestibility of proteins in camel milk in comparison to bovine and human milk using an *in vitro* infant gastrointestinal digestion system. *Food Chem* **374**, 131704.
26. Bakshi S, Paswan VK, Yadav SP, *et al.* (2023) A comprehensive review on infant formula: nutritional and functional constituents, recent trends in processing and its impact on infants' gut microbiota. *Front Nutr* **10**, 1194679.
27. Zenker HE, van Lieshout GA, van Gool MP, *et al.* (2020) Lysine blockage of milk proteins in infant formula impairs overall protein digestibility and peptide release. *Food Funct* **11**, 358–369.
28. Nakai S & Li-Chan E (1987) Effect of clotting in stomachs of infants on protein digestibility of milk. *Food Struct* **6**, 8.
29. Huppertz T & Lambers TT (2020) Influence of micellar calcium phosphate on *in vitro* gastric coagulation and digestion of milk proteins in infant formula model systems. *Int Dairy J* **107**, 104717.

30. De Oliveira SC, Deglaire A, Ménard O, *et al.* (2016) Holder pasteurization impacts the proteolysis, lipolysis and disintegration of human milk under *in vitro* dynamic term newborn digestion. *Food Res Int* **88**, 263–275.
31. Bouzerzour K, Morgan F, Cuinet I, *et al.* (2012) *In vivo* digestion of infant formula in piglets: protein digestion kinetics and release of bioactive peptides. *Br J Nutr* **108**, 2105–2114.
32. Rudloff S & Kunz C (1997) Protein and nonprotein nitrogen components in human milk, bovine milk, and infant formula: quantitative and qualitative aspects in infant nutrition. *J Pediatr Gastroenterol Nutr* **24**, 328–344.
33. Carratù B, Boniglia C, Scalise F, *et al.* (2003) Nitrogenous components of human milk: non-protein nitrogen, true protein and free amino acids. *Food Chem* **81**, 357–362.
34. Khaldi N, Vijayakumar V, Dallas DC, *et al.* (2014) Predicting the important enzymes in human breast milk digestion. *J Agric Food Chem* **62**, 7225–7232.
35. Bourlieu C, Ménard O, Bouzerzour K, *et al.* (2014) Specificity of infant digestive conditions: some clues for developing relevant *in vitro* models. *Crit Rev Food Sci Nutr* **54**, 1427–1457.
36. Cavell B (1981) Gastric emptying in infants fed human milk or infant formula. *Acta Paediatr Scand* **70**, 639–641.
37. Piper DW & Fenton BH (1965) pH stability and activity curves of pepsin with special reference to their clinical importance. *Gut* **6**, 506.
38. Armand M, Hamosh M, Mehta NR, *et al.* (1996) Effect of human milk or formula on gastric function and fat digestion in the premature infant. *Pediatr Res* **40**, 429–437.
39. De Oliveira SC, Deglaire A, Ménard O, *et al.* (2016) Holder pasteurization impacts the proteolysis, lipolysis and disintegration of human milk under *in vitro* dynamic term newborn digestion. *Food Res Int* **88**, 263–275.
40. Norman A, Strandvik B & Ojamäe O (1972) Bile acids and pancreatic enzymes during absorption in the newborn. *Acta Paediatr Scand* **61**, 571–576.
41. Dumont RC & Rudolph CD (1994) Development of gastrointestinal motility in the infant and child. *Gastrointest Endosc Clin N Am* **23**, 655–671.
42. Kauffman RE (2011) Chapter 3. Drug action and therapy in the infant and child. In *Neonatal and Pediatric Pharmacology: Therapeutic Principles in Practice*, pp. 20–30 [YJ Summer & JV Aranda, editors]. Philadelphia: Lippincott Williams and Wilkins.
43. Zhang H, Duan S, Yu Y, *et al.* (2023) Impact of casein-to-whey protein ratio on gastric emptying, proteolysis, and peptidome profile of fermented milk during *in vitro* dynamic gastrointestinal digestion in preschool children. *Food Chem* **405**, 134840.
44. Charton E, Henry G, Cahu A, *et al.* (2023) Ileal digestibility of nitrogen and amino acids in human milk and an infant formula as determined in neonatal minipiglets. *J Nutr* **153**, 1063–1074.
45. Nielsen SD, Beverly RL, Underwood MA, *et al.* (2020) Differences and similarities in the peptide profile of preterm and term mother's milk, and preterm and term infant gastric samples. *Nutrients* **12**, 2825.
46. Wada Y & Lönnerdal B (2020) Bioactive peptides derived from human milk proteins: an update. *Curr Opin Clin Nutr Metab Care* **23**, 217–222.
47. Miltenburg J, Bastiaan-Net S, Hoppenbrouwers T, *et al.* (2024) Gastric clot formation and digestion of milk proteins in static *in vitro* infant gastric digestion models representing different ages. *Food Chem* **432**, 137209.
48. Fusco V, Chieffi D, Fanelli F, *et al.* (2020) Microbial quality and safety of milk and milk products in the 21st century. *Compr Rev Food Sci Food Saf* **19**, 2013–2049.
49. Food and Agriculture Organization (2013) Dietary Protein Quality Evaluation in Human Nutrition: Report of an FAO Expert Consultation Food and Nutrition Paper; 92. Rome: FAO.
50. Liu D, Wang Y, Yu Y, *et al.* (2016) Effects of enzymatic dephosphorylation on infant *in vitro* gastrointestinal digestibility of milk protein concentrate. *Food Chem* **197**, 891–899.

British Journal of Nutrition