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Dairy Foods: A Matrix for Human Health and Precision Nutrition— Effect of processing infant milk formula on protein digestion and gut barrier health (in vitro and preclinical)

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ABSTRACT

The infant gut is immature and permeable with high gastric pH, low protease activities, and underdeveloped intestinal architecture. Protein digestion in the upper gastrointestinal tract of infants is slow and incomplete. During manufacture, infant milk formula (IMF) is typically heat-treated so it is safe for human consumption. This heat treatment causes denaturation and aggregation of milk proteins, and formation of undesirable Maillard reaction products. The aim of this review is to critically summarize the in vitro and preclinical data available on the effect of IMF thermal processing on protein digestion and gut barrier physiology in the immature infant gut. Recent research efforts have focused on reducing thermal loads during IMF manufacturing by sourcing ingredients with low thermal loads, by reducing temperatures during IMF processing itself, and by seeking alternative processing technologies. This review also aims to evaluate whether these thermal reductions have a knock-on effect on protein digestion and gut barrier health in the infant. The ultimate aim is to create a safe next-generation IMF product that more closely mimics human breast milk in its protein digestion kinetics and its ability to promote gut barrier maturity in the infant.
Key words: infant milk formula, processing, protein digestion, gut barrier health, infant

INTRODUCTION

When babies are born their digestive tract is underdeveloped (Jiang et al., 2022). This immaturity of the infant gastrointestinal tract is reflected by higher gastric pH (Bourlieu et al., 2014), lower levels and activities of digestive enzymes (Nguyen et al., 2015; Ménard et al., 2018), immature intestinal architecture (Gleeson et al., 2021), lower numbers of enterocytes, goblet cells, and Paneth cells (Demers-Mathieu, 2022), a more permeable mucus layer (Macierzanka et al., 2014), higher intestinal permeability (Kosek et al., 2017; Musa et al., 2019; Gleeson et al., 2021), and lower diversity of gut microbiota (Yao et al., 2021), compared with the adult gastrointestinal tract.

All of these characteristics combine to result in differences in nutrient digestion and absorption between infant and adult (Gan et al., 2018). At birth, preterm and term infants have a gastric pH ≥7, which is heavily influenced by fed or fasted state and the infant's ability to secrete gastric acid (Bourlieu et al., 2014; Ménard et al., 2018). This is in sharp contrast to pH 1.5 to 2 in the adult stomach (Fujimori, 2020). The optimum pH for activity of the gastric enzyme, pepsin, is pH 2 (Nguyen et al., 2015; Gan et al., 2018). At pH ≥6.5, pepsin activity is reduced, with complete denaturation and inactivation at pH ≥8 (Johnston et al., 2007). Therefore, in the infant stomach, pepsin activity is <10% of the activity reported in adults (Nguyen et al., 2015). In addition, the activities of the small intestine enzymes, trypsin and chymotrypsin, are reported in the infant at 10% to 60% of the adult activity levels (Nguyen et al., 2015). Whether age differences exist in brush border enzyme activities is less well characterized.

The small intestine surface is a mass of villi to increase surface area and therefore maximize nutrient absorptive capacity (Walton et al., 2016). Nutrients must cross the villi epithelium to be absorbed. The epithelium barrier is a single layer of cells composed primarily of absorptive enterocytes, goblet cells, Paneth cells, stem cells, and neuroendocrine cells. The enterocytes, goblet cells, and neuroendocrine cells begin life in the Paneth controlled crypts before maturing and migrating toward the villus tip (Kong et al., 2018). In the small intestine, villi height is lower in infants compared with adults (Gleeson et al., 2021). Mucus-secreting goblet cells prevent adhesion and attack by pathogenic bacteria (Johansson et al., 2013). In the mature gut, goblet cells account for 4% to 12% of

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intestinal epithelial cells, but it is widely accepted that the percentage of goblet cells is much lower in the infant intestine (Demers-Mathieu, 2022). Intestinal permeability is also higher in infants than in adults, with this permeability decreasing as the distance between neighboring cells narrows while the gut matures (Chelakkot et al., 2018; Gleeson et al., 2021). In 72 healthy infants the permeability test of lactulose:mannitol ratio was highest at birth, decreasing significantly during the first month of life (mean ± SD; 1.27 ± 0.73 at d 1 and 0.22 ± 0.21 at 1 mo; $P < 0.05$; Catassi et al., 1995). Neighboring barrier cells connect via tight junctions. The mRNA transcript levels of various tight junction biomarkers (*occludin*, *claudin-3*, *zonula occludens-1*, and *junctional adhesion molecule-A*) were significantly lower in the proximal and distal small intestine of infant mice pups compared with older adult mice ($P < 0.05$; Gleeson et al., 2021). In the infant gut, tight junction protein localization patterns differ from those in adults, with immunofluorescence staining revealing claudin-3 protein localized in the crypts in infant mice compared with in the midvillus and villus tip in adult mice (Gleeson et al., 2021). The infant gut microbiota also has lower microbial diversity compared with the adult (Odamaki et al., 2016; Lugli et al., 2023), and is heavily influenced by environmental factors such as mode of baby delivery, type of feeding, and health status (Hill et al., 2017; Yao et al., 2021).

Up to 6 mo of life, babies will consume either human breast milk or infant formula as a sole food. Breast milk is the optimum source of food, providing not only nutrients but also immunological factors, probiotics, and bioactive components (Henderson et al., 2022; Phosanam et al., 2021). The composition of breast milk is dynamic, changing not only over stage of lactation but also within a feed from foremilk to hindmilk (Martin et al., 2016). From a nutrient perspective, mature breast milk contains approximately 9 to 12 g/L protein, 67 to 78 g/L lactose, 12 to 14 g/L oligosaccharides, and 32 to 40 g/L fat (Ballard and Morrow, 2013; Kim and Yi, 2020). Mature breast milk also contains minerals such as iron (0.3–0.7 mg/L), calcium (200–250 mg/L), phosphorus (120–140 mg/L), magnesium (30–35 mg/L), sodium (150–250 mg/L), chloride (400–450 mg/L), potassium (400–550 mg/L), manganese (3–4 µg/L), iodine (140–150 µg/L), selenium (10–25 µg/L), copper (0.1–0.3 µg/L), and zinc (1–3 µg/L; Kim and Yi, 2020). Whey proteins account for 70% to 80% of total milk protein in early to mid-lactation (Martin et al., 2016), with α-LA accounting for 40% and lactoferrin at 26% of whey protein (Guo and Hendricks, 2008). The CN protein content in breast milk increases from 20% in early lactation to 50% in mature breast milk (Lönnerdal, 2003). The CN in breast milk consists primarily of β-CN and κ-CN but also has trace concentrations of α_{s1}-CN (Basdeki et al., 2021).

Breast milk has a digestible indispensable AA score (**DIAAS**) of 101, determined in 25 d old Yucatan piglets (n = 9) who received breast milk for 6 d (Charton et al., 2023). Complete gastric emptying of breast milk in infants (n = 26, age 4.7 ± 1.7 mo) takes 2.12 ± 1.07 h, as defined by a cross-sectional area of ≤3.07 cm^2, determined by ultrasound (Das et al., 2024). Using simulated infant conditions, digestibility of breast milk was reported at 48.89% at the end of the gastric phase (60 min; Xiao et al., 2023). Under in vitro infant gastric conditions, breast milk samples formed large curds or aggregates in the stomach with particles sizes ranging between 1 and 10 µm and 10 and 1,000 µm (He et al., 2022). Surprisingly, a semidynamic in vitro infant gastrointestinal system observed only 6% protein hydrolysis of breast milk after 120 min gastric digestion but >38% following intestinal digestion (Abrahamse et al., 2022).

It is widely accepted that infants who receive breast milk have a lower incidence of diarrhea and lower risk of developing necrotizing enterocolitis than bottle-fed babies (Lyons et al., 2020). Preterm infants (n = 62, ≤32 wk of gestation) receiving breast milk also had significantly lower intestinal permeability (lactulose:mannitol ratio) compared with preterm infants fed infant formula at 1 wk postpartum (0.076 vs. 0.205, $P = 0.04$; Taylor et al., 2009). Rasmussen et al. (2016) also observed significantly higher brush border membrane aminopeptidase N, aminopeptidase A, lactase, and dipeptidyl peptidase IV activities in the proximal small intestine of preterm pigs (n = 47, 105–106 d gestation) fed breast milk compared with those fed commercial infant formula ($P < 0.05$; Rasmussen et al., 2016). In human enteroid monolayers, exposure to breast milk (for 24 and 72 h) has been shown to increase goblet cell and Paneth cell number and expression of occludin protein levels (Noel et al., 2021). With increases in digestive enzyme capacity, goblet cell and Paneth cell numbers, tight junction markers, and a reduction in permeability, breast milk is beneficial to the intestinal epithelium, helping to mature the infant gut.

In circumstances where breastfeeding is not possible, the World Health Organization recommends the use of infant formula (World Health Organization, 2013). Under Commission Regulation (EU) No. 609/2013 infant formula is described as a "food intended for use by infants during the first months of life and satisfying by itself the nutritional requirements of such infants until the introduction of appropriate complementary feeding" (Commission Regulation, 2013). Before, during, and after processing infant formula is heat-treated. These high thermal treatment steps ensure a microbiologically safe product with an extended shelf life (Wada and Lönnerdal, 2015b). Bovine milk-based infant formulas are the most common, commercially available infant formula on the market (Halabi et al., 2020b; He et al., 2022). Heat treat-

ment of infant milk formula (**IMF**) will induce denaturation and aggregation of milk proteins (Pellegrino et al., 2011), and the formation of undesirable Maillard reaction products (Lund et al., 2022). This differs substantially from breast milk. The objective of this narrative literature review is to evaluate the consequences of IMF heat treatment on protein digestion and gut barrier health in the infant gut, using data generated solely from in vitro and preclinical studies. For comparison purposes, in vitro and preclinical data generated with mildly heat-treated IMF will also be summarized. The hypothesis is that mildly heat-treated IMF may be more similar to breast milk in the context of protein digestion and gut physiology in the infant.

INFANT FORMULA COMPOSITION

When producing first-stage infant formula, manufacturers aim to produce a product with a macronutrient and micronutrient composition that mimics breast milk (He et al., 2022). In addition, and in an attempt to achieve the health promoting attributes of breast milk, manufacturers will formulate first-stage infant formulas with additional ingredients, such as omega-3 fatty acids and omega-6 fatty acids, oligosaccharides, probiotics, prebiotics, taurine, lactoferrin, folic acid, L-carnitine, α-LA, osteopontin, and milk fat globule membrane (Almeida et al., 2021, Bakshi et al., 2023).

Skim milk is the starting ingredient for IMF. To achieve a whey-to-CN ratio of 60:40 (similar to mature breast milk), skim milk powder (**SMP**), whey protein isolate (**WPI**), or whey protein concentrate (**WPC**) or combinations are added (Phosanam et al., 2021; Bakshi et al., 2023). The base formula will also contain lactose, vegetable oil, and vitamins and minerals (Bakshi et al., 2023). Protein composition of bovine milk differs from mature breast milk with a whey-to-CN ratio of 20:80 (Navis et al., 2020b) and the presence of β-LG, which is absent in breast milk (Ballard and Morrow, 2013). By correcting the whey-to-CN ratio with the addition of WPI or WPC, the protein content of IMF (1.8–2.5 g/100 kcal) is typically higher than breast milk (EFSA Panel on Dietetic Products and Allergies, 2014).

IMF PROCESSING

Ingredient suppliers rarely disclose the processing histories of their dairy ingredients but traditionally SMP, WPI, and WPC are routinely subjected to thermal processing before IMF manufacturing (Lin et al., 2018). Then, during IMF processing, these same ingredients undergo a series of heat-processing steps including pasteurization (72°C–75°C for 15 s) and UHT (135°C–150°C for 1–10 s; Dash et al., 2022). Finally, for ease of transport and to extend shelf life, liquid IMF is typically spray dried (inlet temperature 180°C–200°C and outlet temperature 80°C–100°C) to produce an IMF powder (Masum et al., 2020). High thermal loads ensure a microbiologically safe product. Routine monitoring and testing is required in manufacturing facilities for the indicator bacteria, *Enterobacteriaceae* (Commission Regulation [EC] No. 2073/2005; Commission Regulation, 2005). Dried infant formula must not contain *Cronobacter sakazakii* or *Salmonella* spp. (Commission Regulation, 2005).

PROTEIN DENATURATION AND AGGREGATION IN THERMALLY PROCESSED IMF

The effects of heat on milk and milk products have been reviewed previously by Pellegrino et al. (2011). Table 1 summarizes the main effects of heat treatment of IMF ingredients (SMP, WPI, and WPC) and IMF final products on (a) milk protein structure, (b) protein interactions, and (c) Maillard reaction products.

For IMF protein ingredients, Lin et al. (2018) observed that 80.75% of the total whey protein content in skim milk was denatured when this milk was heated at 120°C for 120 s and spray dried at inlet and outlet temperatures of 180°C and 85°C, respectively, to produce SMP (Lin et al., 2018; Table 1). In addition, 86.7% of this denatured whey protein had associated with the CN micelle (Lin et al., 2018). This high-heat-treated SMP had a particle size of 213 nm (Lin et al., 2018). In skim milk heated at 75°C to 100°C, the CN micelle size increased by 30 to 35 nm (Anema and Li, 2003). For WPC, 70% of whey protein was denatured when this ingredient was heated to 73°C for 30 s followed by 80°C for 6 min (Navis et al., 2020b). Only 21% of the protein was soluble (Navis et al., 2020b). Where WPI was preheated to either 72°C for 2 min or 85°C for 2 min, whey protein denaturation was 25.5% and 74.8%, respectively (Joyce et al., 2017). Li et al. (2013) observed that a WPC produced from acid whey that was double pasteurized and spray dried, contained 0.05 g/L native lactoferrin and 0.2 g/L native IgG (Li et al., 2013; Table 1).

Once protein ingredients are sourced and IMF processing begins, it is important to note that protein denaturation and aggregation in heated IMF powders is dependent on β-LG concentration (Crowley et al., 2016). β-lactoglobulin is more heat labile than α-LA because the compact globular structure of α-LA results in higher heat tolerance and a resistance to forming covalent bonds with other proteins (Buggy et al., 2017). Joyce et al. (2017) observed that IMF processed at 85°C for 2 min resulted in 94.7 ± 0.66% denaturation of β-LG (Joyce et al., 2017). This rose to 98 ± 0.22% denaturation when a heat-treated WPI ingredient was used (Joyce et al., 2017). Denaturation of α-LA was lower at 78 ± 1.07%, which rose to

Table 1. Effect of processing on IMF protein ingredients and proteins in IMF[1]

Ingredient or IMF product	Processing parameter	Effect on protein structure, protein interactions, and AGE	Reference
	Ingredient		
2 SMP	High-heat SMP: 120°C for 120 s Pasteurized SMP: 72°C for 15 s	High-heat SMP ↑ whey protein denaturation, ↑ interactions with CN micelle and ↑ particle size vs. pasteurized SMP	Lin et al., 2018
2 WPC from whole milk produced by cold filtration	Extensively heated WPC: 73°C for 30 s and 80°C for 6 min Pasteurized WPC: 73°C for 30 s	Extensively heated WPC ↑ whey protein denaturation and ↑ CML levels vs. pasteurized WPC Pasteurized WPC had 100% native whey protein	Navis et al., 2020b
3 WPC	Double-pasteurized skim milk WPC: 72°C for 15 s and 80°C for 30 s Stored (37°C for 6 wk) double-pasteurized skim milk WPC Pasteurized skim milk WPC (from sweet whey): 72°C for 15 s	Stored double-pasteurized WPC ↑ CML and furosine levels vs. pasteurized and double-pasteurized skim milk WPC	Aasmul-Olsen et al., 2024
3 WPC	Double-pasteurized WPC (from acid whey), membrane filtered and standard spray dried Pasteurized WPC (from acid whey), membrane filtered and low-temperature spray dried Membrane-filtered WPC (from sweet whey), freeze-dried and γ-irradiated	Double-pasteurized WPC: 0.05g/L native lactoferrin and 0.2 g/L native IgG Pasteurized WPC: 0.66 g/L native lactoferrin and 3.8 g/L native IgG Membrane-filtered WPC: 0.49 g/L native lactoferrin and 4.6 g/L native IgG	Li et al., 2013
2 WPI	WPI heated at 85°C for 2 min WPI heated at 72°C for 2 min	WPI heated at 85°C for 2 min ↑ whey protein denaturation vs. WPI heated at 72°C for 2 min	Joyce et al., 2017
	IMF final product		
2 liquid IMF	IMF heated at 85°C for 2 min IMF heated at 72°C for 2 min	IMF heated at 85°C for 2 min ↑ denaturation of β-LG and α-LA and ↑ protein particle size in (with preheated WPI) vs. IMF heated at 72°C for 2 min	Joyce et al., 2017
2 IMF powders produced at pilot-plant scale (100 kg)	Extensively heated IMF: double pasteurization at 72°C for 30 s and 90°C for 2–3 s, evaporated and heated at 85°C for 2 min and spray dried Membrane-filtered IMF: 0.8- and 0.1-µm membranes and spray dried	Extensively heated IMF ↑ whey protein denaturation vs. membrane-filtered IMF (58 ± 0% vs. 6 ± 4%) Extensively heated IMF ↓ bacterial load No difference in CML or furosine	Yu et al., 2021
2 IMF powders produced at laboratory scale	High-temperature IMF: 125°C for 5 s and spray dried Membrane-filtered IMF: 1.4- and 0.2-µm membranes and spray dried	High-temperature IMF ↓ native whey protein vs. membrane-filtered IMF (2.5% vs. 18.3%) High-temperature IMF↓ total bacterial count vs. membrane-filtered IMF (5.6×10^1 vs. 1.3×10^2 cfu/g of powder) No difference in CML between high-temperature IMF and membrane-filtered IMF (3.97 ± 0.7 and 3.65 ± 0.74 µg/g of powder)	Chen et al., 2021
2 IMF powders produced at pilot-plant scale (250 kg)	High-temperature IMF: 125°C for 5 s and spray dried Membrane-filtered IMF: 1.4 and 0.2 µm membranes and spray dried	High-temperature IMF ↓ native whey protein vs. membrane-filtered IMF (4.5% vs. 59.9%) High-temperature IMF↓ total bacterial count vs. membrane-filtered IMF (8.3×10^2 vs. 1.8×10^7 cfu/g of powder)	Chen et al., 2023; Dold et al., 2024
2 liquid IMF produced at laboratory scale	IMF heated at 90°C for 15 s IMF heated at 75°C for 15 s	IMF heated at 90°C for 15 s ↓ native whey protein vs. IMF heated at 75°C for 15 s	Halabi et al., 2020b

Continued

Table 1 (Continued). Effect of processing on IMF protein ingredients and proteins in IMF[1]

Ingredient or IMF product	Processing parameter	Effect on protein structure, protein interactions, and AGE	Reference
2 liquid IMF produced at laboratory scale	IMF with commercial sodium caseinate and WPI in batch sterilized at 110°C for 38 min IMF with sodium caseinate and WPC (acid whey) from pasteurized skim milk in batch sterilized at 110°C for 38 min	In-batch sterilized IMF (with commercial ingredients) ↑ furosine and lysinoalanine levels vs. in-batch sterilized IMF (with sodium caseinate and WPC from pasteurized skim milk) No difference in pyrraline levels	Cattaneo et al., 2017
3 liquid IMF produced at manufacturing scale	UHT IMF: 143°C for 6 s Stored UHT IMF (40°C for 60 d) Pasteurized IMF: 72°C for 10 s	UHT IMF and stored UHT IMF ↑ CML and furosine levels and ↑ protein aggregation vs. pasteurized IMF	Sun et al., 2022

[1]SMP = skim milk powder; WPC = whey protein concentrate; WPI = whey protein isolate; IMF = infant milk formula; AGE = advanced glycation end product; CML = N(6)-carboxymethyllysine; CEL = N(6)-carboxyethyllysine. Up and down arrows signify increases and decreases, respectively.

94.2 ± 0.78% with the heat-treated WPI ingredient (Joyce et al., 2017). Not surprisingly, the use of preheated WPI also resulted in increased protein particle size from 176 ± 4 nm to 278 ± 15 nm (Joyce et al., 2017). Fourier-transform infrared spectroscopy data demonstrated that as IMF powders (11 g of protein/100g IMF powder) are heated beyond 50°C, whey protein structures will switch from β-sheets to β-turns, with no changes in α-helices (Ye et al., 2017). In an extensively heat processed IMF powder (double pasteurization of milk at 72°C for 30 s and 90°C for 2–3 s before evaporation and 85°C for 2 min before spray drying), 58% of whey proteins were denatured (Yu et al., 2021). An IMF powder heated at 125°C for 5 s followed by single-stage spray drying contained 97.5% denatured whey protein (Chen et al., 2021). Interestingly, the WPI ingredient used in this formulation already contained 8.5% denatured protein (Chen et al., 2021). Only 5.19% of the total β-LG content in the IMF was in its native, monomeric form compared with 16.57% of the total α-LA (Chen et al., 2021; Table 1).

In the quest to mimic breast milk, IMF manufacturers have toyed with various α-LA:β-LG ratios. Crowley et al. (2016) observed that unheated IMF, regardless of α-LA:β-LG ratio, had protein particle size similar to CN micelles, whereas IMF heated to 140°C had a protein particle size ranging from 319 ± 80.2 nm for IMF (0.1 α-LA:1 β-LG) to 165 ± 2 nm for IMF (4.6 α-LA:1 β-LG; Crowley et al., 2016). Similarly, after heat treatment at 90°C for 15 s, 81% of whey proteins remained in their native state in a liquid IMF formulated with 0.06 g β-LG/100 g IMF in contrast to 48% in an IMF containing 0.51 g β-LG/100 g IMF (Halabi et al., 2020b; Table 1).

Where IMF ingredients are heat-treated or liquid IMF is thermally processed, spray drying has only a minor effect on whey protein denaturation and aggregation (Oldfield et al., 2005; Yu et al., 2021).

ADVANCED GLYCATION END PRODUCTS IN THERMALLY PROCESSED IMF

The Maillard reaction is a nonenzymatic, complex series of reactions between reducing sugars and AA that occurs at high temperatures (Kumar et al., 2017). The final result is "browning," that is, the formation of advanced glycation end products (**AGE**) such as N(6)-carboxymethyllysine (**CML**), N(6)-carboxyethyllysine (**CEL**), and pyrraline. During thermal processing of IMF, the early-stage Maillard reaction occurs between the carbonyl group of the reducing sugar lactose and an available free amino group, which results in the creation of a Schiff base (Van Boekel, 1998). Rearrangements in the unstable Schiff base leads to the formation of the stable early Maillard reaction products known as Amadori products (Horvat and Jakas, 2004). Detection of furosine

is commonly used as an indicator of early-stage Maillard reaction (Chen et al., 2019; Li et al., 2021b). Furfural compounds such as 5-hydroxymethylfurfural, 5-methyl-2-furaldehyde, 2-furyl-methyl ketone, and 2-furaldehyde are indicators of intermediate Maillard reaction products (Chávez-Servín et al., 2015). Finally, AGE such as CML, CEL, and pyrraline are signals of advanced-stage Maillard reaction (Chen et al., 2019; van der Lugt et al., 2020; Li et al., 2021b). Several studies have reported furosine and CML concentrations in commercially available liquid IMF and IMF powders (Fenaille et al., 2006; Chen et al., 2019). In addition, AGE are commonly quantified during IMF processing and in the final product as an indicator of the extent of Maillard reaction (Chen et al., 2019; Lee et al., 2019; Chen et al., 2021; Yu et al., 2021; Sun et al., 2022; Aasmul-Olsen et al., 2024). Importantly, micronutrients such as vitamin C and iron, added to IMF formulations, can promote the Maillard reaction by acting as precursors (Leclère et al., 2002; Pischetsrieder et al., 2005). However, it must be noted that it is difficult to compare reported levels of Maillard reaction products, including furosine, in final IMF products. Concentrations of these compounds will vary, depending on detection methods used, ingredient quality, protein concentration, and lactose content in the formulation, different IMF processing strategies, and storage conditions.

Table 1 lists the effect of heat treatment of IMF ingredients and IMF final products on Maillard reaction products. Before IMF processing, thermal processing of dairy ingredients will result in the presence of Maillard reaction products where lactose is present. Aasmul-Olsen et al. (2024) reported furosine and CML concentrations of 2,893 ± 105 μg/g of protein and 160 ± 4 μg/g of protein, respectively, in a double-pasteurized WPC (72°C for 15 s and 80°C for 30 s) before incorporation into an IMF suitable for piglets (Aasmul-Olsen et al., 2024). The ingredient contained 13.2% lactose (Aasmul-Olsen et al., 2024). However, CEL levels were below the limits of detection of liquid chromatography (**LC**)-MS/MS (Aasmul-Olsen et al., 2024). Using ultra-fast LC/fluorescence, Navis et al. (2020b) reported approximately 19 μg/mL CML in an extensively heated WPC (73°C for 30 s and 80°C for 6 min; Navis et al., 2020b; Table 1).

Cattaneo et al. (2017), using in-batch sterilization produced liquid IMF by sourcing commercial sodium caseinate and WPI (Cattaneo et al., 2017). This IMF had 6,378 ± 225 μg furosine/g of protein, 55 ± 2 μg pyrraline/g of protein and 999 ± 95 μg lysinoalanine/g of protein (Cattaneo et al., 2017; Table 1).

In UHT-treated liquid IMF, quantified by LC-MS/MS, Sun et al. (2022) reported furosine, CML, and CEL concentrations of 2,798 ± 136 μg/g of protein, 242 ± 9 μg/g of protein and 71 ± 1 μg/g of protein, respectively (Sun et al., 2022). After spray drying, a heat-treated IMF powder contained 3.97 ± 0.7 μg CML/g of powder (Chen et al., 2021). In an extensively heat processed IMF powder, furosine concentration was approximately 5,000 μg/g of protein and CML concentrations was 106 ± 0 μg/g of protein (Yu et al., 2021; Table 1).

Lee et al. (2019) reported a contribution of the spray-drying process to CML content in IMF powder, with an increase from approximately 30 μg/g of protein in the heat-treated liquid IMF to 60 μg/g of protein in the final powder, albeit no processing temperatures before spray drying were given (Lee et al., 2019). Yu et al. (2021) have previously shown no contribution by spray drying to furosine or CML content in IMF with high thermal loads. However, it is well established that spray drying and storage of dairy powders will promote the Maillard reaction (van Lieshout et al., 2020). The level of AGE in the final IMF powder will be determined by the level of protein denaturation during processing, the inlet and outlet temperatures of spray drying, storage temperature, and relative humidity (Zhu et al., 2018; van Lieshout et al., 2020).

INFLUENCE OF THERMAL PROCESSING OF IMF ON PROTEIN DIGESTION IN THE INFANT

Thermally processed IMF will deliver aggregated, denatured proteins and AGE to the infant gut. The protein digestibility (DIAAS) of heat-treated IMF in the infant gut cannot be generalized because it is governed by the extent of these factors in the specific IMF product and the level they interfere with digestive enzymes. Recently, van Lieshout et al. (2020) and Li et al. (2021a) reviewed the effect of these components, within a variety of dairy products, on protein digestion in the adult stomach and small intestine (van Lieshout et al., 2020; Li et al., 2021a). Specifically, Charton et al. (2023) recently reported a DIAAS of 83 for an extensively heat processed IMF powder, which is considerably lower than breast milk at a DIAAS of 101, as determined in young pigs. Table 2 summarizes the effect of IMF processing on protein digestion in the infant gut using data generated from in vitro and preclinical models.

Chen et al. (2022) produced a high-temperature (125°C for 5 s) IMF powder, which was subjected to an in vitro semidynamic digestion mimicking the physiological conditions of the infant stomach (Chen et al., 2022). Once IMF was added to the gastric chamber, the pH rose immediately from 2.5 to 7.57 ± 0.13 (Chen et al., 2022). A large compact curd was formed and remained to the end of the gastric phase (156 min, pH 3.2; Chen et al., 2022). Confocal laser scanning microscopy revealed microstructures of protein aggregates remaining on fat droplet surfaces at 156 min of gastric digestion (Chen et al., 2022). The SDS-PAGE analysis showed

Table 2. Effect of IMF processing on protein digestibility[1]

Product	Processing parameter	Method	Measure of digestibility	Effect on protein digestibility	Reference
2 IMF powders produced at laboratory scale	High-temperature IMF: 125°C for 5 s and spray dried Membrane-filtered IMF: 1.4- and 0.2-µm membranes and spray dried	INFOGEST in vitro semidynamic infant gastric digestion and INFOGEST in vitro static infant gastrointestinal digestion	SDS-PAGE and degree of protein hydrolysis	High-temperature IMF ↑ gastric pH at 0 and 31 min, ↓ protein hydrolysis and ↑ β-LG digestion vs. membrane-filtered IMF High-temperature IMF formed large aggregates in stomach High-temperature IMF ↓ water-soluble material in gastric phase at 0, 31, 62, 94, and 125 min vs. membrane-filtered IMF	Chen et al., 2022
		INFOGEST in vitro static infant gastrointestinal digestion		High-temperature IMF and membrane-filtered IMF had similar protein hydrolysis and no intact proteins visible at 60 min of intestinal digestion	Bavaro et al., 2021
2 IMF powders produced at pilot-plant scale (250 kg)	High-temperature IMF: 125°C for 5 s and spray dried Membrane-filtered IMF: 1.4- and 0.2-µm membranes and spray dried	28-d-old pigs (n = 20) fed IMF diets (35% inclusion) for 28 d	Degree of protein hydrolysis and AA analysis	High-temperature IMF ↓ protein hydrolysis in stomach and duodenum vs. membrane-filtered IMF (stomach: 509 ± 17 vs. 599 ± 45 µmol free amines/g of protein and duodenum: 1,174 ± 124 vs. 1,530 ± 136 µmol free amines/g of protein) High-temperature IMF ↓ protein/peptide content in lumen, ↓ release of Pro in duodenum and ↓ taurine and Trp in jejunum vs. membrane-filtered IMF	Chen et al., 2023
2 IMF powders produced at pilot-plant scale (100 kg)	Extensively heated IMF: double pasteurization at 72°C for 30 s, 90°C for 2–3 s before evaporation and 85°C for 2 min before spray drying Membrane-filtered IMF: 0.8- and 0.1-µm membranes and spray dried	28 d Wistar Han rats (n = 60) fed IMF diets (40% inclusion) for 14 d	Total nitrogen digestibility, DIAAS, and plasma AA concentration	Similar DIAAS for extensively heated IMF and membrane-filtered IMF (0.82 and 0.8) Extensively heated IMF ↓ plasma indispensable and total AA concentrations 1 h after ingestion vs. membrane-filtered IMF No change in dietary nitrogen in stomach and SI in extensively heated IMF vs. membrane-filtered IMF (stomach: 0.14 ± 0.34% vs. 0.04 ± 0.09% and SI: 1 ± 1.66% vs. undetectable)	Calvez et al., 2024
2 liquid IMF produced at laboratory scale	IMF heated at 67.5°C for 200 min or 80°C for 7 min Unheated IMF	INFOGEST in vitro static infant gastrointestinal digestion	SDS-PAGE and AA analysis	Heated IMF↓ protein hydrolysis vs. unheated IMF at 5 min of intestinal digestion No difference in bioaccessibility of AA	Halabi et al., 2020a
2 IMF powders produced at manufacturing scale	IMF dry heated at 70°C for 335 h Unheated IMF	INFOGEST in vitro static infant gastrointestinal digestion	SDS-PAGE and degree of protein hydrolysis	Dry-heated IMF ↓ protein hydrolysis vs. unheated IMF at 60 min of gastric digestion and 120 min of intestinal digestion Dry-heated IMF ↑ peptide length in intestinal digesta vs. unheated IMF	Zenker et al., 2020

Continued

Table 2 (Continued). Effect of IMF processing on protein digestibility[1]

Product	Processing parameter	Method	Measure of digestibility	Effect on protein digestibility	Reference
2 liquid IMF produced at manufacturing scale	UHT IMF: 143°C for 6 s (direct or indirect) Pasteurized IMF: 72°C for 10 s	In vitro static infant gastrointestinal digestion	SDS-PAGE and LC-MS/MS	β-LG undigested in pasteurized IMF UHT IMF ↑ protein aggregates in digesta vs. pasteurized IMF	Ye et al., 2022
2 liquid IMF produced at laboratory scale with commercial sodium caseinate and WPI or sodium caseinate and WPC (acid whey) from pasteurized skim milk	IMF in batch sterilized at 110°C for 38 min Unheated IMF	INFOGEST in vitro static gastrointestinal digestion	Degree of proteolysis calculated based on nitrogen content of a 3 kDa fraction of digesta	IMF with commercial sodium caseinate and WPI: in-batch sterilized IMF ↑ proteolysis vs. unheated IMF (40.3 ± 1.2% vs. 35.6 ± 0.6%) IMF with sodium caseinate and WPC (acid whey) from pasteurized skim milk: in-batch sterilized IMF ↓ proteolysis vs. unheated IMF (54 ± 0.9% vs. 35%± 0.9%)	Cattaneo et al., 2017

[1]IMF = infant milk formula; DIAAS = digestible indispensable amino acid score; SI = small intestine.

that at 125 min intact CN were still present (Chen et al., 2022). By the end of the gastric phase, β-LG bands had weakened considerably but α-LA was still present (Chen et al., 2022). The degree of protein hydrolysis, determined by the o-phthalaldehyde method, at the end of the gastric phase was 845 ± 78 μmol free amines/g of protein (Chen et al., 2022). Not surprisingly, the release of peptides (<10 kDa) from heated IMF digesta increased significantly from the beginning to the end of gastric digestion ($P < 0.05$; Chen et al., 2022). At the end of the intestinal phase, using an in vitro static protocol for infant digestion (Ménard et al., 2018), no intact proteins were visible by SDS-PAGE with this high-temperature IMF (Bavaro et al., 2021). In a follow-on preclinical study, young pigs were fed a diet composed of this high-temperature IMF and lumen samples were collected from 4 different gut locations 180 min after the final meal (Chen et al., 2023). Pigs fed the heated IMF had a degree of protein hydrolysis of 509 ± 17 μmol free amines/g of protein in the stomach and 1,174 ± 124 μmol free amines/g of protein in the duodenum, with an overall value for the stomach, duodenum, jejunum, and ileum of 1,022 ± 43 μmol free amines/g of protein (Chen et al., 2023). The concentration of free AA released was 59.4 ± 3.05 μmol/g of protein in the gastric phase, 171.9 ± 29.7 μmol/g of protein in the duodenum, 211.4 ± 22.75 μmol/g of protein in the jejunum, and 285 ± 28.51 μmol/g of protein in the ileum (Chen et al., 2023). In contrast, Calvez et al. (2024) fed Wistar Han rats (n = 30, 28 d old) with extensively heat-treated IMF at 40% inclusion (Calvez et al., 2024). Levels of dietary nitrogen recovered in the stomach (0.14 ± 0.34%) and the small intestine (1 ± 1.66%) 6 h after ingestion were not influenced by heating (Calvez et al., 2024).

The results of Halabi et al. (2020a) showed that in vitro infant gastric digestion of heat-treated IMF (80°C) formed small curd particles of 10 μm. Thermal treatment of IMF at either 67.5°C or 80°C supported gastric CN hydrolysis with only 6 ± 1% of residual intact CN still present after 30 min of gastric digestion (Halabi et al., 2020a). However, the degree of protein hydrolysis was low in IMF heated at 80°C, quantified at only 2 ± 1% after 60 min of gastric digestion, rising to 14 ± 2% within 5 min of the intestinal phase and finishing at 35 ± 2% at the end of the intestinal phase (Halabi et al., 2020a). The bioaccessibility of AA as a percentage of total AA was lower than expected, with 35.5 ± 1.2% for EAA and 10.3 ± 0.6% for NEAA in IMF heated at 80°C (Halabi et al., 2020a). Lysine, Phe, Tyr, Leu, and Arg were higher than all other AA, and free Pro concentration was negligible (Halabi et al., 2020a). Gómez-Gallego et al. (2016) produced an IMF powder with heat treatment at 70°C and then 105°C before spray drying. An in vitro infant gastrointestinal digestion protocol was performed (Gómez-

Gallego et al., 2016). No intact proteins were visible at the end of the intestinal phase by SDS-PAGE and levels of NPN rose from 0.19 ± 0.02 mg/g before digestion to 0.95 ± 0.2 mg/g after digestion yielding an estimated in vitro digestibility of 45.19 ± 11.29% (Gómez-Gallego et al., 2016). Liquid chromatography-MS/MS identified 87 unique peptides from in vitro digestion of the final IMF product at the end of the intestinal phase (Gómez-Gallego et al., 2016). Cattaneo et al. (2017) reported that the degree of proteolysis, during in vitro infant gastrointestinal of in-batch sterilized liquid IMF, was 40.3 ± 1.2% (Cattaneo et al., 2017). This was calculated based on the nitrogen content of a 3 kDa fraction of digesta (Cattaneo et al., 2017). A standard IMF produced by pasteurization, evaporation, and spray drying underwent an in vitro semidynamic gastric digestion simulating infant digestion (Lambers et al., 2023). The degree of protein hydrolysis ranged from approximately 0.11 to 0.18 μmol free amines/mg of protein from the early stage of gastric digestion at pH 6 to the end of digestion (final pH of 3.5; Lambers et al., 2023). The molecular weight distribution analysis revealed that approximately 53% of peptides were >50 kDa at pH 3.5, indicating low levels of digestion in the gastric phase (Lambers et al., 2023). Ye et al. (2022) produced a liquid IMF by direct or indirect UHT (143°C for 6 s) processing (Ye et al., 2022). This study performed an in vitro static infant gastrointestinal digestion with gastric digestion at pH 4 for 2 h followed by 6 h intestinal digestion at pH 6.5 (Ye et al., 2022). At the end of the gastric phase, aggregates (>260 kDa) in both UHT IMF remained resistant to pepsin (Ye et al., 2022). Following intestinal digestion all protein bands, including aggregates, were digested (Ye et al., 2022). The LC-MS/MS analysis revealed the total number of peptides released during digestion was similar in both direct and indirect UHT treatments (Ye et al., 2022). Interestingly, there were significantly more β-CN peptides in the intestinal digesta from indirect UHT IMF compared with the direct UHT IMF ($P < 0.05$; Ye et al., 2022). Direct UHT processing had a lower heat load indicated by significantly lower levels of lactosylation in released β-LG peptides from direct UHT IMF compared with indirect UHT IMF ($P < 0.05$; Ye et al., 2022).

Previous studies have shown that structural changes to dairy proteins induced by the Maillard reaction can influence protein digestion by exposing or blocking enzymatic cleavage sites (Joubran et al., 2015; Joubran et al., 2017). Zenker et al. (2020) induced a Maillard reaction in a commercially-sourced IMF powder by dry heating to 70°C for 335 h (Zenker et al., 2020). This generated a powder with 2,022 mg furosine/100 g of protein (Zenker et al., 2020). At the end of in vitro infant gastric digestion, the degree of protein hydrolysis was only 4.3 ± 0.2% rising to 29.8 ± 0.8% at the end of the intestinal phase (Zenker et al., 2020). A rapid disappearance in CN bands was observed in the gastric phase whereas intact β-LG was still present at 60 min of intestinal digestion (Zenker et al., 2020). This corresponds to results from peptide identification experiments where fewer whey protein-derived peptides (3.9 ± 0.19%) were identified compared with CN-derived peptides (96.1 ± 0.19%) at the end of the intestinal phase (Zenker et al., 2020). Levels of furosine in the IMF did not influence peptide size distribution at the end of the gastric phase but at the end of the intestinal phase there were significantly more peptides with 15–19 AA, 25–29 AA, and 30–34 AA, and significantly fewer peptides with 5–9 AA compared with the control IMF (308 mg furosine/100 g of protein; $P < 0.05$; Zenker et al., 2020). Overall, the average peptide length was significantly higher in dry-heated IMF compared with the control IMF at the end of the intestinal phase ($P < 0.05$; Zenker et al., 2020). Zenker et al. (2020) calculated that 44.5% of the AA Lys was blocked from enzymatic hydrolysis due to its reaction with lactose (Zenker et al., 2020). This blockage impaired the breakdown of larger peptides (Zenker et al., 2020).

IMPACT OF THERMAL PROCESSING OF IMF ON GUT BARRIER HEALTH

Recently, researchers have used both in vitro and preclinical approaches to examine the relationship between thermally processed IMF and gut barrier health (Li et al., 2013; Navis et al., 2020a; Navis et al., 2020b; Bavaro et al., 2021; Sun et al., 2022; Aasmul-Olsen et al., 2024; Dold et al., 2024). Table 3 summarizes the results of these studies. In many cases, differences observed in gut barrier response have been attributed to higher levels of Maillard reaction products in IMF with high thermal loads (Sun et al., 2022; Aasmul-Olsen et al., 2024). Individual AGE compounds can interact with the gut barrier directly to mediate an adverse effect (Guibourdenche et al., 2021; Wu et al., 2022). However, Maillard reaction products in thermally processed IMF may not be the sole contributor to barrier response, with protein structure also playing a role (Li et al., 2013; Navis et al., 2020a; Navis et al., 2020b; Bavaro et al., 2021; Dold et al., 2024). Indeed it is likely that both protein structure and AGE levels will modulate digestive enzyme accessibility to milk protein cleavage sites, resulting in differences in bioactive peptides released during digestion. This difference in bioactive capacity may also drive gut response.

Sun et al. (2022) fed preterm pigs UHT liquid IMF for 5 d by an orogastric feeding tube. A wide range of Maillard reaction products and cross-linked AA were quantified in the UHT IMF (Sun et al., 2022). To further increase these levels, the UHT IMF was stored at 40°C for 60 d before the intervention trial (Sun et al., 2022). The ileal mucosa

Table 3. Impact of IMF processing on gut barrier health[1]

Product	Processing parameter	Method	Gut barrier health assessment	Impact on gut barrier health	Reference
3 liquid IMF produced at manufacturing scale	UHT IMF: 143°C for 6 s Stored UHT IMF (40°C for 60 d) Pasteurized IMF: 72°C for 10 s	Preterm pigs (n = 56, 106-d gestation) received IMF by orogastric feeding tube for 5 d	Histology, intestinal permeability (lactulose: mannitol ratio), and brush border enzyme activity	UHT IMF ↓ SI villus height and ↑ distal SI crypt depth vs. pasteurized IMF Stored UHT IMF ↓ SI villus height, ↑ SI crypt depth and ↓ proximal and middle SI lactase activity vs. pasteurized IMF No difference in lactulose:mannitol ratio	Sun et al., 2022
3 IMF powders produced with different WPC	Double-pasteurized skim milk WPC: 72°C for 15 s and 80°C for 30 s Stored (37°C for 6 wk) double-pasteurized skim milk WPC Pasteurized skim milk WPC (from sweet whey): 72°C for 15 s	Preterm piglets (n = 72, 105-d gestation) received IMF by orogastric feeding tube for 5 d	Histology, AGE quantified in distal SI tissue by LC-MS/MS, and brush border enzyme activity	IMF with stored double-pasteurized WPC ↑ CML and furosine in distal SI tissue vs. IMF with pasteurized WPC and double-pasteurized WPC No change in villus height, crypt depth, and brush border enzymes	Aasmul-Olsen et al., 2024
2 IMF powders produced at pilot-plant scale (250 kg)	High-temperature IMF: 125°C for 5 s and spray dried Membrane-filtered IMF: 1.4- and 0.2-µm membranes and spray dried	28-d-old pigs (n = 20) fed IMF diets (35% inclusion) for 28 d	Histology, RT-qPCR, Western blot, acidic mucus levels, and brush border enzyme activity	In duodenum high-temperature IMF ↓ brush border lactase activity and ↓ *claudin-1*, *mucin-1*, and *mucin-2* mRNA transcripts vs. membrane-filtered IMF In jejunum high-temperature IMF ↓ goblet cell number, ↓ acidic mucus levels, ↓ mucin-2 protein, and ↑ *RAGE* mRNA transcripts vs. membrane-filtered IMF No change in villus height or crypt depth	Dold et al., 2024
2 IMF powders produced at laboratory scale	High-temperature IMF: 125°C for 5 s and spray dried Membrane-filtered IMF: 1.4- and 0.2-µm membranes and spray dried	INFOGEST in vitro static infant gastrointestinal digestion and 21-d-old Caco-2 monolayers treated with 200 µg of protein/cm^2	TEER and Western blot	High-temperature IMF ↓ TEER and claudin-1 protein in Caco-2 monolayers vs. membrane-filtered IMF	Bavaro et al., 2021
3 liquid IMF produced with different WPC	IMF with double-pasteurized WPC (from acid whey) IMF with pasteurized WPC (from acid whey) IMF with membrane-filtered WPC (from sweet whey)	Preterm pigs (n = 55, 105-d gestation) received IMF by orogastric feeding tube for 5 d	Histology, Western blot, intestinal permeability (lactulose:mannitol ratio), inflammatory biomarkers, and brush border enzyme activity	IMF with double-pasteurized WPC ↓ proximal SI villus height vs. IMF with pasteurized WPC and IMF with membrane-filtered WPC IMF with double-pasteurized WPC ↑ lactulose:mannitol ratio vs. IMF with membrane filtered WPC No change in inflammatory biomarkers No change in brush border enzymes	Li et al., 2013
2 liquid IMF produced with different WPC	IMF with extensively heated WPC: 73°C for 30 s and 80°C for 6 min IMF with pasteurized WPC: 73°C for 30 s	Preterm (n = 34, 106-d gestation) and near-term (n = 18, 113-d gestation) piglets received IMF by orogastric feeding tube for 5 d	Histology, brush border enzyme activity, intestinal permeability (lactulose: mannitol ratio), and inflammatory biomarkers	IMF with extensively heated WPC ↑ crypt depth, ↓ colonic goblet cells, ↓ intestinal alkaline phosphatase activity, ↑ lactulose:mannitol ratio and ↑ IL-8, IL-1β, and TNF-α vs. IMF with pasteurized WPC	Navis et al., 2020a,b

[1]IMF = infant milk formula; WPC = whey protein concentrate; AGE = advanced glycation end product; SI = small intestine; RT-qPCR = reverse transcription quantitative real-time PCR; TEER = transepithelial electrical resistance; CML = N(6)-carboxymethyllysine; RAGE = receptor for advanced glycation end products; IL-8 = interleukin-8; IL-1β = interleukin-1β; TNF-α = tumor necrosis factor α.

of pigs who received stored UHT IMF had furosine levels >450 µg/g of dry weight and CML levels >130 µg/g of dry weight (Sun et al., 2022). Preterm pigs fed the stored UHT IMF had significantly lower villus height at the proximal small intestine compared with those fed the UHT IMF ($P < 0.05$), but similar villus height in the middle and distal small intestine (Sun et al., 2022). Pigs who received either UHT IMF or stored UHT IMF had similar crypt depth and brush border lactase activity in the small intestine (Sun et al., 2022). Gut permeability (lactulose: mannitol ratio) did not decrease by increasing AGE levels in the IMF (Sun et al., 2022). Also, no changes occurred in intestinal mRNA transcript levels of pathogen recognition receptors, acute inflammatory response, and oxidative stress markers with increasing levels of AGE in IMF (Sun et al., 2022). However, dietary intervention with stored UHT IMF resulted in significant increases in mRNA transcript levels of AGE receptors compared with UHT IMF ($P < 0.05$; Sun et al., 2022).

Preterm piglets were fed for 5 d, by orogastric feeding tube, an IMF produced with a double-pasteurized WPC (Aasmul-Olsen et al., 2024). Again, in this study storage of the WPC ingredient was used to increase levels of furosine and CML in the final IMF product (Aasmul-Olsen et al., 2024). In contrast to the results of Sun et al. (2022), there was no significant effect of processing on villus height and crypt depth ($P > 0.05$; Aasmul-Olsen et al., 2024). Brush border aminopeptidase N, aminopeptidase A, lactase, sucrase, maltase, and dipeptidyl peptidase IV activities were all similar in the small intestine of piglets fed the IMF with double-pasteurized WPC or stored double-pasteurized WPC (Aasmul-Olsen et al., 2024). In the distal small intestine, tissue furosine and CML levels were significantly higher in piglets fed IMF with stored double-pasteurized WPC (2,894 ± 427 µg/g of protein and 639 ± 67 µg/g of protein) compared with those piglets fed the IMF with double-pasteurized WPC (1,301 ± 188 µg/g of protein and 331 ± 38 µg/g of protein; $P < 0.01$; Aasmul-Olsen et al., 2024).

We have recently fed young pigs a high-temperature IMF (3.97 µg CML/g; 35% inclusion in the diet) for 28 d (Chen et al., 2023). Both the duodenum and jejunum were assessed for gut physiology and digestive enzyme capacity (Dold et al., 2024). For example, in the duodenum of pigs fed high-temperature IMF diets, villus height was 449.60 ± 24.72 µm, crypt depth was 366.78 ± 23.63 µm, and goblet cell number was 30.81 ± 1.67 (Dold et al., 2024). In the duodenal lumen, trypsin activity was 0.91 ± 0.2 U/mg of protein and in the duodenal brush border membrane vesicles aminopeptidases N activity was 7.44 ± 0.69 U/mg of protein, aminopeptidase A activity was 155.17 ± 22.83 mU/mg of protein, intestinal alkaline phosphatase levels were 167.44 ± 30.49 U/mg of protein and lactase activity was 110.75 ± 7.21 nmol glucose/mg of protein (Dold et al., 2024). Previously, treatment of polarized human epithelial cell monolayers (Caco-2) with in vitro gastrointestinal digested high-temperature IMF resulted in a significant reduction in monolayer integrity (transepithelial electrical resistance [**TEER**]) compared with untreated control cell monolayers ($P < 0.05$; Bavaro et al., 2021). However, this barrier disruption did not alter levels of the tight junction proteins occludin, claudin-4, and zonula occludens-1 or junctional adhesion molecule-A (Bavaro et al., 2021).

Li et al. (2013) described a study where preterm pigs received an IMF formulated with double-pasteurized, spray-dried WPC for 5 d by orogastric feeding tube (Li et al., 2013). Maillard reaction products were not determined in the final formulation (Li et al., 2013). In the proximal small intestine these preterm pigs had a villus height of 440 ± 47 µm (Li et al., 2013). Brush border enzyme activities were determined in small intestinal tissue of these preterm pigs with an aminopeptidase N activity of 5.9 ± 1.4 U/g of tissue, aminopeptidase A activity of 2.5 ± 0.6 U/g of tissue, lactase activity of 9.7 ± 0.8 U/g of tissue, sucrase activity of 0.15 ± 0.01 U/g of tissue, maltase activity of 3.1 ± 0.5 U/g of tissue, and dipeptidyl peptidase IV activity of 2.3 ± 0.7 U/g of tissue (Li et al., 2013). The inflammatory biomarker, IL-8 in the distal small intestinal tissue of preterm pigs was approximately 58 pg/mg of tissue (Li et al., 2013). The urinary lactulose: mannitol ratio was >0.5 (Li et al., 2013).

In a 5-d intervention trial, preterm and near-term piglets received increasing doses, by orogastric feeding tube, of an IMF produced using an extensively heated WPC (Navis et al., 2020a,b). This WPC had approximately 19 µg CML/mL (Navis et al., 2020b). The colon crypt depth in the piglets from both age groups fed this IMF formulated with extensively heated WPC was >200 µm (Navis et al., 2020a).

ALTERNATIVES TO THERMAL PROCESSING FOR IMF PRODUCTION

To preserve native proteins and reduce AGE levels in IMF, recent research efforts have focused on reducing thermal loads by (a) sourcing mildly processed protein ingredients with lower thermal loads (i.e., sweet whey), (b) by decreasing IMF processing temperatures or looking to alternative processing technologies (membrane filtration and UV irradiation), or both, and (c) by eliminating spray-drying steps (i.e., liquid IMF).

Food authorities are likely to limit AGE concentrations in IMF in the near future. Certainly, the EU Commission Regulation (EU) No. 2017/2158 has already set the benchmark level for the presence of acrylamide in "baby foods" at 40 µg/kg of BW (Commission Regulation, 2017). Acrylamide is a toxic, potentially carcinogenic,

byproduct of the Maillard reaction (Augustine and Bent, 2022). Sourcing ingredients manufactured with lower thermal loads and lowering the temperature during IMF processing will result in lower levels of AGE (Navis et al., 2020b; Aasmul-Olsen et al., 2024; Table 1). From an ingredient perspective, Navis et al. (2020b) noted that pasteurized WPC (73°C for 30 s) had significantly lower levels of CML compared with an extensively heated WPC (73°C for 30 s and 80°C for 6 min; $P < 0.05$; Navis et al., 2020b). The same pasteurized WPC had close to 100% native whey protein compared with only 30% in the extensively heated WPC (Navis et al., 2020b). In this pasteurized WPC, 72% soluble protein remained following processing compared with only 21% in the extensively heated WPC (Navis et al., 2020b). In a pasteurized WPC (72°C for 15 s) there was significantly lower furosine and CML levels compared with a double-pasteurized WPC (72°C for 15 s and 80°C for 30 s) stored at 37°C for 6 wk (3,172 ± 231 and 182 ± 13 μg/g of protein vs. 6,166 ± 115 and 399 ± 14 μg/g of protein; $P < 0.05$; Aasmul-Olsen et al., 2024). No distinctive protein aggregates were observed by SDS-PAGE in the pasteurized WPC compared with large disulfide-linked protein aggregates seen in the double-pasteurized WPC (Aasmul-Olsen et al., 2024). Lin et al. (2018) observed significantly lower levels of whey protein denaturation in a pasteurized SMP versus a high-heat-treated SMP (5.44% vs. 80.75%; $P < 0.05$; Lin et al., 2018). A significant reduction in particle size to 179 nm was seen in pasteurized SMP versus 213 nm in the SMP with high-heat ($P < 0.05$; Lin et al., 2018). Where WPC is produced using sweet whey rather than acid whey, lower temperatures are often employed. The study by Cattaneo et al. (2017) produced sodium caseinate and WPC by skim milk acidification before IMF processing (Cattaneo et al., 2017). Furosine (3,492 ± 110 μg/g of protein) and lysinoalanine (385 ± 43 μg/g of protein) were significantly reduced compared with IMF formulated with commercial ingredients ($P < 0.05$; Cattaneo et al., 2017). There was no change in pyrraline levels (Cattaneo et al., 2017; Table 1). A WPC, produced using membrane filtration (sweet whey) and sterilized by γ-irradiation, had higher amounts of native lactoferrin (0.49 g/L) and native IgG (4.6 g/L) compared with a double-pasteurized WPC from acid whey (Li et al., 2013; Table 1).

Lowering the temperature during IMF processing is also an option (Lund et al., 2022; Sun et al., 2022). Reducing the thermal load from 90°C for 15 s to 72°C for 15 s increased retention of native whey proteins in liquid IMF to 98% and 99%, respectively (Halabi et al., 2020b; Table 1). Pasteurized liquid IMF (72°C for 10 s) had lower furosine and CML concentrations (1,797 ± 59 μg/g of protein and 38 ± 2 μg/g of protein) compared with the UHT liquid IMF (143°C for 6 s; 2,798 ± 136 μg/g of protein and 242 ± 9 μg/g of protein; Sun et al., 2022). The CEL concentrations were similar between pasteurized and UHT IMF (Sun et al., 2022). Not surprisingly, storage of the same UHT liquid at 40°C for 60 d increased furosine, CML, and CEL concentrations (Sun et al., 2022). Sodium dodecyl sulfate-PAGE revealed less protein aggregation in pasteurized liquid IMF compared with UHT-treated liquid IMF and stored UHT liquid IMF (Sun et al., 2022). An IMF powder produced with pasteurized skim milk and WPC (72°C for 15 s) had a CML concentration of 143 ± 15 μg/g of protein, a CEL concentration of 8.99 ± 1.93 μg/g of protein, and pyrraline levels of 36.2 ± 2.9 μg/g of protein (Lund et al., 2022). However, in these studies the total bacterial load of these low thermally processed IMF was not reported, which is critical to determine whether a product is safe for human consumption.

Membrane filtration technology using filters to trap bacteria has been used recently to produce IMF at pilot-plant scale (Chen et al., 2021; Yu et al., 2021). Yu et al. (2021) produced 100 kg of IMF powder (whey:CN ratio of 60:40) from fresh bovine milk using 0.8- and 0.1-μm pore size membranes (Yu et al., 2021). Reverse-phase HPLC revealed significantly lower levels of whey protein denaturation in the membrane-filtered IMF powder (6 ± 4% denatured whey protein) compared with the IMF powder produced using extensive heat processing (58% denatured whey protein; $P < 0.05$; Yu et al., 2021; Table 1). Surprisingly, using LC-MS/MS, Yu et al. (2021) found no significant differences in AGE (furosine and CML) concentrations between IMF at different stages of production, from mixing to final powder ($P > 0.05$; Yu et al., 2021). In the final IMF powder collected after spray drying, furosine levels were comparable between the membrane-filtered and extensively heat processed IMF powders (approximately 4,800 and 5,000 μg furosine/g of protein; Yu et al., 2021). The CML concentrations were 88 ± 2 μg/g of protein in the membrane-filtered IMF and 106 ± 0 μg/g of protein in the extensively heat processed IMF (Yu et al., 2021). From a safety perspective, membrane-filtered IMF contained 3.63×10^2 cfu/g of powder compared with only 0.95×10^2 cfu/g of powder in the extensively heated IMF powder (Yu et al., 2021), albeit absent of *C. sakazakii* and *Salmonella* spp., adhering to the specifications outlined under Commission Regulation (EC) No. 2073/2005 on microbiological criteria for foodstuffs (Commission Regulation, 2005).

In our group, a ceramic 1.4-μm microfiltration membrane followed by a polyvinylidene difluoride polymeric 0.2-μm membrane was used to produce an IMF powder (whey:CN ratio of 60:40) at laboratory scale (Chen et al., 2021). A control, high-temperature IMF was also produced by heating at 125°C for 5 s (Chen et al., 2021). Both homogenized IMF were spray dried at 185°C (inlet temperature) and 80°C (outlet temperature; Chen et al.,

2021). There was no significant difference in CML concentrations in both spray-dried IMF powders (3.65 ± 0.74 µg/g of membrane-filtered IMF powder vs. 3.97 ± 0.7 µg/g of high-temperature IMF powder; $P > 0.05$; Chen et al., 2021; Table 1). Taken together with the results of Yu et al. (2021), this may indicate that where thermal load is low before entering the dryer, spray drying then becomes a major contributor to CML levels in IMF.

At pilot-plant scale (250 kg), this membrane-filtered IMF had a significant increase in native whey protein content (59.9%, 4.02 g native whey protein) compared with high-temperature IMF (4.5%, 0.3 g native whey protein; $P < 0.001$; Chen et al., 2021; Chen et al., 2023). There was no loss in protein by membrane filtration with no differences in total protein content (10.9 g total protein/100 g of membrane-filtered IMF vs. 11.4 g total protein/100 g of high-temperature IMF; Chen et al., 2021; Chen et al., 2023). In agreement with Yu et al. (2021), pathogenic bacteria were absent in the final powders, but the total bacterial load was significantly higher in membrane-filtered IMF (1.8×10^7 cfu/g of powder) compared with high-temperature IMF (8.3×10^2 cfu/g of powder; $P < 0.05$; Dold et al., 2024).

Proof of principle research has demonstrated that UV irradiation can reduce the bacteria load from commercial IMF powder spiked with *C. sakazakii* (Liu et al., 2012). However, manufacturing IMF at pilot-plant scale using UV irradiation as an alternative to thermal processing is still under development.

WILL REDUCTION IN THERMAL PROCESSING OF IMF ALTER PROTEIN DIGESTIBILITY?

Limiting the exposure of ingredients and IMF to high thermal loads during processing may influence protein digestion in the upper infant gut (Cattaneo et al., 2017; Halabi et al., 2020a; Navis et al., 2020b; Zenker et al., 2020; Bavaro et al., 2021; Ye et al., 2022; Chen et al., 2022; Chen et al., 2023; Calvez et al., 2024; Table 2).

Producing IMF with a low-heat WPC ingredient supported faster gastric emptying of IMF compared with IMF produced with extensively heat-treated WPC (Navis et al., 2020b; Table 2). Both preterm and near-term piglets received an IMF formulated with pasteurized WPC for 5 d (Navis et al., 2020b). At 60 min post feeding, these piglets had significantly lower gastric content (approximately 15 g/kg and 30 g/kg) compared with piglets that received an IMF with extensively heated WPC (approximately 35 g/kg and 45 g/kg; $P < 0.01$; Navis et al., 2020b). In another study, the rate of protein hydrolysis differed during in vitro intestinal digestion between unheated IMF and heated IMF (Halabi et al., 2020a). At 5 min intestinal digestion, unheated IMF had a significantly higher degree of protein hydrolysis versus IMF heated at 67.5°C or 80°C (24 ± 2% vs. 14 ± 2%; $P < 0.05$; Halabi et al., 2020a). However, by the end of intestinal digestion, the degree of protein hydrolysis was similar (35 ± 2%; Halabi et al., 2020a). Unheated and IMF heat-treated at 80°C had comparable bioaccessibility of EAA (34.1 ± 1.5% vs. 35.5 ± 1.2%) and NEAA (10.2 ± 0.7% vs. 10.3 ± 0.6%) at the end of the intestinal phase (Halabi et al., 2020a; Table 2). Zenker et al. (2020) produced an unheated IMF powder with relatively low furosine levels (308 mg furosine/100 g of protein). This IMF powder had a significantly higher degree of protein hydrolysis following gastric (5.9 ± 0.4%) and intestinal (46.8 ± 3.9%) digestion compared with the dry-heated IMF powder with 2,022 mg furosine/100 g of protein ($P < 0.001$; Zenker et al., 2020).

Using a semidynamic infant in vitro gastric digestion model, protein digestion of membrane-filtered IMF was tracked (Chen et al., 2022). Once membrane-filtered IMF was added to the gastric phase, an increase in pH from 2.5 to 7.38 ± 0.02 was observed (Chen et al., 2022). By the end of gastric digestion (156 min, pH 3.2), a significantly higher degree of protein hydrolysis was recorded in the membrane-filtered IMF (1,338 ± 253 µmol free amines/g of protein) versus the high-temperature IMF (845 ± 78 µmol free amines/g of protein; $P < 0.05$; Chen et al., 2022). The membrane-filtered IMF had a fragmented curd throughout in vitro gastric digestion (Chen et al., 2022). From 94 min gastric digestion, a clear phase separation could be observed as fat droplets began to separate from protein aggregates, indicating that membrane-filtered IMF had a less dense curd than high-temperature IMF (Chen et al., 2022). Still, SDS-PAGE analysis showed intact β-LG was still present in membrane-filtered IMF at the end of gastric digestion (Chen et al., 2022). No significant difference was observed in the release of peptides <10 kDa from either membrane-filtered IMF or high-temperature IMF at the end of the gastric phase ($P > 0.05$; Chen et al., 2022). In the follow-up pig trial, 180 min after a final feed of a membrane-filtered IMF-based diet, young pigs had a significantly higher degree of protein hydrolysis in the lumen collected from the stomach compared with stomach contents of pigs fed high-temperature IMF diets (599 ± 45 µmol free amines/g of protein vs. 509 ± 17 µmol free amines/g of protein; $P < 0.05$; Chen et al., 2023). In the stomach of these pigs there was a significantly higher concentrations of water-soluble proteins and peptides compared with high-temperature IMF fed pigs ($P < 0.05$; Chen et al., 2023). The total concentration of free AA released from the stomach of pigs fed diets with

membrane-filtered IMF or high-temperature IMF were comparable (56.5 ± 4.6 μmol/g of protein vs. 59.4 ± 3.05 μmol/g of protein; Chen et al., 2023).

In the duodenum, there was a significant increase in the degree of protein hydrolysis from pigs fed membrane-filtered IMF diets (1,530 ± 136 μmol free amines/g of protein) compared with pigs fed diets with high-temperature IMF (1,174 ± 124 μmol free amines/g of protein); $P < 0.05$; Chen et al., 2023). The total concentration of free AA released was similar in the duodenum of pigs in both IMF treatment groups (Chen et al., 2023). However, membrane-filtered IMF fed pigs had a significantly higher Pro concentration in the duodenum compared with high-temperature IMF fed pigs (7.8 ± 2.61 μmol/g of protein vs. 2.2 ± 0.9 μmol/g of protein; $P < 0.05$; Chen et al., 2023). There was a similar level of protein hydrolysis recorded in the jejunum and ileum of pigs in both IMF treatment groups (Chen et al., 2023). In the jejunum, taurine and Trp concentrations were both significantly higher in pigs fed the membrane-filtered IMF diets compared with pigs fed high-temperature IMF diets (4.7 ± 1.55 and 4.9 ± 0.37 μmol/g of protein, respectively, vs. 1.5 ± 0.37 and 3.5 ± 0.48 μmol/g of protein; $P < 0.05$; Chen et al., 2023). Similar amounts of total free AA were released from the ileum of membrane-filtered IMF fed pigs (251.5 ± 33.1 μmol/g of protein) and pigs fed high-temperature IMF (285 ± 28.51 μmol/g of protein; Chen et al., 2023). Pigs fed membrane-filtered IMF did have lower concentrations of Tyr in the ileum compared with high-temperature IMF fed pigs (8.8 ± 0.98 μmol/g of protein vs. 11.5 ± 0.86 μmol/g of protein; $P < 0.05$; Chen et al., 2023). Overall, membrane-filtered IMF fed pigs had a higher total degree of protein hydrolysis in the gastrointestinal tract compared with high-temperature IMF fed pigs (1,173 ± 46 μmol free amines/g of protein vs. 1,022 ± 43 μmol free amines/g of protein; $P < 0.05$) and higher concentrations of water-soluble proteins and peptides ($P < 0.001$; Chen et al., 2023). However, some discrepancies between in vitro and preclinical experiments did exist, with an in vitro static infant gastrointestinal digestion reporting similar levels of proteolysis at the end of intestinal digestion for membrane-filtered and high-temperature IMF (471 ± 36.5 μmol free amines/mg of protein vs. 481 ± 7.7 μmol free amines/mg of protein; Bavaro et al., 2021). In vitro, at the end of the intestinal phase, Lys, Leu, and Tyr were the most abundant AA released from membrane-filtered IMF compared with Lys, Leu, and Phe from high-temperature IMF (Bavaro et al., 2021).

From a peptide-profiling perspective, there is evidence that processing will affect individual peptides released during digestion. Although processing parameters were not disclosed, following in vitro gastrointestinal digestion, Wada and Lönnerdal (2015a) identified 39 bioactive peptides in intestinal digesta from a standard IMF powder compared with only 9 in the intestinal digesta from an extensively hydrolyzed IMF powder (Wada and Lönnerdal, 2015a). Bavaro et al. (2021) treated Caco-2 cell monolayers with IMF digesta for 4 h. Analysis by LC-MS/MS identified 488 dairy peptides in the apical chambers common to both membrane-filtered and high-temperature IMF, with 112 peptides unique to membrane-filtered IMF and 97 peptides unique to high-temperature IMF (Bavaro et al., 2021). Bavaro et al. (2021) did not determine if this resulted in differences in peptide bioactive capacity. Interestingly, the bioactive β-casomorphin-7 opioid peptide (Claustre et al., 2002; Trompette et al., 2003; Zoghbi et al., 2006), which is released from β-CN following gastrointestinal digestion, was identified in both IMF (Bavaro et al., 2021).

Calvez et al. (2024) calculated a DIAAS of 82 for a membrane-filtered IMF powder, which was similar to 80 for an extensively, heat-treated IMF powder. In a preclinical study by Calvez et al. (2024), Wistar Han rats (n = 30, 28-d old) were fed a diet containing this membrane-filtered IMF at 40% inclusion for 14 d. There was no significant effect of IMF processing on dietary nitrogen levels in the stomach ($P = 0.5$) or the small intestine ($P = 0.17$) 6 h after final meal ingestion (Calvez et al., 2024). However, there were differences observed in AA bioavailability, with Calvez et al. (2024) observing significant increases in postprandial indispensable and total AA concentrations in rats fed membrane-filtered IMF within 1 h, compared with rats fed thermally processed IMF ($P < 0.001$). No significant effect of processing on AA bioavailability was observed at any other time point ($P > 0.05$; Calvez et al., 2024).

However, lowering thermal processing may not always speed up digestion. An in vitro infant digestion of a pasteurized liquid IMF (72°C for 10 s) resulted in visible β-LG and α-LA protein bands on SDS-PAGE at the end of the gastric phase, while β-LG was the only whey protein still present at the end of intestinal digestion (Ye et al., 2022). In contrast, all proteins in the intestinal digesta of UHT-treated liquid IMF were completely degraded (Ye et al., 2022). A similar number of peptides were released from the intestinal digesta of pasteurized liquid IMF and UHT IMF (Ye et al., 2022; Table 2). In another study, Cattaneo et al. (2017) reported that the degree of proteolysis was significantly lower in an unheated IMF (35.6 ± 0.6%) compared with an IMF produced by in-batch sterilization at 110°C for 38 min (40.3 ± 1.2%; $P <$ 0.05) albeit this was reversed when ingredients (sodium

caseinate and WPC) were processed in-house rather than purchased commercially (sodium caseinate and WPI; Cattaneo et al., 2017).

EFFECTS OF LOWER HEAT TREATMENT DURING IMF PRODUCTION ON GUT BARRIER

Several studies have focused on whether or not reduced thermal processing of IMF will benefit gut physiology and gut barrier health (Li et al., 2013; Navis et al., 2020a,b; Bavaro et al., 2021; Sun et al., 2022; Dold et al., 2024; Table 3).

In a study by Sun et al. (2022), preterm pigs received pasteurized IMF for 5 d by orogastric feeding tube. These pigs had significantly higher villus height and lower crypt depth in the proximal, middle, and distal small intestine compared with pigs receiving 60 d stored UHT liquid IMF ($P < 0.05$; Sun et al., 2022). Intestinal permeability was unaffected by IMF processing (Sun et al., 2022).

In another study by Li et al. (2013), preterm pigs receiving an IMF formulated with membrane-filtered WPC for 5 d by orogastric feeding tube had significantly increased villus height in the proximal small intestine compared with pigs receiving IMF produced with double-pasteurized WPC (667 ± 46 μm vs. 440 ± 47 μm; $P < 0.05$; Li et al., 2013). Interestingly, Western blot analysis revealed that claudin-4 protein levels were significantly lower in pigs receiving IMF with membrane-filtered WPC compared with the pigs that received IMF formulated with double-pasteurized WPC ($P < 0.05$; Li et al., 2013). The urinary lactulose:mannitol ratio was significantly lower in pigs that received the IMF with membrane-filtered WPC (>0.1) compared with pigs receiving the IMF produced with double-pasteurized WPC (>0.5; $P < 0.05$; Li et al., 2013). Inflammatory biomarkers IL-8, tumor necrosis factor α (0.6 ± 0.7 pg/mg of tissue), and IL-1 β (3.7 ± 0.5 pg/mg of tissue) in the distal small intestine of preterm pigs were unaffected by processing type (Li et al., 2013).

Preterm and near-term piglets received an IMF produced with pasteurized WPC by orogastric feeding (Navis et al., 2020a,b). Crypt depth was significantly lower in the colon of preterm ($P < 0.05$) and near-term ($P < 0.01$) piglets receiving IMF with pasteurized WPC compared with piglets receiving an IMF with extensively heated WPC (Navis et al., 2020a). Using periodic acid-Schiff staining, Navis et al. (2020a) reported an observational increase in colonic goblet cell number in piglets receiving the IMF made with pasteurized WPC compared with those piglets that received an IMF with extensively heated WPC, although no difference was observed in colonic mRNA transcript levels of *mucin-1* and *mucin-2*. *Mucin-2* mRNA transcripts were also unaffected by processing type in the distal small intestine (Navis et al., 2020a). Intestinal permeability was influenced by processing type, with near-term piglets receiving IMF with pasteurized WPC having a significantly lower urinary lactulose:mannitol ratio compared with piglets receiving IMF with extensively heated WPC (>0.07 vs. > 0.2; $P < 0.05$; Navis et al., 2020b). Interleukin-8 protein levels and mRNA transcripts were significantly reduced in the colon of preterm piglets that received IMF with pasteurized WPC compared with piglets that received IMF with extensively heated WPC ($P < 0.05$; Navis et al., 2020a). Near-term piglets receiving IMF with pasteurized WPC also had significantly lower mRNA transcript levels of *IL-8* ($P < 0.05$; Navis et al., 2020a). Interleukin-1β mRNA transcript levels were significantly lower in the distal small intestine of preterm piglets and colon of near-term piglets receiving IMF with pasteurized WPC ($P < 0.05$; Navis et al., 2020a). Protein concentrations and mRNA transcript levels of tumor necrosis factor α were significantly lower in near-term piglets that received IMF with pasteurized WPC compared with piglets that received IMF with extensively heated WPC ($P < 0.05$; Navis et al., 2020a).

In preterm piglets fed for 5 d by orogastric feeding tube an IMF produced with a pasteurized WPC, there was no difference in villus height or crypt depth compared with piglets fed an IMF produced with a stored double-pasteurized WPC (Aasmul-Olsen et al., 2024).

For our preclinical study with 28-d-old pigs, there were also no significant differences observed in villus height, crypt depth, or mucosa thickness in the upper small intestine (duodenum and jejunum) of pigs fed membrane-filtered or high-temperature IMF ($P > 0.05$; Dold et al., 2024). However, there was a significant increase in goblet cell number in the jejunum of pigs fed membrane-filtered IMF diets compared with high-temperature IMF diets (17.39 ± 1.43 vs. 11.7 ± 1.27; $P < 0.05$; Dold et al., 2024). In addition, acidic mucus levels and mucin-2 protein concentrations in the jejunum were significantly increased in membrane-filtered IMF fed pigs compared with pigs fed high-temperature IMF diets ($P < 0.05$; Dold et al., 2024). A significant increase in mRNA transcript levels of *claudin-1* ($P < 0.001$), *mucin-1* ($P < 0.01$), and *mucin-2* ($P < 0.001$) was measured in the duodenal mucosal scrapings of pigs fed membrane-filtered IMF diets versus pigs fed high-temperature IMF diets (Dold et al., 2024). In parallel, in in vitro experiments where Caco-2 polarized cell monolayers were treated with intestinal digesta from membrane-filtered IMF, TEER values and claudin-1 protein concentrations were significantly increased compared with high-temperature IMF treatment ($P < 0.05$; Bavaro et al., 2021). Other biomarkers were unaffected by treatment type, in the pig study mRNA transcripts of *occludin*, *claudin-2*, *claudin-4*, *zonula occludens-1*, and

Table 4. Sensitivity of gut biomarkers to high-temperature processing

Biomarker (no. of studies)	Sensitive	Occasionally sensitive	Insensitive or resistant	Reference
Villus height (n = 4)		↓ in villus height in 2 of 4 studies		Li et al., 2013; Sun et al., 2022; Aasmul-Olsen et al., 2024; Dold et al., 2024
Crypt depth (n = 4)		↑ in crypt depth in 2 of 4 studies		Navis et al., 2020b; Sun et al., 2022; Aasmul-Olsen et al., 2024; Dold et al., 2024
Tight junctions (n = 3)		↓ in tight junction mRNA transcripts and protein levels in 2 of 3 studies		Li et al., 2013; Bavaro et al., 2021; Dold et al., 2024
Goblet cell number (n = 2)	↓ in goblet cell number in 2 of 2 studies			Navis et al., 2020a; Dold et al., 2024
Mucins (n = 2)		↓ in mucin mRNA transcripts and protein levels in 1 of 2 studies		Navis et al., 2020a; Dold et al., 2024
Intestinal permeability (n = 3)		↑ in intestinal permeability (lactulose:mannitol ratio) in 2 of 3 studies		Li et al., 2013; Navis et al., 2020b; Sun et al., 2022
Aminopeptidase activity (n = 5)			↑ in aminopeptidase N activity in 1 of 5 studies (no change in aminopeptidase A activity in all 5 studies)	Li et al., 2013; Navis et al., 2020b; Sun et al., 2022; Aasmul-Olsen et al., 2024; Dold et al., 2024
Intestinal alkaline phosphatase activity (n = 2)		↓ in intestinal alkaline phosphatase activity in 1 of 2 studies		Navis et al., 2020a; Dold et al., 2024
Lactase activity (n = 5)		↓ in lactase activity in 2 of 5 studies		Li et al., 2013; Navis et al., 2020b; Sun et al., 2022; Aasmul-Olsen et al., 2024; Dold et al., 2024

junctional adhesion molecule-A were similar (Dold et al., 2024) and in vitro occludin, claudin-4, zonula occludens-1, and junctional adhesion molecule-A protein levels were unaffected by processing (Bavaro et al., 2021). Interestingly, in the pig study, a significant reduction in mRNA transcript levels for the receptor for AGE was also detected in the jejunum of membrane-filtered IMF fed pigs compared with high-temperature IMF fed pigs ($P < 0.05$; Dold et al., 2024), even though both IMF had similar CML concentrations (Chen et al., 2021).

From a digestive capacity perspective in the gut, Sun et al. (2022) reported a significant increase in brush border lactase activity in the proximal and middle small intestine of pigs fed the pasteurized liquid IMF compared with stored UHT liquid IMF ($P < 0.05$; Sun et al., 2022). No differences were reported in activities of the other brush border enzymes analyzed (aminopeptidase N, aminopeptidase A, sucrase, maltase, and dipeptidyl peptidase IV; Sun et al., 2022). In a study by Li et al. (2013) processing of WPC used in the formulation of IMF had no significant effect on brush border aminopeptidase N, aminopeptidase A, lactase, sucrase, maltase, and dipeptidyl peptidase IV activities ($P > 0.05$). For a study by Navis et al. (2020a) brush border intestinal alkaline phosphatase activity was significantly higher in the colon of preterm piglets receiving IMF with pasteurized WPC compared with piglets receiving the IMF with extensively heated WPC ($P < 0.05$), with similar levels recorded in the colon of near-term piglets (Navis et al., 2020a). For preterm and near-term piglets, similar levels of intestinal alkaline phosphatase activity were recorded in the distal small intestine, regardless of IMF diet (Navis et al., 2020a). Aminopeptidase N, aminopeptidase A, and lactase in the middle small intestine were also unaffected by IMF processing (Navis et al., 2020b). In the trial of Aasmul-Olsen et al. (2024) brush border aminopeptidase N, aminopeptidase A, lactase, sucrase, maltase, and dipeptidyl peptidase IV activities were similar between treatment types. Consistent with the findings of Sun et al. (2022), we have also recently measured a significant increase in lactase activity in isolated duodenal brush border membrane vesicles in pigs fed membrane-filtered IMF diets compared with pigs fed high-temperature IMF diets (166.59 ± 22.09 nmol glucose/mg of protein vs. 110.75 ± 7.21 nmol glucose/mg of protein; $P < 0.05$; Dold et al., 2024). In addition, duodenal trypsin activities were significantly higher in membrane-filtered IMF fed pigs compared with pigs fed high-temperature IMF diets (1.36 ± 0.19 mU/mg of protein vs. 0.91 ± 0.2 mU/mg of protein; $P < 0.05$; Dold et al., 2024). However, in the duodenum, brush border aminopeptidase N activity was significantly lower in pigs fed membrane-filtered IMF compared with pigs on high-temperature IMF diets (5.23 ± 0.26 U/mg of protein vs. 7.44 ± 0.69 U/mg of protein; $P < 0.05$; Dold et al., 2024). Processing type had no significant effect on duodenal brush border aminopeptidase A or intestinal alkaline phosphatase activities and no effect on the digestive enzyme capacity of the jejunum ($P > 0.05$; Dold et al., 2024).

By comparing gut responses to IMF produced with different thermal loads, it is apparent that several gut barrier biomarkers are sensitive to IMF thermal processing (Table 4). These include villus height, crypt depth, tight junction proteins, goblet cell number, mucus levels, brush border enzyme activities, and intestinal permeability. Further work is needed to determine whether the main driver of gut responses to processing is IMF protein structure or IMF AGE levels or a contribution from both. Mechanistic studies are required to investigate the response of gut cells to individual AGE compounds and individual bioactive peptides.

CONCLUSIONS

In general, in vitro and preclinical studies have revealed that thermal processing of IMF reduces protein digestibility and alters gut physiology in the young gut. A major limitation to date is that all studies have been performed in vitro or preclinical. How this data correlates to the human infant requires follow-up intervention studies in babies, which is challenging. Research progress is also hampered by the failure of IMF manufacturers to disclose processing temperatures. A full profile of AGE, quantified by standardized methods, should be given with each IMF product to allow accurate comparisons. Processing improvements, at pilot-plant scale, are also required to develop alternative technologies for IMF processing (i.e., UV irradiation and membrane filtration), whilst ensuring the production of a safe product. Ultimately, the impact of any IMF product on protein digestion and gut barrier physiology in the infant gut must be compared with breast milk.

NOTES

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Nonstandard abbreviations used: AGE = advanced glycation end product; CEL = N(6)-carboxyethyllysine; CML = N(6)-carboxymethyllysine; DIAAS = digestible indispensable amino acid score; IMF = infant milk formula; LC = liquid chromatography; RAGE = receptor for advanced glycation end products; RT-qPCR = reverse transcription quantitative real-time PCR; SI = small intestine; SMP = skim milk powder; TEER = transepithelial electrical resistance; WPC = whey protein concentrate; WPI = whey protein isolate.

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