Interpretive Summary

Effect of processing infant milk formula on protein digestion and gut barrier health (*in vitro* and preclinical). During manufacture, infant milk formula (**IMF**) is heated to ensure it is safe for human consumption. However, heat treatment of IMF changes the native structure of milk proteins and generates undesirable advanced glycation endproducts. This review summarizes the data generated from *in vitro* and preclinical studies on the consequences of these events on protein digestibility and gut barrier health in the infant. Lowering processing temperatures or using alternative processing methods provides an opportunity to produce an IMF which contains native milk proteins and reduced levels of advanced glycation endproducts. How these next generation IMF products fare during upper gut transit are also reviewed.

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 if 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.

Keywords: Infant milk formula, processing, protein digestion, gut barrier health, infant.

Abbreviations: AGE = advanced glycation endproduct; CEL = N(6)-carboxyethyllysine; CML = N(6)-carboxymethyllysine; DIAAS = digestible indispensable amino acid score; IMF = infant milk formula; LC = liquid chromatography; SMP = skim milk powder; TEER = transepithelial electrical resistance; WPC = whey protein concentrate; WPI = whey protein isolate.

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 to 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., 2017). At birth, preterm and term infants have a gastric pH $\geq$ 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 - 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., 2017). At pH $\geq$ 6.5, pepsin activity is reduced with complete denaturation and inactivation at $\geq$ 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 - 60% of the adult activity levels (Nguyen et al., 2015). Whether there are age differences 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 towards the villus tip (Kong et al., 2018). In the small intestine, villi height are lower in infants compared to 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 - 12% of intestinal epithelial cells but it is widely accepted that the percentage of goblet cells is much lower in infants intestine (Demers-Mathieu, 2022). Intestinal permeability is also higher in infants than in adults with this permeability decreasing as the distance between neighboring cells narrow, whilst 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 (1.27 ± 0.73 at d 1 and 0.22 ± 0.21 at 1 month) ($P < 0.05$) (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 to 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 to in the mid-villus and villus tip in adult mice (Gleeson et al., 2021). The infant gut microbiota also has lower microbial diversity compared to 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 six months 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., 2021,

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 - 12 g/L protein, 67 - 78 g/L lactose, 12 - 14 g/L oligosaccharides and 32 - 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), phosphorous (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 - 80% of total milk protein in early to mid-lactation (Martin et al., 2016), with α-LA accounting for 40% (Kim and Yi, 2020) and lactoferrin at 26% of whey protein (Guo and Hendricks, 2008). The casein protein content in breast milk increases from 20% in early lactation to 50% in mature breast milk (Lönnerdal, 2003). The casein in breast milk consists primarily of β-caseins and κ-caseins but also has trace concentrations of α_{s1}-caseins (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 months) 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 - 10 μm and 10 - 1000 μm (He et al., 2022). Surprisingly, a semi-dynamic *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).

127 It is widely accepted that infants who receive breast milk have a lower incidence of diarrhea
128 and lower risk of developing necrotizing enterocolitis than bottle-fed babies (Lyons et al.,
129 2020). Preterm infants (n = 62, ≤ 32 weeks of gestation) receiving breast milk also had
130 significantly lower intestinal permeability (lactulose:mannitol ratio) compared to preterm
131 infants fed infant formula at one week postpartum (0.076 *vs* 0.205, $P = 0.04$) (Taylor et al.,
132 2009). Rasmussen et al. (2016) also observed significantly higher brush border membrane
133 aminopeptidase N, aminopeptidase A, lactase and dipeptidyl peptidase IV activities in the
134 proximal small intestine of preterm pigs (n = 47, 105 - 106 d gestation) fed breast milk
135 compared to those fed commercial infant formula ($P < 0.05$) (Rasmussen et al., 2016). In
136 human enteroid monolayers, exposure to breast milk (for 24 and 72 h) has been shown to
137 increase goblet cell and Paneth cell number and expression of occludin protein levels (Noel et
138 al., 2021). With increases in digestive enzyme capacity, goblet cell and Paneth cell numbers,
139 tight junction markers and a reduction in permeability, breast milk is beneficial to the
140 intestinal epithelium, helping to mature the infant gut.

141 In circumstances where breastfeeding is not possible, the World Health Organization
142 recommends the use of infant formula (World Health Organization, 2013). Under
143 Commission Regulation (EU) No. 609/2013 infant formula is described as a "food intended
144 for use by infants during the first months of life and satisfying by itself the nutritional
145 requirements of such infants until the introduction of appropriate complementary feeding"
146 (Commission Regulation, 2013). Before, during and after processing infant formula is heat-
147 treated. These high thermal treatment steps ensure a microbiological safe product with an
148 extended shelf life (Wada and Lönnerdal, 2015b). Bovine milk based infant formulae are the
149 most common, commercially available infant formula on the market (Halabi et al., 2020b, He
150 et al., 2022). Heat treatment of infant milk formula (**IMF**) will induce denaturation and
151 aggregation of milk proteins (Pellegrino et al., 2011), and the formation of undesirable

Maillard reaction products (Lund et al., 2021). This differs substantially to 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 pre-clinical studies. For comparison purposes, *in vitro* and pre-clinical 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 mimicking 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 formulae 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 casein ratio (60:40) similar to mature breast milk, skim milk powder (**SMP**), whey protein isolate (**WPI**) and/or whey protein concentrate (**WPC**) are added (Phosanam et al., 2021, Bakshi et al., 2023). The base formula will also contain lactose, vegetable oil, vitamins and minerals (Bakshi et al., 2023). Protein composition of bovine milk differs to mature breast milk with a whey to casein 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 casein ratio with the addition of WPI and/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).

INFANT MILK FORMULA PROCESSING

Ingredient suppliers rarely disclose the processing histories of their dairy ingredients but traditionally SMP, WPI and WPC are routinely subjected to thermal processing prior to IMF manufacturing (Lin et al., 2018). Then, during IMF processing, these same ingredients undergo a series of heat processing steps including pasteurization (72 - 75 °C for 15 s) and UHT (135 - 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 - 200 °C and outlet temperature 80 - 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 INFANT MILK FORMULA

Effects of heat on milk and milk products have been reviewed previously by Pellegrino et al. (2011) (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 casein 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 to 100 °C, the casein micelle size increased by 30 - 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-LG is more heat labile than α-LA, as 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 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 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, 90 °C for 2 - 3 s before evaporation and 85 °C for 2 min before spray-drying), 58 ± 0% 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 to 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 casein 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 ENDPRODUCTS IN THERMALLY PROCESSED INFANT MILK FORMULA

The Maillard reaction is a non-enzymatic, complex series of reactions between reducing sugars and AA that occurs at high temperatures (Kumar et al., 2017). The final result is "browning" i.e. the formation of advanced glycation endproducts (**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 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 or intermediates (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. Prior to 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 2893 ± 105 µg/g protein and 160 ± 4 µg/g protein, respectively in a double pasteurized WPC (72 °C/15 s and 80 °C/30 s) prior to incorporation into an IMF formula 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 liquid chromatography/fluorescence, Navis et al. (2020b) reported approximately 19 μg/mL CML in an extensively heated WPC (73 °C/30 s and 80 °C/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 6378 ± 225 μg furosine/g protein, 55 ± 2 μg pyrraline/g protein and 999 ± 95 μg lysinoalanine/g 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 2798 ± 136 μg/g protein, 242 ± 9 μg/g protein and 71± 1 μg/g protein, respectively (Sun et al., 2022). After spray drying, the heat-treated IMF powder contained 3.97 ± 0.7 μg CML/g powder (Chen et al., 2021). In the extensively, heat processed IMF powder, furosine concentration was approximately 5000 μg/g protein and CML concentrations was 106 ± 0 μg/g protein (Yu et al., 2021) (Table 1).

Lee et al. (2019) reported a contribution of spray drying process to CML content in IMF powder, with an increase from approximately 30 μg/g protein in the heat-treated liquid IMF to 60 μg/g protein in the final powder, albeit no processing temperatures prior to spray drying were given (Lee et al., 2019). Yu et al. (2021) has previously shown no contribution by spray drying to furosine or CML content in IMF with high thermal loads (Yu et al., 2021). 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 INFANT MILK FORMULA 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 as 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 impact 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, considerably lower than breast milk at a DIAAS of 101, as determined in young pigs (Charton et al., 2023). 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 IMF (125 °C/5 s) powder which was subjected to an *in vitro* semi-dynamic 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 gastric digestion (Chen et al., 2022). SDS-PAGE analysis showed that at 125 min intact caseins 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 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 four different gut locations, 180 min after final meal (Chen et al., 2023). Pigs fed the heated IMF had a degree of protein hydrolysis of 509 ± 17 μmol free amines/g protein in the stomach and 1174 ± 124 μmol free amines/g protein in the duodenum, with an overall value for the stomach, duodenum, jejunum and ileum of 1022 ± 43 μmol free amines/g protein (Chen et al., 2023). The concentration of free AA released was 59.4 ± 3.05 μmol/g protein in the gastric phase, 171.9 ± 29.7 μmol/g protein in the duodenum, 211.4 ± 22.75 μmol/g protein in the jejunum and 285 ± 28.51 μmol/g 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).

Halabi et al. (2020a) results showed that *in vitro* infant gastric digestion of heat-treated IMF (80 °C) formed small curd particles of 10 μm (Halabi et al., 2020a). Thermal treatment of IMF at either 67.5 °C or 80 °C supported gastric casein hydrolysis with only 6 ± 1% of residual intact caseins still present after 30 min 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 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). Lys, Phe, Tyr, Leu and Arg were higher than all other AA whilst 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 (Gómez-Gallego et al., 2016). 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 nonprotein nitrogen 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). LC-MS/MS identified 87 unique peptides from *in vitro* digestion of the final IMF product at 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* semi-dynamic 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 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. (2021) produced a liquid IMF by direct or indirect UHT (143 °C/6 s) processing (Ye et al., 2021). 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., 2021). At the end of gastric phase, aggregates (> 260 kDa) in both UHT IMF remained resistant to pepsin (Ye et al., 2021). Following intestinal digestion all protein bands, including aggregates were digested (Ye et al., 2021). LC-MS/MS analysis

revealed the total number of peptides released during digestion were similar in both direct and indirect UHT treatments (Ye et al., 2021). Interestingly, there were significantly more β-casein peptides in the intestinal digesta from indirect UHT IMF compared to the direct UHT IMF ($P < 0.05$) (Ye et al., 2021). 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 to indirect UHT IMF ($P < 0.05$) (Ye et al., 2021).

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 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 2022 mg furosine/100 g 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 casein bands were observed in the gastric phase whereas intact β-LG was still present at 60 min 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 to casein 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 to the control IMF (308 mg furosine/100 g protein) ($P < 0.05$) (Zenker et al., 2020). Overall, the average peptide length was significantly higher in dry heated IMF compared to 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 INFANT MILK FORMULA ON GUT BARRIER HEALTH

Recently, researchers have used both *in vitro* and preclinical approaches to examine the relationship between thermal 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 (Sun et al., 2022). 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 prior to the intervention trial (Sun et al., 2022). The ileal mucosa of pigs who received stored UHT IMF had furosine levels > 450 μg/g dry weight and CML levels > 130 μg/g dry weight (Sun et al., 2022). Preterm pigs fed the stored UHT IMF had

significantly lower villus height at the proximal small intestine compared to 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). There were also no changes 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 to 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) (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 (2894 ± 427 μg/g protein and 639 ± 67 μg/g protein) compared to those piglets fed the IMF with double pasteurized WPC (1301 ± 188 μg/g protein and 331 ± 38 μg/g protein) ($P < 0.01$) (Aasmul-Olsen et al., 2024).

We have recently fed young pigs a high-temperature IMF diet (at 35% inclusion) 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 protein and in the duodenal brush border membrane vesicles aminopeptidases N activity was 7.44 ± 0.69 U/mg protein, aminopeptidase A activity was 155.17 ± 22.83 mU/mg protein, intestinal alkaline phosphatase levels were 167.44 ± 30.49 U/mg protein and lactase activity was 110.75 ± 7.21 nmol glucose/mg 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 to untreated control cell monolayers ($P < 0.05$) (Bavaro et al., 2021). However, this barrier disruption did not alter levels of tight junction proteins occludin, claudin-4, 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 and the mucosal proportion accounted for 62.8 ± 5% (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 tissue, aminopeptidase A activity of 2.5 ± 0.6 U/g tissue, lactase activity of 9.7 ± 0.8 U/g tissue, sucrase activity of 0.15 ± 0.01 U/g tissue, maltase activity of 3.1 ± 0.5 U/g tissue and dipeptidyl peptidase IV activity of 2.3 ± 0.7 U/g tissue (Li et al., 2013). The inflammatory biomarker, IL-8 in the distal small intestinal tissue of preterm pigs

468 was approximately 58 pg/mg tissue (Li et al., 2013). Urinary lactulose:mannitol ratio was >
469 0.5 (Li et al., 2013).

470 In a 5 d intervention trial, preterm and near-term piglets received increasing doses, by
471 orogastric feeding tube, of an IMF produced using an extensively heated WPC (Navis et al.,
472 2020a, Navis et al., 2020b). This WPC had approximately 19 μg CML/mL (Navis et al.,
473 2020b). The colon crypt depth in the piglets from both age groups fed this IMF formulated
474 with extensively heated WPC, was > 200 μm (Navis et al., 2020a).

475 ALTERNATIVES TO THERMAL PROCESSING FOR INFANT MILK FORMULA
476 PRODUCTION

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

482 Food authorities are likely to limit AGE concentrations in IMF in the near future. Certainly,
483 the EU Commission Regulation (EU) No. 2017/2158 has already set the benchmark level for
484 the presence of acrylamide in "baby foods" at 40 μg/kg body weight (Commission
485 Regulation, 2017). Acrylamide is a toxic, potentially carcinogenic, by-product of the Maillard
486 reaction (Augustine and Bent, 2022). Sourcing ingredients manufactured with lower thermal
487 loads and lowering the temperature during IMF processing will result in lower levels of AGE
488 (Navis et al., 2020b, Aasmul-Olsen et al., 2024) (Table 1). From an ingredient perspective,
489 Navis et al. (2020b) noted that pasteurized WPC (73 °C/30 s) had significantly lower levels
490 of CML compared to an extensively heated WPC (73 °C/30 s and 80 °C/6 min) ($P < 0.05$)
491 (Navis et al., 2020b). The same pasteurized WPC had close to 100% native whey protein

compared to only 30% in the extensively heated WPC (Navis et al., 2020b). In this pasteurized WPC, 72% soluble protein remained following processing compared to only 21% in the extensively heated WPC (Navis et al., 2020b). In a pasteurized WPC (72 °C/15 s) there was significantly lower furosine and CML levels compared to a double pasteurized WPC (72 °C/15 s and 80 °C/30 s) stored at 37 °C for 6 weeks (3172 ± 231 and 182 ± 13 μg/g protein *vs* 6166 ± 115 and 399 ± 14 μg/g protein) ($P < 0.05$) (Aasmul-Olsen et al., 2024). No distinctive protein aggregates were observed by SDS-PAGE in the pasteurized WPC compared to 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 prior to IMF processing (Cattaneo et al., 2017). Furosine (3492 ± 110 μg/g protein) and lysinoalanine (385 ± 43 μg/g protein) were significantly reduced compared to 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 to 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., 2021, 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/10 s) had lower furosine and CML concentrations (1797 ± 59 µg/g protein and 38 ± 2 µg/g protein) compared to the UHT liquid IMF (143 °C/6 s) (2798 ± 136 µg/g protein and 242 ± 9 µg/g protein) (Sun et al., 2022). 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). SDS-PAGE revealed less protein aggregation in pasteurized liquid IMF compared to 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/15 s) had a CML concentration of 143 ± 15 µg/g protein, a CEL concentration of 8.99 ± 1.93 µg/g protein and pyrraline levels of 36.2 ± 2.9 µg/g protein (Lund et al., 2021). However, in these studies the total bacterial load of these low thermally processed IMF was not reported which is critical to determine if a product is safe for human consumption.

Membrane filtration technology using filters to trap bacteria has been utilized 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:casein 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 to the IMF powder produced using extensive heat processing (58 ± 0% 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 4800 and 5000 µg furosine/g protein) (Yu et al., 2021). CML concentrations were 88 ± 2 µg/g protein in the membrane

filtered IMF and 106 ± 0 μg/g 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 powder compared to only 0.95×10^2 CFU/g 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 employed to produce an IMF powder (whey:casein 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 membrane filtered IMF powder *vs* 3.97 ± 0.7 μg/g high-temperature IMF powder) ($P > 0.05$) (Chen et al., 2021) (Table 1). Taken together with Yu et al (2021) results, 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 to 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 membrane filtered IMF *vs* 11.4 g total protein/100 g high-temperature IMF) (Chen et al., 2021, Chen et al., 2023). In agreement with Yu et al (2021) (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 powder) compared to high-temperature IMF (8.3×10^2 CFU/g powder) ($P < 0.05$) (Dold et al., 2024).

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

WILL REDUCTION IN THERMAL PROCESSING OF INFANT MILK FORMULA 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., 2021, 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 to 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 to 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

588 bioaccessibility of EAA (34.1 ± 1.5% *vs* 35.5 ± 1.2%) and NEAA (10.2 ± 0.7% *vs* 10.3 ±
589 0.6%) at the end of the intestinal phase (Halabi et al., 2020a) (Table 2). Zenker et al. (2020)
590 produced an unheated IMF powder with relatively low furosine levels (308 mg furosine/100
591 g protein) (Zenker et al., 2020). This IMF powder had a significantly higher degree of protein
592 hydrolysis following gastric (5.9 ± 0.4%) and intestinal (46.8 ± 3.9%) digestion compared to
593 the dry heated IMF powder with 2022 mg furosine/100 g protein ($P < 0.001$) (Zenker et al.,
594 2020).

595 Using a semi-dynamic infant *in vitro* gastric digestion model, protein digestion of membrane
596 filtered IMF was tracked (Chen et al., 2022). Once membrane filtered IMF was added to the
597 gastric phase, an increase in pH from 2.5 to 7.38 ± 0.02 was observed (Chen et al., 2022). By
598 the end of gastric digestion (156 min, pH 3.2), a significantly higher degree of protein
599 hydrolysis was recorded in the membrane filtered IMF (1338 ± 253 μmol free amines/g
600 protein) versus the high-temperature IMF (845 ± 78 μmol free amines/g protein) ($P < 0.05$)
601 (Chen et al., 2022). The membrane filtered IMF had a fragmented curd throughout *in vitro*
602 gastric digestion (Chen et al., 2022). From 94 min gastric digestion, a clear phase separation
603 could be observed as fat droplets began to separate from protein aggregates, indicating
604 membrane filtered IMF had a less dense curd than high-temperature IMF (Chen et al., 2022).
605 Although, SDS-PAGE analysis showed intact β-LG was still present in membrane filtered
606 IMF at the end of gastric digestion (Chen et al., 2022). No significant difference was
607 observed in the release of peptides < 10 kDa from either membrane filtered IMF or high-
608 temperature IMF at the end of the gastric phase ($P > 0.05$) (Chen et al., 2022). In the follow
609 up pig trial, 180 min after a final feed of a membrane filtered IMF based diet, young pigs had
610 a significantly higher degree of protein hydrolysis in the lumen collected from the stomach
611 compared to stomach contents of pigs fed high-temperature IMF diets (599 ± 45 μmol free
612 amines/g protein *vs* 509 ± 17 μmol free amines/g protein) ($P < 0.05$) (Chen et al., 2023). In

613 the stomach of these pigs there was a significantly higher concentrations of water-soluble
614 proteins and peptides compared to high-temperature IMF fed pigs ($P < 0.05$) (Chen et al.,
615 2023). The total concentration of free AA released from the stomach of pigs fed diets with
616 membrane filtered IMF or high-temperature IMF were comparable (56.5 ± 4.6 μmol/g
617 protein *vs* 59.4 ± 3.05 μmol/g protein) (Chen et al., 2023).

618 In the duodenum, there was a significant increase in the degree of protein hydrolysis from
619 pigs fed membrane filtered IMF diets (1530 ± 136 μmol free amines/g protein) compared to
620 pigs fed diets with high-temperature IMF (1174 ± 124 μmol free amines/g protein) ($P < 0.05$)
621 (Chen et al., 2023). The total concentration of free AA released were similar in the duodenum
622 of pigs in both IMF treatment groups (Chen et al., 2023). However, membrane filtered IMF
623 fed pigs had a significantly higher Pro concentration in the duodenum compared to high-
624 temperature IMF fed pigs (7.8 ± 2.61 μmol/g protein *vs* 2.2 ± 0.9 μmol/g protein) ($P < 0.05$)
625 (Chen et al., 2023). There was a similar level of protein hydrolysis recorded in the jejunum
626 and ileum of pigs in both IMF treatment groups (Chen et al., 2023). In the jejunum, taurine
627 and Trp concentrations were both significantly higher in pigs fed the membrane filtered IMF
628 diets compared to pigs fed high-temperature IMF diets (4.7 ± 1.55 and 4.9 ± 0.37 μmol/g
629 protein *vs* 1.5 ± 0.37 and 3.5 ± 0.48 μmol/g protein) ($P < 0.05$) (Chen et al., 2023). Similar
630 amounts of total free AA were released from the ileum of membrane filtered IMF fed pigs
631 (251.5 ± 33.1 μmol/g protein) and pigs fed high-temperature IMF (285 ± 28.51 μmol/g
632 protein) (Chen et al., 2023). Pigs fed membrane filtered IMF did have lower concentrations
633 of Tyr in the ileum compared to high-temperature IMF fed pigs (8.8 ± 0.98 μmol/g protein *vs*
634 11.5 ± 0.86 μmol/g protein) ($P < 0.05$) (Chen et al., 2023). Overall, membrane filtered IMF
635 fed pigs had a higher total degree of protein hydrolysis in the gastrointestinal tract compared
636 to high-temperature IMF fed pigs (1173 ± 46 μmol free amines/g protein *vs* 1022 ± 43 μmol
637 free amines/g protein) ($P < 0.05$) and higher concentrations of water-soluble proteins and

638 peptides ($P < 0.001$) (Chen et al., 2023). However, some discrepancies between *in vitro* and
639 preclinical experiments did exist with an *in vitro* static infant gastrointestinal digestion
640 reporting similar levels of proteolysis at the end of intestinal digestion for membrane filtered
641 and high-temperature IMF (471 ± 36.5 µmol free amines/mg protein *vs* 481 ± 7.7 µmol free
642 amines/mg protein) (Bavaro et al., 2021). *In vitro*, at the end of the intestinal phase, Lys, Leu
643 and Tyr were the most abundant AA released from membrane filtered IMF compared to Lys,
644 Leu and Phe from high-temperature IMF (Bavaro et al., 2021).

645 From a peptide profiling perspective, there is evidence that processing will affect individual
646 peptides released during digestion. Although processing parameters were not disclosed,
647 following *in vitro* gastrointestinal digestion, Wada and Lönnerdal (2015a) identified 39
648 bioactive peptides in intestinal digesta from a standard IMF powder compared to only 9 in the
649 intestinal digesta from an extensively hydrolysed IMF powder (Wada and Lönnerdal, 2015a).
650 Bavaro et al. (2021) treated Caco-2 cell monolayers with IMF digesta for 4 h (Bavaro et al.,
651 2021). LC-MS/MS analysis identified 488 dairy peptides in the apical chambers common to
652 both, membrane filtered and high-temperature IMF with 112 peptides unique to membrane
653 filtered IMF and 97 peptides unique to high-temperature IMF (Bavaro et al., 2021). Bavaro et
654 al. (2021) did not determine if this resulted in differences in peptide bioactive capacity
655 (Bavaro et al., 2021). Interestingly, the bioactive β-Casomorphin-7 opioid peptide (Claustre
656 et al., 2002, Trompette et al., 2003, Zoghbi et al., 2006), released from β-casein following
657 gastrointestinal digestion, was identified in both IMF (Bavaro et al., 2021).

658 Calvez et al. (2024) calculated a DIAAS of 82 for a membrane filtered IMF powder similar to
659 80 for an extensively, heat-treated IMF powder (Calvez et al., 2024). In a preclinical study by
660 Calvez et al. (2024), Wistar Han rats (n = 30, 28 d old) were fed a diet containing this
661 membrane filtered IMF at 40% inclusion for 14 d (Calvez et al., 2024). 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 to rats fed thermal processed IMF ($P < 0.001$) (Calvez et al., 2024). 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 /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., 2021). In contrast, all proteins in the intestinal digesta of UHT treated liquid IMF were completely degraded (Ye et al., 2021). A similar number of peptides were released from the intestinal digesta of pasteurized liquid IMF and UHT IMF (Ye et al., 2021) (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 to an IMF produced by 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 INFANT MILK FORMULA 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, Navis et al., 2020b, Bavaro et al., 2021, Sun et al., 2022, Dold et al., 2024) (Table 3).

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

691 In another study by Li et al. (2013), preterm pigs receiving an IMF formulated with
692 membrane filtered WPC for 5 d by orogastric feeding tube had significantly increased villus
693 height in the proximal small intestine compared to pigs receiving IMF produced with double
694 pasteurized WPC (667 ± 46 μm *vs* 440 ± 47 μm) ($P < 0.05$) (Li et al., 2013). Interestingly,
695 western blot analysis revealed that claudin-4 protein levels were significantly lower in pigs
696 receiving IMF with membrane filtered WPC compared to the pigs that received IMF
697 formulated with double pasteurized WPC ($P < 0.05$) (Li et al., 2013). Urinary
698 lactulose:mannitol ratio was significantly lower in pigs that received the IMF with membrane
699 filtered WPC (> 0.1) compared to pigs receiving the IMF produced with double pasteurized
700 WPC (> 0.5) ($P < 0.05$) (Li et al., 2013). Inflammatory biomarkers IL-8, tumor necrosis
701 factor alpha (0.6 ± 0.7 pg/mg tissue) and IL-1 beta (3.7 ± 0.5 pg/mg tissue) in the distal small
702 intestine of preterm pigs were unaffected by processing type (Li et al., 2013).

703 Preterm and near-term piglets received an IMF produced with pasteurized WPC by orogastric
704 feeding (Navis et al., 2020a, Navis et al., 2020b). Crypt depth was significantly lower in the
705 colon of preterm ($P < 0.05$) and near-term ($P < 0.01$) piglets receiving IMF with pasteurized
706 WPC compared to piglets receiving an IMF with extensively heated WPC (Navis et al.,
707 2020a). Using periodic acid-Schiff staining, Navis et al. (2020a) reported an observational
708 increase in colonic goblet cell number in piglets receiving the IMF made with pasteurized
709 WPC compared to those piglets that received an IMF with extensively heated WPC, although

710 no difference was observed in colonic mRNA transcript levels of *mucin-1* and *mucin-2*
711 (Navis et al., 2020a). *Mucin-2* mRNA transcripts were also unaffected by processing type in
712 the distal small intestine (Navis et al., 2020a). Intestinal permeability was influenced by
713 processing type, with near-term piglets receiving IMF with pasteurized WPC having a
714 significantly lower urinary lactulose:mannitol ratio compared to piglets receiving IMF with
715 extensively heated WPC (> 0.07 *vs* > 0.2) ($P < 0.05$) (Navis et al., 2020b). IL-8 protein levels
716 and mRNA transcripts were significantly reduced in the colon of preterm piglets that received
717 IMF with pasteurized WPC compared to piglets that received IMF with extensively heated
718 WPC ($P < 0.05$) (Navis et al., 2020a). Near-term piglets receiving IMF with pasteurized
719 WPC also had significantly lower mRNA transcript levels of *IL-8* ($P < 0.05$) (Navis et al.,
720 2020a). *IL-1 beta* mRNA transcript levels were significantly lower in the distal small
721 intestine of preterm piglets and colon of near-term piglets receiving IMF with pasteurized
722 WPC ($P < 0.05$) (Navis et al., 2020a). Protein concentrations and mRNA transcript levels of
723 tumor necrosis factor alpha were significantly lower in near-term piglets that received IMF
724 with pasteurized WPC compared to piglets that received IMF with extensively heated WPC
725 ($P < 0.05$) (Navis et al., 2020a).

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

729 For our preclinical study with 28 d old pigs, there were also no significant differences
730 observed in villus height, crypt depth or mucosa thickness in the upper small intestine
731 (duodenum and jejunum) of pigs fed membrane filtered or high-temperature IMF ($P > 0.05$)
732 (Dold et al., 2024). However, there was a significant increase in goblet cell number in the
733 jejunum of pigs fed membrane filtered IMF diets compared to high-temperature IMF diets

734 (17.39 ± 1.43 *vs* 11.7 ± 1.27) ($P < 0.05$) (Dold et al., 2024). In addition, acidic mucus levels
735 and mucin-2 protein concentrations in the jejunum were significantly increased in membrane
736 filtered IMF fed pigs compared to pigs fed high-temperature IMF diets ($P < 0.05$) (Dold et
737 al., 2024). A significant increase in mRNA transcript levels of *claudin-1* ($P < 0.001$), *mucin-1*
738 ($P < 0.01$) and *mucin-2* ($P < 0.001$) was measured in the duodenal mucosal scrapings of pigs
739 fed membrane filtered IMF diets versus pigs fed high-temperature IMF diets (Dold et al.,
740 2024). In parallel, in *in vitro* experiments where Caco-2 polarized cell monolayers were
741 treated with intestinal digesta from membrane filtered IMF, TEER values and claudin-1
742 protein concentrations were significantly increased compared to high-temperature IMF
743 treatment ($P < 0.05$) (Bavaro et al., 2021). Other biomarkers were unaffected by treatment
744 type, in the pig study mRNA transcripts of *occludin*, *claudin-2*, *claudin-4*, *zonula occludens-*
745 *1* and *junctional adhesion molecule-A* were similar (Dold et al., 2024) and *in vitro* occludin,
746 claudin-4, zonula occludens-1 and junctional adhesion molecule-A protein levels were
747 unaffected by processing (Bavaro et al., 2021). Interestingly, in the pig study, a significant
748 reduction in mRNA transcript levels for the *receptor for advanced glycation endproducts* was
749 also detected in the jejunum of membrane filtered IMF fed pigs compared to high-
750 temperature IMF fed pigs ($P < 0.05$) (Dold et al., 2024), even though both IMF had similar
751 CML concentrations (Chen et al., 2021).

752 From a digestive capacity perspective in the gut, Sun et al (2022) reported a significant
753 increase in brush border lactase activity in the proximal and middle small intestine of pigs fed
754 the pasteurized liquid IMF compared to stored UHT liquid IMF ($P < 0.05$) (Sun et al., 2022).
755 No differences were reported in activities of the other brush border enzymes analysed
756 (aminopeptidase N, aminopeptidase A, sucrase, maltase and dipeptidyl peptidase IV) (Sun et
757 al., 2022). In Li et al. (2013) study processing of WPC used in the formulation of IMF had no
758 significant effect on brush border aminopeptidase N, aminopeptidase A, lactase, sucrase,

759 maltase and dipeptidyl peptidase IV activities ($P > 0.05$) (Li et al., 2013). For Navis et al.
760 (2020a) study brush border intestinal alkaline phosphatase activity was significantly higher in
761 the colon of preterm piglets receiving IMF with pasteurized WPC compared to piglets
762 receiving the IMF with extensively heated WPC ($P < 0.05$), with similar levels recorded in
763 the colon of near-term piglets (Navis et al., 2020a). For preterm and near-term piglets, similar
764 levels of intestinal alkaline phosphatase activity were recorded in the distal small intestine,
765 regardless of IMF diet (Navis et al., 2020a). Aminopeptidase N, aminopeptidase A and
766 lactase in the middle small intestine were also unaffected by IMF processing (Navis et al.,
767 2020b). In Aasmul-Olsen et al. (2024) trial brush border aminopeptidase N, aminopeptidase
768 A, lactase, sucrase, maltase and dipeptidyl peptidase IV activities were similar between
769 treatment types (Aasmul-Olsen et al., 2024). Consistent with Sun et al. (2022) (Sun et al.,
770 2022) findings, we have also recently measured a significant increase in lactase activity in
771 isolated duodenal brush border membrane vesicles in pigs fed membrane filtered IMF diets
772 compared to pigs fed high-temperature IMF diets (166.59 ± 22.09 nmol glucose/mg protein
773 *vs* 110.75 ± 7.21 nmol glucose/mg protein) ($P < 0.05$) (Dold et al., 2024). In addition,
774 duodenal trypsin activities were significantly higher in membrane filtered IMF fed pigs
775 compared to pigs fed high-temperature IMF diets (1.36 ± 0.19 mU/mg protein *vs* 0.91 ± 0.2
776 mU/mg protein) ($P < 0.05$) (Dold et al., 2024). However, in the duodenum, brush border
777 aminopeptidase N activity was significantly lower in pigs fed membrane filtered IMF
778 compared to pigs on high-temperature IMF diets (5.23 ± 0.26 U/mg protein *vs* 7.44 ± 0.69
779 U/mg protein) ($P < 0.05$) (Dold et al., 2024). Processing type had no significant impact on
780 duodenal brush border aminopeptidase A or intestinal alkaline phosphatase activities and no
781 effect on the digestive enzyme capacity of the jejunum ($P > 0.05$) (Dold et al., 2024).

782 By comparing gut responses to IMF produced with different thermal loads, it is apparent,
783 there are a number of gut barrier biomarkers 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.

CONCLUSIONS

In general, *in vitro* and preclinical studies have revealed that protein hydrolysis, peptide profiles and release of free AA can be reduced by thermal processing. IMF thermal processing can also reduce villus height, tight junction expression, goblet cell number, mucus levels and brush border enzyme activities whilst increasing crypt depth 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. From a gut epithelial prospective, further mechanistic studies are also required to investigate the response of gut cells to individual AGE compounds and individual bioactive peptides.

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 ingredient suppliers and 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 ingredients and IMF (i.e. ultraviolet irradiation and membrane filtration), whilst ensuring the production of a microbiologically safe IMF product. Ultimately, the impact of any IMF product on protein digestion and gut barrier physiology in the infant gut must be compared to breast milk.

ACKNOWLEDGEMENTS

This work was funded by Science Foundation Ireland (SFI) and the Department of Agriculture, Food, and the Marine on behalf of the Government of Ireland under Grant No. 16/RC/3835 VistaMilk. Cathal A. Dold is in receipt of a Teagasc Walsh Scholarship (No. 2019220).

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Conflicts of interest statement

The authors declare that there are no conflicts of interest.

1172 **Table 1.** Effect of processing on infant milk formula protein ingredients and proteins in infant milk formula

Ingredient or IMF product[1]	Processing parameters	Effect on protein structure, protein interactions & AGE[2]	Reference
Ingredient			
2 SMP	High-heat SMP: 120 °C/120 s Pasteurized SMP: 72 °C/15 s	High-heat SMP ↑ whey protein denaturation, ↑ interactions with casein micelle & ↑ particle size *vs* pasteurized SMP	(Lin et al., 2018)
2 WPC from whole milk produced by cold filtration	Extensively heated WPC: 73 °C/30 s & 80 °C/6min Pasteurized WPC: 73 °C/30 s	Extensively heated WPC ↑ whey protein denaturation & ↑ 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/15 s & 80 °C/30 s Stored (37 °C/6 weeks) double pasteurized skim milk WPC Pasteurized skim milk WPC (from sweet whey): 72 °C/15 s	Stored double pasteurized WPC ↑ CML & furosine levels *vs* pasteurized & double pasteurized skim milk WPC	(Aasmul-Olsen et al., 2024)
3 WPC	Double pasteurized WPC (from acid whey), membrane filtered & standard pray dried Pasteurized WPC (from acid whey), membrane filtered & low temperature spray dried Membrane filtered WPC (from sweet whey), freeze dried & γ-irradiated	Double pasteurized WPC: 0.05g/L native lactoferrin & 0.2 g/L native IgG Pasteurized WPC: 0.66 g/L native lactoferrin & 3.8 g/L native IgG Membrane filtered WPC: 0.49 g/L native lactoferrin & 4.6 g/L native IgG	(Li et al., 2013)
2 WPI	WPI heated at 85 °C/2 min WPI heated at 72 °C/2 min	WPI heated at 85 °C/2 min ↑ whey protein denaturation *vs* WPI heated at 72 °C/2 min	(Joyce et al., 2017)
IMF final product			
2 liquid IMF	IMF heated at 85 °C/2 min IMF heated at 72 °C/2 min	IMF heated at 85 °C/2 min ↑ denaturation of β-LG & α-LA & ↑ protein particle size in (with preheated WPI) *vs* IMF heated at 72 °C/2 min	(Joyce et al., 2017)
2 IMF powders produced at pilot-plant scale (100 kg)	Extensively heated IMF: double pasteurization at 72 °C/30 s & 90 °C/2 - 3 s, evaporated & heated at 85 °C/2 min & spray-dried Membrane filtered IMF: 0.8 & 0.1 µm membranes & 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/5 s & spray dried Membrane filtered IMF: 1.4 & 0.2 µm membranes & 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 powder) No difference in CML between high-temperature IMF & membrane filtered IMF (3.97 ± 0.7 & 3.65 ± 0.74 µg/g powder)	(Chen et al., 2021)
2 IMF powders produced at pilot-plant scale (250 kg)	High-temperature IMF: 125 °C/5 s & spray dried Membrane filtered IMF: 1.4 & 0.2 µm membranes & 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 powder)	(Chen et al., 2023, Dold et al., 2024)
2 liquid IMF produced at laboratory scale	IMF heated at 90 °C/15 s IMF heated at 75 °C/15 s	IMF heated at 90 °C/15 s ↓ native whey protein *vs* IMF heated at 75 °C/15 s	(Halabi et al., 2020b)
2 liquid IMF produced at laboratory scale	IMF with commercial sodium caseinate & WPI in batch sterilized at 110 °C/38 min IMF with sodium caseinate & WPC (acid whey) from pasteurized skim milk in batch sterilized at 110 °C/38 min	In batch sterilized IMF (with commercial ingredients) ↑ furosine & lysinoalanine levels *vs* in-batch sterilized IMF (with sodium caseinate & 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/6 s Stored UHT IMF (40 °C/60 d) Pasteurized IMF: 72 °C/10 s	UHT IMF & stored UHT IMF↑ CML & furosine levels & ↑ 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.

[2] AGE = advanced glycation endproduct; CML = N(6)-carboxymethyllysine; CEL = N(6)-carboxyethyllysine.

1175 **Table 2.** Effect of infant milk formula processing on protein digestibility

Product[1]	Processing parameters	Methods	Measure of digestibility[2]	Effect on protein digestibility[3]	Reference
2 IMF powders produced at laboratory scale	High-temperature IMF: 125 °C/5 s & spray dried Membrane filtered IMF: 1.4 & 0.2 µm membranes & spray dried	INFOGEST *in vitro* semi-dynamic infant gastric digestion & INFOGEST *in vitro* static infant gastrointestinal digestion	SDS-PAGE & degree of protein hydrolysis	High-temperature IMF ↑ gastric pH at 0 & 31 min, ↓ protein hydrolysis & ↑ 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 & 125 min *vs* membrane filtered IMF	(Chen et al., 2022)
		INFOGEST *in vitro* static infant gastrointestinal digestion		High-temperature IMF & membrane filtered IMF had similar protein hydrolysis & no intact proteins visible at 60 min intestinal digestion	(Bavaro et al., 2021)
2 IMF powders produced at pilot-plant scale (250 kg)	High-temperature IMF: 125 °C/5 s & spray dried Membrane filtered IMF: 1.4 & 0.2 µm membranes & spray dried	28 d old pigs (n = 20) fed IMF diets (35% inclusion) for 28 d	Degree of protein hydrolysis & AA analysis	High-temperature IMF ↓ protein hydrolysis in stomach & duodenum *vs* membrane filtered IMF (stomach: 509 ± 17 *vs* 599 ± 45 µmol free amines/g protein & duodenum: 1174 ± 124 *vs* 1530 ± 136 µmol free amines/g protein) High-temperature IMF ↓ protein/peptide content in lumen, ↓ release of Pro in duodenum & ↓ taurine & 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/30 s, 90 °C/2 - 3 s before evaporation & 85 °C/2 min before spray-drying	28 d old Wistar Han rats (n = 60) fed IMF diets (40% inclusion) for 14 d	Total nitrogen digestibility, DIAAS & plasma AA concentration	Similar DIAAS for extensively heated IMF & membrane filtered IMF (0.82 & 0.8) Extensively heated IMF ↓ plasma indispensable & total AA concentrations 1 h after ingestion *vs* membrane filtered IMF No change in dietary nitrogen in stomach & SI in extensively heated IMF *vs* membrane	(Calvez et al., 2024)

	Membrane filtered IMF: 0.8 & 0.1 µm membranes & spray dried			filtered IMF (stomach: 0.14 ± 0.34% *vs* 0.04 ± 0.09% & SI: 1 ± 1.66% *vs* undetectable)	
2 liquid IMF produced at laboratory scale	IMF heated at 67.5 °C/200 min or 80 °C/7 min Unheated IMF	INFOGEST *in vitro* static infant gastrointestinal digestion	SDS-PAGE & AA analysis	Heated IMF↓ protein hydrolysis *vs* unheated IMF at 5 min 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/335 h Unheated IMF	INFOGEST *in vitro* static infant gastrointestinal digestion	SDS-PAGE & degree of protein hydrolysis	Dry heated IMF ↓ protein hydrolysis *vs* unheated IMF at 60 min gastric digestion & 120 min intestinal digestion Dry heated IMF ↑ peptide length in intestinal digesta *vs* unheated IMF	(Zenker et al., 2020)
2 liquid IMF produced at manufacturing scale	UHT IMF: 143 °C/6 s (direct or indirect) Pasteurized IMF: 72 °C/10 s	*In vitro* static infant gastrointestinal digestion	SDS-PAGE & LC-MS/MS	β-LG undigested in pasteurized IMF UHT IMF ↑ protein aggregates in digesta *vs* pasteurized IMF	(Ye et al., 2021)
2 liquid IMF produced at laboratory scale with commercial sodium caseinate & WPI or sodium caseinate & WPC (acid whey) from pasteurized skim milk	IMF in batch sterilized at 110 °C/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 & WPI: in batch sterilized IMF ↑ proteolysis *vs* unheated IMF (40.3 ± 1.2% *vs* 35.6 ± 0.6%) IMF with sodium caseinate & 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.

[2]DIAAS = digestible indispensable amino acid score.

[3] SI = small intestine.

1179 **Table 3.** Impact of infant milk formula processing on gut barrier health

Product[1]	Processing parameters[2]	Methods	Gut barrier health assessment[3]	Impact on gut barrier health[4]	Reference
3 liquid IMF produced at manufacturing scale	UHT IMF: 143 °C/6 s Stored UHT IMF (40 °C/60 d) Pasteurized IMF: 72 °C/10 s	Preterm pigs (n = 56, 106 d gestation) received IMF by orogastric feeding tube for 5 d	Histology, intestinal permeability (lactulose:mannitol ratio) & brush border enzyme activity	UHT IMF ↓ SI villus height & ↑ distal SI crypt depth *vs* pasteurized IMF Stored UHT IMF ↓ SI villus height, ↑ SI crypt depth & ↓ proximal & 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/15 s & 80 °C/30 s Stored (37 °C/6 weeks) double pasteurized skim milk WPC Pasteurized skim milk WPC (from sweet whey): 72 °C/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 & brush border enzyme activity	IMF with stored double pasteurized WPC ↑ CML & furosine in distal SI tissue *vs* IMF with pasteurized WPC & double pasteurized WPC No change in villus height, crypt depth & brush border enzymes	(Aasmul-Olsen et al., 2024)
2 IMF powders produced at pilot-plant scale (250 kg)	High-temperature IMF: 125 °C/5 s & spray dried Membrane filtered IMF: 1.4 & 0.2 µm membranes & spray dried	28 d old pigs (n = 20) fed IMF diets (35% inclusion) for 28 d	Histology, RT-qPCR, western blot, acidic mucus levels & brush border enzyme activity	In duodenum high-temperature IMF ↓ brush border lactase activity & ↓ *claudin-1*, *mucin-1* & *mucin-2* mRNA transcripts *vs* membrane filtered IMF In jejunum high-temperature IMF ↓ goblet cell number, ↓ acidic mucus levels, ↓ mucin-2 protein & ↑ *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/5 s & spray dried Membrane filtered IMF: 1.4 & 0.2 µm membranes & spray dried	INFOGEST *in vitro* static infant gastrointestinal digestion & 21 d old Caco-2 monolayers treated with 200 µg protein/cm^2	TEER & western blot	High-temperature IMF ↓ TEER & 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 & brush border enzyme activity	IMF with double pasteurized WPC ↑ lactulose:mannitol ratio & ↓ proximal SI villus height *vs* IMF with pasteurized WPC & IMF with membrane filtered WPC IMF with double pasteurized WPC ↑ distal SI claudin-4 protein *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/30 s & 80 °C/6min IMF with pasteurized WPC: 73 °C/30 s	Preterm (n=34, 106 d gestation) & 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) & inflammatory biomarkers	IMF with extensively heated WPC ↓ colonic goblet cells, ↓ intestinal alkaline phosphatase activity, ↑ lactulose:mannitol ratio & ↑ IL-8, IL-1β & TNF-α *vs* IMF with pasteurized WPC	(Navis et al., 2020a, Navis et al., 2020b)

[1]IMF = infant milk formula.

[2]WPC = whey protein concentrate.

[3]AGE = advanced glycation endproduct; SI = small intestine; RT-qPCR = reverse transcription quantitative real-time PCR; TEER = transepithelial electrical resistance.

[4]CML = CML = N(6)-carboxymethyllysine; RAGE = receptor for advanced glycation endproducts; IL-8 = interleukin-8; IL-1β = interleukin-1 beta; TNF-α = tumor necrosis factor.

1186 **Table 4.** Sensitivity of gut biomarkers to high temperature processing

Biomarker (n = 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 & 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 & 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 3 of 5 studies		(Li et al., 2013, Navis et al., 2020b, Sun et al., 2022, Aasmul-Olsen et al., 2024, Dold et al., 2024)

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