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UCC
University College Cork, Ireland
Coláiste na hOllscoile Corcaigh

Ollscoil na hÉireann, Corcaigh

National University of Ireland, Cork

School of Food and Nutritional Sciences

University College Cork, Ireland

Head of School: Prof. Alan Kelly

Supervisor: Dr. Aylin W. Sahin

Teagasc Food Research Centre

Moorepark, Fermoy, Co. Cork, Ireland

Supervisor: Dr. Linda Giblin

Influence of infant milk formula processing on protein digestion and gut barrier health *in vitro* and *in vivo*

Thesis presented by

Cathal Dold, BSc (Hons)

for the degree of

Doctor of Philosophy

December 2024

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Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism and intellectual property.

Signed: 

Name: Cathal Dold

Date: 4th December 2024

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Publications and Awards

Publications

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Conference presentations

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Abstract

Thermal treatment is essential during infant milk formula (IMF) processing to produce a safe product. However, high thermal loads during IMF processing denature native milk proteins, increase protein aggregation and promote the Maillard reaction, with the generation of advanced glycation end products. Denatured and aggregated dairy proteins will be digested differently in the infant gut compared to native dairy proteins. The degree of glycation will also alter digestion. Employing lower temperatures or non-thermal processing methods, or both, should produce IMF with a higher native protein content and less advanced glycation end products.

Previously, in our laboratory, a split-stream process using membrane filtration technology with a ceramic 1.4 µm microfiltration membrane and a 0.2 µm polyvinylidene difluoride polymeric membrane was employed to produce an IMF (MEM-IMF) with significantly higher levels of native whey protein compared to a thermally processed IMF (125 °C for 5 s) (HT-IMF).

The objective of this thesis was to examine the gastrointestinal responses of the young gut *in vitro*, *in vivo* and *ex vivo* to MEM-IMF. The hypothesis was that IMF produced using membrane filtration technology will increase protein digestibility in the upper gastrointestinal tract and support gut barrier health compared to HT-IMF.

Young pigs, at 28 days old, were blocked by sex, weight and litter origin and assigned to one of two treatments (n = 14 per treatment) and fed (1) a starter diet containing 35% MEM-IMF or (2) a starter diet containing 35% HT-IMF. Pigs, 28 days later, were sacrificed 180 mins after final feeding for the collection of gastric, duodenal, jejunum and ileal contents. Analysis revealed a significant increase in the degree of protein hydrolysis, release of free amino acids and an increase in water-soluble proteins and peptides in the gastrointestinal tract of young pigs fed MEM-IMF diets compared to pigs fed HT-IMF diets ($P < 0.05$). MEM-IMF fed pigs had a

higher number of goblet cells, acidic mucus and mucin-2 protein in the jejunum compared to pigs fed HT-IMF ($P < 0.05$). In the duodenum, MEM-IMF fed pigs had higher trypsin activity in the gut lumen, higher mRNA transcript levels of *claudin-1* in the mucosal scrapings and higher lactase activity in brush border membrane vesicles compared to those pigs fed HT-IMF ($P < 0.05$).

To further investigate these gut responses, *in vitro* gut barrier models were employed (i.e. polarised monolayers of human enterocytes (Caco-2) and goblet cells (HT29-MTX)). *Ex vivo* jejunal MEM-IMF and *in vitro* digested MEM-IMF significantly reduced the ability of pathogenic bacteria to adhere to HT29-MTX monolayers, compared to HT-IMF test samples ($P < 0.05$). This benefit is likely attributed to changes in mucus biomarkers. *Ex-vivo* jejunal MEM-IMF increased mRNA transcript levels of *mucin-2* and *mucin-5AC* in Caco-2/HT29-MTX monolayers compared to jejunal HT-IMF ($P < 0.05$). *In vitro* digested MEM-IMF increased mucin-2 protein and mRNA transcripts of *mucin-1* and *mucin-2* in Caco-2/HT29-MTX monolayers compared to *in vitro* digested HT-IMF. This mucus modulation is likely supported by the bioaccessible MEM-IMF peptide, α_{s1}-casein ^{23}FFVAP^{27}, released during *in vitro* gastrointestinal digestion of MEM-IMF but not HT-IMF. The α_{s1}-casein ^{23}FFVAP^{27} peptide increased mucin-1 and mucin-2 protein and mRNA transcripts of *mucin-5AC* in HT29MTX monolayers compared to untreated monolayers ($P < 0.05$).

In conclusion, MEM-IMF differs to HT-IMF as it (a) has faster protein digestion in a young gut, (b) promotes intestinal digestive capacity, (c) significantly increases goblet cell numbers and mucin biomarkers in the jejunum and (d) significantly reduces pathogen adhesion to an *in vitro* gut barrier. These gut responses for MEM-IMF are similar to gut responses reported with human breast milk. Therefore, we can consider MEM-IMF superior to HT-IMF. As such, MEM-IMF is a promising candidate in efforts to produce an IMF product that more closely resembles breast milk.

Keywords: Infant milk formula, membrane filtration, high-temperature, protein digestion, intestinal barrier health.

Glossary of Terms

AA: amino acid

ADFI: average daily feed intake

ADG: average daily gain

AGE: advanced glycation end product

ANOVA: analysis of variance

ATCC: American Type Culture Collection

ATOH1: atonal BHLH transcription factor 1

BCA: bicinchoninic acid

BCM-7: β-casomorphin-7

BHI: brain heart infusion

BSA: bovine serum albumin

cDNA: complementary DNA

CEL: N(6)-carboxyethyllysine

CFU: colony-forming unit

CLDN-1: claudin-1

CLDN-2: claudin-2

CLDN-4: claudin-4

CML: N(6)-carboxymethyllysine

CN: casein

DIAAS: digestible indispensable amino acid score

DMEM: Dulbecco’s Modified Eagle Medium

EAA: essential amino acid

FBS: fetal bovine serum

FCE: feed conversion efficiency

GLP-1: glucagon-like peptide 1

HBSS: Hanks’ Balanced Salt Solution

HPLC: high performance liquid chromatography

HT: high-temperature

Ig: immunoglobulin

IL-6: interleukin 6

IL-8: interleukin 8

IMF: infant milk formula

JAM-A: junctional adhesion molecule-A

KLF4: Krüppel-like factor 4

LC: liquid chromatography

MALDI-TOF MS: Matrix Assisted Laser Deionisation Time of Flight mass spectrometry

MEM: membrane filtration

MS: mass spectrometry

MUC-1: mucin-1

MUC-2: mucin-2

MUC-5AC: mucin-5AC

NEAA: non-essential amino acid

OCLN: occludin

OPA: o-phthaldialdehyde

PAGE: polyacrylamide gel electrophoresis

PBS: phosphate buffered saline

RAGE: receptor for advanced glycation end products

RPLP0: ribosomal protein lateral stalk subunit P0

RT-qPCR: reverse transcription quantitative real-time PCR

SD: standard deviation

SDS: sodium dodecyl sulfate

SEC: size exclusion chromatography

SEM: standard error of the mean

SGID: simulated gastrointestinal digestion

SI: small intestine

SID: standardised ileal digestible

SMP: skim milk powder

TEER: transepithelial electrical resistance

TNF-α: tumor necrosis factor alpha

TSA: Tryptic Soy Agar

UHT: ultra-high temperature

VRBGA: Violet Red Bile Glucose Agar

WPC: whey protein concentrate

WPI: whey protein isolate

XLD: Xylose Lysine Deoxycholate

ZO-1: zona occludens-1

α-LA: α-lactalbumin

β-LG: β-lactoglobulin

Chapter 1

Effect of processing infant milk formula on protein digestion and gut barrier health (*in vitro* and preclinical)

Literature Review

A version of this chapter was published in the Journal of Dairy Science as:

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Published version of this chapter is presented in Appendix B.

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.

1.1. 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 (Ménard et al., 2018; Nguyen et al., 2015), 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 (Gleeson et al., 2021; Kosek et al., 2017; Musa et al., 2019) 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 to 2 in the adult stomach (Fujimori, 2020). The optimum pH for activity of the gastric enzyme, pepsin, is pH 2 (Gan et al., 2017; Nguyen et al., 2015). 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 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 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 the 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 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 (1.27 ± 0.73 at day 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 (Lugli et al., 2023; Odamaki et al., 2016), 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 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 & Morrow, 2013; Kim & 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 & Yi, 2020). Whey proteins account for 70 - 80% of total milk protein in early to mid-lactation (Martin et al., 2016), with α-lactalbumin (α-LA) accounting for 40% and lactoferrin at 26% of total milk protein (Guo & 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 day old Yucatan piglets (n = 9) who received breast milk for 6 day (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, gastric digestion of total protein in 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).

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 weeks of gestation) receiving breast milk also had significantly lower intestinal permeability (lactulose:mannitol ratio) compared to preterm infants fed infant formula at one week 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 days gestation) fed breast milk compared to 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 numbers 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 microbiological safe product with an extended shelf life (Y. Wada & B. Lönnerdal, 2015). Bovine milk based infant formulae are the most common, commercially available infant formula on the market (Amira Halabi et al., 2020; He et al., 2022). Heat treatment 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., 2021). Denaturation refers to the unfolding, disruption and loss of protein structure caused by stress factors (i.e. temperature) with subsequent protein aggregation occurring following protein denaturation when the unfolded protein molecules accumulate to form aggregates (Butreddy 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 the purpose of this literature review, high-heat treatment is defined as any product produced at temperatures above pasteurisation temperature. 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.

1.2. 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 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 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 (Bakshi et al., 2023; Phosanam et al., 2021). 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-lactoglobulin (β-LG), which is absent in breast milk (Ballard & Morrow, 2013). By correcting the whey to casein ratio with the addition of either 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 & Allergies, 2014).

1.3. 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 pasteurisation (72 - 75 °C for 15 s) and ultra-high temperature (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).

1.4. 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 1.1 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.

Table 1.1. Effect of processing on infant milk formula protein ingredients and proteins in infant milk formula

Ingredient or IMF product[1]	Processing parameter	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/6 min 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, Schwebel, et al., 2020)
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 pasteurisation at 72 °C/30 s & 90 °C/2 - 3 s, evaporated & heated at 85 °C/2 min & spray-dried IMF produced by membrane filtration: 0.8 & 0.1 µm membranes & spray dried	Extensively heated IMF ↑ whey protein denaturation *vs* IMF produced by membrane filtration (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	High-temperature IMF ↓ native whey protein *vs* IMF produced by membrane filtration (2.5% *vs* 18.3%)	(Chen et al., 2021)

	IMF produced by membrane filtration: 1.4 & 0.2 µm membranes & spray dried	High-temperature IMF↓ total bacterial count *vs* IMF produced by membrane filtration (5.6×10^1 *vs* 1.3×10^2 CFU/g powder) No difference in CML between high-temperature IMF & IMF produced by membrane filtration (3.97 ± 0.7 & 3.65 ± 0.74 µg/g powder)	
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	(Amira Halabi et al., 2020)
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 end product; CML = N(6)-carboxymethyllysine; CEL = N(6)-carboxyethyllysine.

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 1.1). 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 & 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, Schwebel, et al., 2020). Only 21% of the protein was soluble (Navis, Schwebel, et al., 2020). 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 immunoglobulin (Ig) G (Li et al., 2013) (Table 1.1).

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 higher 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 pasteurisation 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 1.1).

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 (Amira Halabi et al., 2020) (Table 1.1).

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).

1.5. Advanced glycation end products 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 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 & Jakas, 2004). Detection of furosine is commonly used an indicator of early stage Maillard reaction (Chen et al., 2019; Y. Li et al., 2021). 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; Y. Li et al., 2021; van der Lugt et al., 2020). Several studies have reported furosine and CML concentrations in commercially available liquid IMF and IMF powders (Chen et al., 2019; Fenaille et al., 2006). In addition, AGE are commonly quantified during IMF processing and in the final product as an indicator of the extent of Maillard reaction (Aasmul-Olsen et al., 2024; Chen et al., 2021; Chen et al., 2019; Lee et al., 2019; Sun et al., 2022; Yu et al., 2021). 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 1.1 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 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/30 s and 80 °C/6 min) (Navis, Schwebel, et al., 2020) (Table 1.1).

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 1.1).

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, heat-treated IMF powder contained 3.97 ± 0.7 µg CML/g powder (Chen et al., 2021). In 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 1.1).

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 (van Lieshout et al., 2020; Zhu et al., 2018).

1.6. 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 (Li et al., 2021; van Lieshout et al., 2020). 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 1.2 summarizes the effect of IMF processing on protein digestion in the infant gut using data generated from *in vitro* and preclinical models.

Table 1.2. Effect of infant milk formula processing on protein digestibility

Product[1]	Processing parameters	Method	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 IMF produced by membrane filtration: 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* IMF produced by membrane filtration 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* IMF produced by membrane filtration	(Chen et al., 2022)
		INFOGEST *in vitro* static infant gastrointestinal digestion		High-temperature IMF & IMF produced by membrane filtration 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 (100 kg)	Extensively heated IMF: double pasteurisation at 72 °C/30 s, 90 °C/2 - 3 s before evaporation & 85 °C/2 min before spray-drying IMF produced by membrane filtration: 0.8 & 0.1 µm membranes & spray dried	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 & IMF produced by membrane filtration (0.82 & 0.8) Extensively heated IMF ↓ plasma indispensable & total AA concentrations 1 h after ingestion *vs* IMF produced by membrane filtration No change in dietary nitrogen in stomach & SI in extensively heated IMF *vs* IMF produced by membrane filtration (stomach: 0.14 ± 0.34% *vs* 0.04 ± 0.09% & SI: 1 ± 1.66% *vs* undetectable)	(Calvez et al., 2024)
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	(A. Halabi et al., 2020)

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 (35 ± 0.9% *vs* 54 ± 0.9%)	(Cattaneo et al., 2017)

[1]IMF = infant milk formula.

[2]DIAAS = digestible indispensable amino acid score.

[3] SI = small intestine.

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). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (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 contrast, Calvez et al. (2024) fed Wistar Han rats (n = 30, 28 days 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 (A. Halabi et al., 2020). 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 (A. Halabi et al., 2020). 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 (A. Halabi et al., 2020). 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 (A. Halabi et al., 2020). Lysine, phenylalanine, tyrosine, leucine and arginine were higher than all other AA whilst free Pro concentration was negligible (A. Halabi et al., 2020). 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 pasteurisation, 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).

1.7. 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 (Aasmul-Olsen et al., 2024; Bavaro et al., 2021; Li et al., 2013; Navis, Muncan, et al., 2020; Navis, Schwebel, et al., 2020; Sun et al., 2022). Table 1.3 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 (Aasmul-Olsen et al., 2024; Sun et al., 2022). 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 (Bavaro et al., 2021; Li et al., 2013; Navis, Muncan, et al., 2020; Navis, Schwebel, et al., 2020). 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.

Table 1.3. Impact of infant milk formula processing on gut barrier health

Product[1]	Processing parameter[2]	Method	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 laboratory scale	High-temperature IMF: 125 °C/5 s & spray dried IMF produced by membrane filtration: 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* IMF produced by membrane filtration	(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 ↓ proximal SI villus height *vs* IMF with pasteurized WPC & IMF with membrane filtered WPC IMF with double pasteurized WPC ↑ lactulose:mannitol ratio *vs* 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	(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	No change in brush border enzymes IMF with extensively heated WPC ↑ crypt depth, ↓ colonic goblet cells, ↓ intestinal alkaline phosphatase activity, ↑ lactulose:mannitol ratio & ↑ IL-8, IL-1β & TNF-α *vs* IMF with pasteurized WPC	(Navis, Muncan, et al., 2020; Navis, Schwebel, et al., 2020)

[1]IMF = infant milk formula.

[2]WPC = whey protein concentrate.

[3]AGE = advanced glycation end product; 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 end products; IL-8 = interleukin-8; IL-1β = interleukin-1 beta; TNF-α = tumor necrosis factor.

Sun et al. (2022) fed preterm pigs UHT liquid IMF for 5 day 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 days 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 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). 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 days, 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 (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).

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

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

this IMF formulated with extensively heated WPC, was > 200 µm (Navis, Muncan, et al., 2020).

1.8. Alternatives to thermal processing for infant milk formula 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 ultraviolet 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 body weight (Commission Regulation, 2017). Acrylamide is a toxic, potentially carcinogenic, by-product of the Maillard reaction (Augustine & Bent, 2022). Sourcing ingredients manufactured with lower thermal loads and lowering the temperature during IMF processing will result in lower levels of AGE (Aasmul-Olsen et al., 2024; Navis, Schwebel, et al., 2020) (Table 1.1). From an ingredient perspective, Navis et al. (2020b) noted that pasteurized WPC (73 °C/30 s) had significantly lower levels of CML compared to an extensively heated WPC (73 °C/30 s and 80 °C/6 min) ($P < 0.05$) (Navis, Schwebel, et al., 2020). The same pasteurized WPC had close to 100% native whey protein compared to only 30% in the extensively heated WPC (Navis, Schwebel, et al., 2020). In this pasteurized WPC, 72% soluble protein remained following processing compared to only 21% in the extensively heated WPC (Navis, Schwebel, et al., 2020). 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 1.1). 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 1.1).

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 (Amira Halabi et al., 2020) (Table 1.1). 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 days 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 IMF powder produced by membrane filtration (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 1.1). 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 IMF produced by membrane filtration and 106 ± 0 µg/g protein in the extensively, heat processed IMF (Yu et al., 2021). From a safety perspective, the IMF produced by membrane filtration 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 IMF powder produced by membrane filtration *vs* 3.97 ± 0.7 μg/g high-temperature IMF powder) ($P > 0.05$) (Chen et al., 2021) (Table 1.1). 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 IMF produced by membrane filtration had a significant increase in native whey protein content (4.02 g native whey protein) compared to high-temperature IMF (0.3 g native whey protein) (Chen et al., 2021). There was no loss in protein by membrane filtration with no differences in total protein content (10.9 g total protein/100 g IMF produced by membrane filtration *vs* 11.4 g total protein/100 g high-temperature IMF) (Chen et al., 2021).

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.

1.9. 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 (Bavaro et al., 2021; Calvez et al., 2024;

Cattaneo et al., 2017; Chen et al., 2022; A. Halabi et al., 2020; Navis, Schwebel, et al., 2020; Ye et al., 2021; Zenker et al., 2020) (Table 1.2).

Producing IMF with a low heat WPC ingredient supported faster gastric emptying of IMF compared to IMF produced with extensively heat-treated WPC (Navis, Schwebel, et al., 2020) (Table 1.2). Both preterm and near-term piglets received an IMF formulated with pasteurized WPC for 5 days (Navis, Schwebel, et al., 2020). 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, Schwebel, et al., 2020). In another study, the rate of protein hydrolysis differed during *in vitro* intestinal digestion between unheated IMF and heated IMF (A. Halabi et al., 2020). 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%) ($P < 0.05$) (A. Halabi et al., 2020). However, by the end of intestinal digestion, the degree of protein hydrolysis was similar (35 ± 2%) (A. Halabi et al., 2020). 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 (A. Halabi et al., 2020) (Table 1.2). Zenker et al. (2020) produced an unheated IMF powder with relatively low furosine levels (308 mg furosine/100 g protein) (Zenker et al., 2020). 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 to the dry heated IMF powder with 2022 mg furosine/100 g protein ($P < 0.001$) (Zenker et al., 2020).

Using a semi-dynamic infant *in vitro* gastric digestion model, protein digestion of IMF produced by membrane filtration was tracked (Chen et al., 2022). Once the IMF produced by membrane filtration 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 IMF produced by membrane filtration (1338 ± 253 µmol free amines/g protein) versus the high-temperature IMF (845 ± 78 µmol free amines/g protein) ($P < 0.05$) (Chen et al., 2022). The IMF produced by membrane filtration 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 the IMF produced by membrane filtration had a less dense curd than high-temperature IMF (Chen et al., 2022). Although, SDS-PAGE analysis showed intact β-LG was still present in the IMF produced by membrane filtration at the end of gastric digestion (Chen et al., 2022). No significant difference was observed in the release of peptides < 10 kDa from either the IMF produced by membrane filtration or high-temperature IMF at the end of the gastric phase ($P > 0.05$) (Chen et al., 2022).

In vitro static infant gastrointestinal digestion reported similar levels of proteolysis at the end of intestinal digestion for membrane filtered and high-temperature IMF (471 ± 36.5 µmol free amines/mg protein *vs* 481 ± 7.7 µmol free amines/mg protein) (Bavaro et al., 2021). *In vitro,* at the end of the intestinal phase, lysine, leucine and tyrosine were the most abundant AA released from the IMF produced by membrane filtration compared to lysine, leucine and phenylalanine 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 to only 9 in the intestinal digesta from an extensively hydrolysed IMF powder (Yasuaki Wada & Bo Lönnerdal, 2015). Bavaro et al. (2021) treated Caco-2 cell monolayers with IMF digesta for 4 h (Bavaro et al., 2021). LC-MS/MS analysis identified 488 dairy peptides in the apical chambers common to both, membrane filtered and high-temperature IMF with 112 peptides

unique to the IMF produced by membrane filtration 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 (Bavaro et al., 2021). Interestingly, the bioactive β-Casomorphin-7 opioid peptide (Claustre et al., 2002; Trompette et al., 2003; Zoghbi et al., 2006), released from β-casein following gastrointestinal digestion, was identified in both IMF (Bavaro et al., 2021).

Calvez et al. (2024) calculated a DIAAS of 82 for an IMF powder produced by membrane filtration similar to 80 for an extensively, heat-treated IMF powder (Calvez et al., 2024). In a preclinical study by Calvez et al. (2024), Wistar Han rats (n = 30, 28 days old) were fed a diet containing this IMF produced by membrane filtration at 40% inclusion for 14 days (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 IMF produced by membrane filtration 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 1.2). 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 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).

1.10. 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 (Bavaro et al., 2021; Li et al., 2013; Navis, Muncan, et al., 2020; Navis, Schwebel, et al., 2020; Sun et al., 2022) (Table 1.3).

In a study by Sun et al. (2022), preterm pigs received pasteurized IMF for 5 days by orogastric feeding tube (Sun et al., 2022). These pigs had significantly higher villus height and lower crypt depth in the proximal, middle and distal small intestine compared to pigs receiving 60 day 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 days by orogastric feeding tube had significantly higher villus height in the proximal small intestine compared to 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 to the pigs that received IMF formulated with double pasteurized WPC ($P < 0.05$) (Li et al., 2013). Urinary lactulose:mannitol ratio was significantly lower in pigs that received the IMF with membrane filtered WPC (> 0.1) compared to 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 alpha (0.6 ± 0.7 pg/mg tissue) and IL-1

beta (3.7 ± 0.5 pg/mg 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, Muncan, et al., 2020; Navis, Schwebel, et al., 2020). 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 to piglets receiving an IMF with extensively heated WPC (Navis, Muncan, et al., 2020). Using periodic acid-Schiff staining, Navis et al. (2020a) reported an observational increase in colonic goblet cell numbers in piglets receiving the IMF made with pasteurized WPC compared to 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* (Navis, Muncan, et al., 2020). *Mucin-2* mRNA transcripts were also unaffected by processing type in the distal small intestine (Navis, Muncan, et al., 2020). 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 to piglets receiving IMF with extensively heated WPC (> 0.07 *vs* > 0.2) ($P < 0.05$) (Navis, Schwebel, et al., 2020). IL-8 protein levels and mRNA transcripts were significantly reduced in the colon of preterm piglets that received IMF with pasteurized WPC compared to piglets that received IMF with extensively heated WPC ($P < 0.05$) (Navis, Muncan, et al., 2020). Near-term piglets receiving IMF with pasteurized WPC also had significantly lower mRNA transcript levels of *IL-8* ($P < 0.05$) (Navis, Muncan, et al., 2020). *IL-1 beta* 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, Muncan, et al., 2020). Protein concentrations and mRNA transcript levels of tumor necrosis factor alpha were significantly lower in near-term piglets that received IMF with pasteurized WPC compared to

piglets that received IMF with extensively heated WPC ($P < 0.05$) (Navis, Muncan, et al., 2020).

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

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 to stored UHT liquid IMF ($P < 0.05$) (Sun et al., 2022). No differences were reported in activities of the other brush border enzymes analysed (aminopeptidase N, aminopeptidase A, sucrase, maltase and dipeptidyl peptidase IV) (Sun et al., 2022). In Li et al. (2013) study 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 ($P > 0.05$) (Li et al., 2013). For Navis et al. (2020a) study brush border intestinal alkaline phosphatase activity was significantly higher in the colon of preterm piglets receiving IMF with pasteurized WPC compared to piglets receiving the IMF with extensively heated WPC ($P < 0.05$), with similar levels recorded in the colon of near-term piglets (Navis, Muncan, et al., 2020). 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, Muncan, et al., 2020). Aminopeptidase N, aminopeptidase A and lactase in the middle small intestine were also unaffected by IMF processing (Navis, Schwebel, et al., 2020). In Aasmul-Olsen et al. (2024) trial brush border aminopeptidase N, aminopeptidase A, lactase, sucrase, maltase and dipeptidyl peptidase IV activities were similar between treatment types (Aasmul-Olsen et al., 2024).

By comparing gut responses to IMF produced with different thermal loads, it is apparent, there are a number of gut barrier biomarkers sensitive to IMF thermal processing (Table 1.4). These include villus height, crypt depth, tight junction proteins, goblet cell numbers, 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.

Table 1.4. Sensitivity of gut biomarkers to high temperature processing

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

1.11. Conclusion

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., ultraviolet 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 to breast milk.

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Research Objectives

The primary objective of this thesis was to examine how infant milk formula (IMF) processing influences protein digestion and intestinal barrier health utilising both *in vitro* and *in vivo* models. Chapter 1 comprehensively reviewed the most recent research on how IMF manufacturing influences protein digestion and gut barrier health in the upper gut. Focusing solely on *in vitro* and preclinical models, this literature review critically examined how thermal processing of IMF denatures milk proteins, reduces protein digestibility and damages the intestinal barrier. This review also discusses how alternative processing measures such as reducing thermal treatments and utilising membrane filtration technology can be employed to retain the native structure of milk proteins, increase protein hydrolysis in the upper gut and protect the intestinal barrier.

For this thesis, a split-stream process using membrane filtration technology with a ceramic 1.4 µm microfiltration membrane and a 0.2 µm polyvinylidene difluoride polymeric membrane was employed to produce an IMF (MEM-IMF). An IMF powder was also produced using standard, high temperature IMF processing at 125 °C for 5 s. Chapter 2 used a dietary intervention with young pigs, 28 days old, to examine if reduced heat treatment during IMF processing could influence growth performance and promote protein digestibility in the stomach, duodenum, jejunum and ileum. In a follow-on study, Chapter 3 examined if MEM-IMF diets fed to young pigs influenced digestive and brush border membrane enzyme activity in the upper small intestine (duodenum and jejunum). In addition, this chapter investigated if reduced thermal processing of IMF changed gut barrier physiology by examining gut barrier structure and mucus production in the pig's upper small intestine.

The objective of Chapter 4 was to determine how lumen samples collected from the jejunum of young pigs and *in vitro* gastrointestinal digesta samples from MEM-IMF or HT-IMF

influences intestinal barrier health in epithelial cells *in vitro*. This chapter investigated how IMF processing influences tight junction and mucus biomarkers in a Caco-2/HT29-MTX intestinal barrier model, protects against pathogenic bacteria adhesion to mucus secreting goblet cells and influences satiety hormone secretion from enteroendocrine cells. This chapter also set out to identify potentially bioactive milk peptides in digesta collected following *in vitro* gastrointestinal digestion of MEM-IMF and investigate if individually peptides influence mucus biomarkers in an *in vitro* mucosal barrier model.

Chapter 5 summarises the main findings from this thesis and how this work contributes and adds additional information to existing knowledge. This general discussion also highlights the strengths and weaknesses of this thesis and potential avenues for future research.

Chapter 2

Membrane filtration processing of infant milk formula alters protein digestion in young pigs

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Published version of this chapter is presented in Appendix B.

Authorship contribution statement

Cathal Dold performed protein and amino acid analysis experiments, performed statistical analysis, contributed to drafting of the original manuscript and reviewing and editing of final manuscript.

Abstract

Reducing heat treatment during infant milk formula (IMF) processing is desirable to produce a product that more closely resembles breast milk. By employing membrane filtration, we produced an IMF (60:40 whey to casein ratio) at pilot plant scale (250 kg). IMF produced by membrane filtration (MEM-IMF) had a significantly higher content of native whey protein (59.9%) compared to a high-temperature IMF (HT-IMF) (4.5%) ($P < 0.001$). Pigs, at 28 days old, were blocked by sex, weight and litter origin and assigned to one of two treatments (n = 14 per treatment): (1) starter diet containing 35% of HT-IMF powder or (2) starter diet containing 35% of MEM-IMF powder for 28 days. Body weight and feed intake were recorded weekly. Pigs at day 28 post-weaning were sacrificed 180 min after their final feeding, for the collection of gastric, duodenal, jejunal and ileal contents (n = 10 per treatment). MEM-IMF diet resulted in more water-soluble proteins and higher levels of protein hydrolysis in the digesta at various gut locations compared to HT-IMF ($P < 0.05$). In the jejunal digesta, a higher concentration of free amino acids were present post MEM-IMF consumption (247 ± 15 μmol/g protein) compared to HT-IMF (205 ± 21 μmol/g protein). Overall, average daily weight gain, average dairy feed intake and feed conversion efficiency were similar for pigs fed either MEM-IMF or HT-IMF diets, but significant differences were observed at particular intervention periods. In conclusion, reducing heat treatment during processing of IMF influenced protein digestion and revealed minor effects on growth parameters providing *in vivo* evidence that babies who are fed with IMF processed by membrane filtration are likely to have different protein digestion kinetics but minimal effect on overall growth trajectories as babies fed IMF processed by traditional thermal processing.

Keywords: Protein digestion, whey protein, infant formula, membrane filtration, *in vivo* digestion, infant formula absorption.

2.1. Introduction

Human breast milk is considered the global "gold standard" when it comes to early life nutrition for infants. However, infant milk formula (IMF) becomes the primary diet for developing infants when breastfeeding is not an option for women or when milk is unavailable (World Health Organization, 2001, 2003). The composition of IMF is formulated to closely mimic that of breast milk, with regard to macronutrient (protein, fat and carbohydrate) and micronutrients (minerals and vitamins), and thereby provide the essential nutrients and bioactive components which are required for adequate growth and development of newborn babies (Masum, Chandrapala, et al., 2020). Nonetheless, several studies have shown that IMF has a lower nutritional value and functional value than breast milk (Chen et al., 2019; Masum, Huppertz, et al., 2020; Patelarou et al., 2012). Industrially manufactured IMF undergoes intensive thermal treatment during processing to guarantee microbiological safety but this is cause for concern, as high temperatures induce Maillard reactions, disrupt the mineral balance and damage vitamins (Wazed et al., 2020). Furthermore, proteins in IMF (mainly β-lactoglobulin (β-LG) and α-lactalbumin (α-LA)) undergo considerable conformational modifications (unfolding, denaturation and aggregation) (Chen et al., 2019). As such, the proteins present in IMF differ in conformation compared to those in breast milk, and this may impact the digestion and overall physiological effect of these proteins once consumed (van Lieshout et al., 2020).

There is scope to improve the nutritional quality of IMF by using alternative processing technologies. Membrane filtration is one such alternative technology, which has recently been assessed for IMF manufacturing (Bavaro et al., 2021; Chen et al., 2021; Yu et al., 2021). In particular, membrane filtration is an emerging alternative to thermal processing, as it greatly reduces the thermal load necessary during formula preparation. Our group produced laboratory scale IMF from raw bovine skim milk with added whey protein isolate using a split-stream process incorporating a 1.4 μm microfiltration, followed by a 0.2 μm microfiltration (Chen et

al., 2021). A control IMF was also produced using standard high-temperature treatment (HT-IMF) without membrane filtration. The IMF produced by membrane filtration (MEM-IMF) powder had significantly higher amounts of native (1.1 g/100 g powder *vs* 0.18 g/100 g powder) and monomeric (1.48 g/100 g powder *vs* 0.46 g/100 g powder) whey proteins compared to HT-IMF. MEM-IMF also exhibited a lower degree of protein aggregation compared to HT-IMF. A comparison of microbial and the Maillard reaction by-product marker, N(6)-carboxymethyllysine, in MEM-IMF suggested that it was safe and could be manufactured at scale. The laboratory scale MEM-IMF and HT-IMF underwent *in vitro* static and semi-dynamic infant gastrointestinal digestion (Bavaro et al., 2021; Chen et al., 2022). The *in vitro* gastric digestion results suggested that the higher native whey protein content in the MEM-IMF led to accelerated disintegration of gastric curd with more free amine groups released during digestion, and potentially, faster gastric emptying (Chen et al., 2022). Differences in digestion of the proteins for MEM-IMF and HT-IMF, resulted in different peptides at the end of the static *in vitro* intestinal phase (Bavaro et al., 2021). This in turn led to different peptides in the basolateral chamber of the *in vitro* gut barrier model (Caco-2 polarised monolayers) treated for 4 h with either MEM-IMF or HT-IMF (Bavaro et al., 2021), indicating IMF processing alters peptide bioavailability *in vitro*.

The present study aimed to investigate whether or not reduced heat treatment during IMF processing altered protein digestion in the gut, by performing a dietary intervention study with young pigs over 28 days. MEM-IMF and HT-IMF were produced at pilot plant scale (250 kg for each powdered IMF). Pigs are the preferred animal model for gastrointestinal research because they have similar gut size and digestive enzyme activity to humans (Guilloteau et al., 2010). Use of pig models is particularly important for paediatric nutrition research due to limitations in the number of intervention trials that can be conducted in human infants, and their value in assessing infant formulae and milk substitutes (Miller & Ullrey, 1987).

Previously, a review by Sun et al (2023) concluded that a human year is equal to approximately 121.7 pig days (Sun et al., 2023). Therefore, pigs used in this study are representative of a 6-month old infant.

Our hypothesis is that MEM-IMF with a higher native whey protein content will elevate protein degradation and increase amino acid concentration in the upper gastrointestinal tract, compared to HT-IMF. This may, in turn, increase dietary protein bioavailability which could potentially affect porcine growth rate. Monitoring growth trajectories is important as ultimately, babies who are fed with IMF processed by membrane filtration should have similar growth trajectories as babies fed IMF processed by traditional thermal processing or preferably similar trajectories to breast fed babies.

2.2. Materials and Methods

2.2.1. Materials

IMF powders used in this study were produced in Moorepark Technology Limited (Ireland). Raw bovine whole milk was collected from the dairy farm at the Teagasc, Animal and Grassland Research and Innovation Centre (Ireland). The fat was removed centrifugally at 50 °C. Whey protein isolate (protein content of 91.6 %) was obtained from Agropur (USA). Lactose was sourced from Glanbia Ingredients Ireland (Ireland). Rapeseed oil was supplied by Triby Trading Ltd. (Ireland).

The ratio of macronutrients for MEM-IMF and HT-IMF, protein: lactose: fat, were 1.29:7:3.49 (Chen et al., 2021). The protein had a whey protein to casein ratio of 60:40 for IMF. This ratio produces a stage one IMF suitable for infants aged 0 - 6 months. IMF powders were manufactured independently in a single batch (250 kg). The processing schemes for the production of MEM-IMF and HT-IMF were comprehensively described previously (Chen et al., 2021).

2.2.2. Total protein profile and quantification of native whey proteins in IMF powders

IMF powder samples were measured for their total protein content, protein profiles and native whey protein content using the methods described previously (Chen et al., 2021). All analysis was performed in triplicate. Total protein content was determined using the Kjeldahl method (ISO, 2001), applying a nitrogen to protein conversion factor of 6.25. Protein profiles and native whey protein content were measured using reverse-phase HPLC, as described previously (Chen et al., 2021).

2.2.3. Animals, treatments and housing

The animal study was performed between June and August 2020, at the Teagasc Pig Development Department (Ireland). Ethical approval for this study was granted by the Teagasc Animal Ethics Committee (approval No. TAEC2020-248), and the project was authorised by the Health Products Regulatory Authority (project authorisation No. AE19132/P111). The experiment was conducted in accordance with Irish legislation (SI No. 543/2012) and the EU Directive 2010/63/EU for animal experimentation, and was in compliance of the ARRIVE guidelines. All pigs remained clinically healthy during the study, and no adverse events were observed in any of the experimental groups.

A total of 28 (18 male and 10 female) newly weaned pigs of the same genetic background (Maternal line: Large White × Landrace; PIC Hermitage, Ireland; Terminal line: Tempo, Premier Pig Genetics Ltd., Ireland) were selected for use in the feeding study. At weaning (d 27 ± 0.7 of age) pigs were blocked on the basis of sex, body weight (8.3 ± 0.59 kg), and litter origin. Within block, individual pigs were randomly assigned to one of two dietary treatments (n = 14 pigs per treatment) until d 28 post-weaning: (1) starter diet containing 35% of HT-IMF powder or (2) starter diet containing 35% of MEM-IMF powder. Each pig was individually penned in fully slatted pens (pen dimensions: 2.4 m × 1.8 m) and each pen was equipped with a single-spaced stainless steel feeder (30 cm wide; O'Donovan Engineering, Ireland) and a drinker bowl (BALP, France). Three sources of environmental enrichment (a hanging wooden post, hessian material and an EASYFIX LUNA 86 rubber toy) were used, with two provided at any one time.. Room temperature was maintained at 28 °C for the first 7 d post-weaning and gradually reduced to 22 °C by d 28 post-weaning. Artificial lighting was provided daily from 08:00 to 17:00 h. The pigs were observed closely at least 3 times daily. Pig growth and feed intake were monitored from d 1 to d 28 post-weaning.

2.2.4. Diets and feeding

Diets were formulated to meet or exceed the National Research Council (National Research Council, 2012) requirements for weanling pigs (Table 2.1). Diets were formulated to be isonitrogenous and isoenergetic and to contain the same standardised ileal digestible (SID) lysine content. As a result, deviation of less than 5% between diets occurred with a difference of 3.72% for maize, 0.58% for soya Hi-Pro, and 4.35% for barley. The diets were manufactured on site and fed in meal form. From d 0 to d 28 post-weaning, pigs were fed twice daily at 08:00 and 17:00 h and *ad libitum* access to water was provided at all times from one bowel drinker per pen. All pigs remained healthy throughout the course of the trial.

Table 2.1. Composition of experimental diets (on air dry basis as fed)

	Experimental Diets[1]	
	MEM-IMF	**HT-IMF**
Ingredients (g/kg)		
IMF powder	350	350
Maize	102.53	65.33
Soya Hi-Pro	271.67	265.90
Barley	235.80	279.31
Limestone flour	8	8
Dicalcium phosphate	11	11
Salt feed grade	3	3
Lysine HCL (78.8)	4.91	4.85
L-Threonine	3	2.90
DL-Methionine	3.08	3.10
L-Valine	1.90	1.60
L-Tryptophan	1	0.90
Vitamin and trace mineral premix[2]	3	3
Phytase[3]	0.10	0.10
Celite[4]	1	1
Nutrient composition (g/kg)		
DM[5]	912.47	913.87
Protein[5]	210	209.88
Ash[5]	51.29	51.10
Fat[6]	101.05	101.88

Fibre[6]	23.50	24.46
Starch + Sugar[6]	424.92	427.01
Sugar[6]	236.12	239.36
Starchew[6]	188.80	187.66
NDF[6]	79.09	82.86
ADF[6]	28.63	29.76
Moisture[6]	87.53	86.13
DE, MJ DE/kg[6]	16.4	16.4
NE, MJ NE/kg[6]	5.7	5.7
Total lysine[6]	16.5	16.5
SID lysine[6]	15.3	15.3

[1]MEM-IMF; infant milk formula produced by membrane filtration; HT-IMF, high-temperature infant milk formula; DM, dry matter; NDF, neutral detergent fibre; ADF, acid detergent fibre; DE, digestible energy; NE, net energy.

[2]Vitamin and mineral premix provided per kilogram of complete diet: Cu from copper sulphate, 15mg; Fe from ferrous sulphate monohydrate, 90 mg; Zn from zinc oxide, 120 mg; Mn from manganese oxide, 47 mg; Ca from calcium iodate, 0.6 mg; Se from sodium selenite, 0.3 mg; vitamin A as retinyl acetate, 1.5 mg; vitamin D_3 as cholecalciferol, 12.5 µg; vitamin E as DL-alpha-tocopheryl acetate, 90 mg; vitamin K, 4 mg; vitamin B_{12}, 15 µg; riboflavin, 2 mg; nicotinic acid, 12 mg; pantothenic aid, 10 mg; choline chloride, 250 mg; vitamin B_1, 2 mg; and vitamin B_6, 3 mg.

[3]The diets contained 500 phytase units (FTU) per kilogram feed from RONOZYME HiPhos (DSM, Belfast, UK).

2.2.5. Data and sample collection

2.2.5.1. Pig growth performance

Pigs (n = 28) were individually weighed on d 0, 7, 14, 21, and 27 post-weaning using an electronic weighing scales (O'Donovan Engineering, Ireland). Pigs were fasted overnight before weighing and all pigs were weighed between 11:00 and 13:00 h. Feed intake was recorded on an individual basis from d 0 to d 27 post-weaning. This data was used to determine the average daily gain (ADG), average daily feed intake (ADFI), and feed conversion efficiency (FCE) for each individual pig:

- Average daily gain (ADG; g/day) = ((final weight (kg) - initial weight (kg)) / No. of days) × 1000
- Average daily feed intake (ADFI; g/day) = ((feed provided (kg) - Feed remaining at end of experiment (kg) - waste feed removed (kg)) / No. of days) × 1000
- Feed conversion efficiency (FCE; g/g) = ADFI (g/day) / ADG (g/day)

2.2.5.2. Apparent total tract digestibility

Celite (1 g/kg) was added to each diet during the milling process as a source of acid insoluble ash for use as a marker for determination of apparent total tract digestibility (McCarthy et al., 1977). Representative feed samples were collected from feed hoppers on d 23 post-weaning and freshly voided faecal samples were collected from a subsample of pigs (n = 10 pigs per treatment) on d 24, 25, and 26 post-weaning. Samples were stored frozen at -20 °C. Prior to analysis for determination of apparent total tract digestibility, faecal samples were freeze dried at -50 °C for 5 d and feed and dried faecal samples were milled through a 2 mm screen (Christy and Norris Hammer Mill, UK).

2.2.5.3. Post slaughter sample collection

On d 28 post-weaning, 3 h after pigs received their final feed, a subsample of pigs (same pigs as sampled for apparent total tract digestibility; n = 10 pigs per treatment) were humanely euthanized by captive bolt stunning, followed immediately by exsanguination.

Following slaughter, the entire digestive tract was removed by blunt dissection and digesta samples were recovered aseptically from the pyloric region of the stomach, duodenum (50 cm caudal from the pyloric junction), jejunum (2.5 m caudal from the pyloric junction), and ileum (15 cm cranial from the ileocaecal valve). Digesta samples were stored in sterile 155 ml collection pots (Alpack Ltd., Ireland), weighed, and subsequently stored at -20 °C until analysis. Prior to laboratory analyses, digesta samples were freeze dried at -50 °C for 7 d and dried digesta samples were finely ground using a pestle and mortar.

2.2.6. Laboratory analysis

2.2.6.1. Apparent total tract digestibility

The dry matter content of feed and faeces was determined after overnight drying at 105 °C (18 h minimum). The crude ash content was determined after ignition of a weighed sample in a muffle furnace (Nabertherm, Germany) at 550 °C for 5.5 h (Thiex et al., 2019). Crude protein content was determined as nitrogen × 6.25 using a LECO FP 528 instrument (LECO Instruments Ltd., UK) (Thiex, 2019). The concentration of acid and insoluble ash in feed, faecal and ileal digesta samples was determined according to the method of McCarthy et al. (1977) (McCarthy et al., 1977).

2.2.6.2. Total amino acid profile

Analysis of the total amino acid profile in feed and faecal samples were performed by Sciantec Analytical Services Ltd. (UK) according to the method described previously (Otter, 2012).

2.2.6.3. Total crude protein content determination

The crude protein content in the digesta samples collected from the stomach, duodenum, jejunum and ileum was determined using the LECO FP 528 instrument (LECO Instruments Ltd., UK). A nitrogen-protein transformation factor of 6.25 was applied. Each measurement was performed in triplicate on different days.

2.2.6.4. Quantification of protein hydrolysis

The level of protein hydrolysis was estimated by measuring the quantity of primary amines in samples using the standardised o-phthaldialdehyde (OPA) spectrophotometric assay in 96-well plates. The OPA assay and sample preparation were described previously (Bavaro et al., 2021; Mulet-Cabero et al., 2019). Various concentrations of standard $_{L}$-Leucine solutions (prepared in phosphate buffered saline (PBS)) in the range 0 to 10 mM were used to prepare a standard calibration curve. Both the standard and samples (10 µl) were placed into each corresponding well on the microtitre plates and mixed with 200 µl of OPA reagent. The plates were covered with aluminium foil and placed in a dark environment to allow the reaction to develop for 15 min. The absorbance was measured at 340 nm using a multi-mode microplate reader (Synergy HT, BioTek Instruments Inc., USA). The concentration of free amino groups was corrected by protein content in samples. Each measurement was carried out in triplicate on different days.

2.2.6.5. Free amino acid analysis

Free amino acid analysis was conducted on freeze dried gastrointestinal (stomach, duodenum, jejunum and ileum) digesta content from the young pigs. The method used was described previously (Bavaro et al., 2021). Briefly, each test sample was mixed using a vortex and then 750 µl of 24% TCA was added to 750 µl of mixed digesta sample. This mixture was again mixed thoroughly using a vortex and then allowed to stand in an ice-bath for 10 min. The mixture was centrifuged for 10 min at 14000 rpm. The supernatant was collected in a separate tube, and stored in -80 °C until analysis. Free amino acids were quantified in the supernatant

samples using a Jeol JLC-500/V amino acid analyser (Jeol Ltd., UK) fitted with a Jeol Na^+ high-performance cation exchange column.

2.2.6.6. Molecular mass distribution of native proteins and peptides by size exclusion chromatography-high performance chromatography

Size exclusion chromatography (SEC) HPLC was utilised to estimate the molecular mass distribution of proteins and peptides in the soluble fraction of digesta collected from the stomach, duodenum, jejunum and ileum. Digesta was dissolved at 2.5% (*w/v*) in Milli-Q water at 4 °C for 30 min. Solutions were then filtered using 0.22 μm filters to remove excess micellar casein fraction, fat and insoluble protein aggregates. Samples were run on a Waters 2695 HPLC with ultraviolet/Visible detector (Waters UV2489) at a wavelength of 214 nm to detect peptide bonds present in proteins and peptides. A TSKgel 2000SW, 7.8mm × 600 mm column (Tosh Biosciences GmbH, Germany) was used. Sample (20 μl) was injected into the column. Separation was performed by isocratic elution using a 0.1% (*v/v*) TFA in 30% (*v/v*) acetonitrile with 0.5 ml per min flowrate. Several molecular weight markers (GE Healthcare, USA) were run on the SEC-HPLC in advance to generate a molecular mass calibration curve; phenylalanine (165 Da), histidine-tyrosine-leucine (294 Da), bacitracin (1.4 kDa), aprotinin (6.5 kDa), α-LA (14.4 kDa), β-LG (18.3 kDa), carbonic anhydrase (29 kDa) and bovine serum albumin (67 kDa). Integration and data analysis was undertaken using the Waters EMPOWER® software (Waters Corporation, USA).

2.2.7. Statistical analysis

All data are reported as means ± standard error of the mean (SEM) of at least three or more biological replicates for each treatment. Data was statistically analysed using the IBM SPSS Statistics Version 27 (IBM Statistics, USA). The protein prolife of IMFs, pig growth and feed intake (body weight, ADG, ADFI and FCE), and apparent total tract digestibility were analysed

using one-way analysis of variance (ANOVA). Molecular mass distribution of water-soluble proteins and peptides were analysed within the gastric and intestinal phase using a generalised estimating equation for repeated measures with "gastrointestinal location" (stomach, duodenum, jejunum and ileum), "molecular mass" (molecular weight intervals: 28 - 65 kDa, 18 - 28 kDa, 10 - 18 kDa, 0.5 - 10 kDa, 0.2 - 0.5 kDa, 0.1 - 0.2 kDa, and < 0.1 kDa) and "diets" (MEM-IMF and HT-IMF) as fixed effects, peak area of HPLC at each molecular weight interval as a dependent variable, and replicates of measurement as random effect. The concentration of free amino groups and the concentration of free amino acids were investigated using generalised estimating equation with "gastrointestinal location" and "diets" as fixed effects; concentration of free amine groups and individual free amino acid of protein in digesta as dependent variables. The Wald chi-square analysis was used to determine the effect of diet on overall gastrointestinal location. Post hoc tests were performed using contrast analysis. Results were considered statistically significant when $P \leq 0.05$ and were considered as tendencies when $0.05 < P < 0.10$.

2.3. Results

2.3.1. Native whey protein content in IMF

Pilot plant production of MEM-IMF resulted in a powder in which 59.9% of the total whey content was native whey, as determined by HPLC. In contrast, the HT-IMF had 0.3 g native whey protein per 100 g of IMF powder, which accounted for 4.5% of total whey protein content, significantly lower than MEM-IMF ($P < 0.001$).

2.3.2. Growth performance and feed intake of young pigs

Performance and feed intake (body weight, ADG, ADFI and FCE) of young pigs (28 days old) who were fed the experimental diets is described in Table 2.2. Pig body weight was similar at all time points for both treatment groups ($P > 0.05$). Pigs fed the MEM-IMF diet had higher ADG between d 0 and d 7 of the intervention compared to HT-IMF ($P < 0.01$). However, between d 21 and d 27, HT-IMF fed pigs tended to have a higher ADG than MEM-IMF fed pigs ($P = 0.07$). Similar to ADG, the ADFI of pigs in the MEM-IMF treatment group was higher than that of the HT-IMF group between d 0 and d 7 ($P < 0.001$). The HT-IMF fed pigs tended to have a higher ADFI between d 21 and d 27 than MEM-IMF fed pigs ($P = 0.06$). Between d 7 and d 14, the FCE of pigs fed HT-IMF was significantly improved compared to the MEM-IMF group ($P = 0.03$). The FCE of pigs between d 0 and d 7 and after d 14 was unaffected by dietary treatment ($P > 0.05$).

Table 2.2. Effect of infant milk formula processing technique on pig body weight, average daily gain, average daily feed intake, and feed conversion efficiency from day 0 to day 27 post-weaning

Measure	Dietary Treatment		SEM	*P*-value
	MEM-IMF	HT-IMF		
BW (kg)				
Day 0	8.3	8.43	0.21	0.63
Day 7	9.7	9.4	0.22	0.16
Day 14	12.8	12.5	0.33	0.44
Day 21	16.6	16.5	0.46	0.97
Day 27	21.0	21.4	0.61	0.47
ADG (g/d)				
Day 0 - day 7	200	140	22.6	0.01
Day 7 - day 14	435	444	31.5	0.78
Day 14 - day 21	537	572	28.6	0.24
Day 21 - day 27	736	815	41.3	0.07
Day 0 - day 27	468	480	10.4	0.54
ADFI (g/d)				
Day 0 - day 7	270	201	17.9	0.01
Day 7 - day 14	486	462	26.7	0.38
Day 14 - day 21	655	679	30.9	0.43
Day 21 - day 27	881	959	39.4	0.06
Day 0 - day 27	561	561	11.3	0.99
FCE (g/g)				
Day 0 - day 7	1.38	1.25	0.183	0.46
Day 7 - day 14	1.13	1.05	0.036	0.03
Day 14 - day 21	1.22	1.19	0.040	0.49
Day 21 - day 27	1.20	1.19	0.044	0.89
Day 0 - day 27	1.20	1.17	0.012	0.36

MEM-IMF; infant milk formula produced by membrane filtration; HT-IMF, high-temperature infant milk formula; BW, body weight; ADG, average daily gain; ADFI, average daily feed

intake; FCE, feed conversion efficiency. $P < 0.05$ indicates statistical significance. Values represent means ± SEM.

2.3.3. Apparent total tract digestibility

The effects of IMF processing on apparent total tract digestibility of dry matter, ash, crude protein, and organic matter are presented in Table 2.3. Pigs in the HT-IMF treatment group had a higher apparent total tract digestibility of dry matter ($P < 0.001$) and crude protein ($P < 0.001$) compared to pigs fed MEM-IMF. Similarly, pigs fed HT-IMF had a higher apparent total tract digestibility of organic matter compared to those fed MEM-IMF ($P = 0.005$). There was no effect of dietary treatment on the apparent total tract digestibility of ash ($P > 0.05$).

Table 2.3. Effect of IMF processing technique on apparent total tract digestibility of dry matter, ash, crude protein and organic matter

	Dietary Treatment			
ATTD	**MEM-IMF**	**HT-IMF**	**SEM**	***P*-value**
Dry Matter (%)	75	79	0.667	< 0.001
Ash (%)	41	49	1.702	0.61
Crude Protein (%)	56	70	1.737	< 0.001
Organic Matter (%)	78	82	0.562	0.005

MEM-IMF; infant milk formula produced by membrane filtration; HT-IMF, high-temperature infant milk formula; ATTD, apparent total tract digestibility. *P* value of < 0.05 indicates significance. Values represent means ± SEM.

2.3.4. Proteolysis of IMF

The *in vivo* proteolysis of the IMF products was characterised by measuring the protein material in digesta collected from the stomach, duodenum, jejunum and ileum. Three complementary methods were used: (i) appearance of water-soluble proteins and peptides by SEC-HPLC, (ii) the measurement of the available amine groups by the OPA method, and (iii) the concentration of free amino acids.

2.3.4.1. Appearance of soluble proteins and peptides by SEC-HPLC

Water-soluble protein fractions and peptides present in the stomach, duodenum, jejunum and ileum lumen of pigs fed MEM-IMF or HT-IMF diets were separated by SEC-HPLC. The molecular weight distribution of the gut proteins and peptides are depicted in Figure 2.1. Insoluble materials, casein micelles, fat, and large protein aggregates cannot be analysed by HPLC and were removed by 0.22 μm syringe filtration. There was a rapid increase in low molecular weight peptides when food moved from the stomach to the small intestine. Overall, the amount of water-soluble protein and peptide content was higher in the intestinal lumen of pigs fed MEM-IMF diets than that of pigs fed HT-IMF diets ($P < 0.001$). The three largest molecular mass groups (28 - 65 kDa, 18 - 28 kDa and 10 - 18 kDa), which are associated with water-soluble protein aggregates and monomeric proteins (bovine serum albumin, β-LG and α-LA), were higher in MEM-IMF digesta from jejunal and ileal samples compared to the HT-IMF treatment. There tended to be a higher concentration of peptides in the ranges of 0.5 - 10 kDa ($P = 0.07$) and 0.2 - 0.5 kDa ($P = 0.09$) in the stomach, 0.5 - 10 kDa ($P = 0.08$) in the duodenum, 0.2 - 0.5 kDa ($P = 0.1$), 0.1 - 0.2 kDa ($P = 0.09$) and 0.01 - 0.1 kDa ($P = 0.1$) in the jejunum of MEM-IMF derived digesta compared to HT-IMF. Moreover, the MEM-IMF digesta showed elevated concentrations of peptides within the 0.01 - 0.1 kDa range in the ileum ($P < 0.01$). However, very few peptide bonds are present at ≤ 1 kDa.

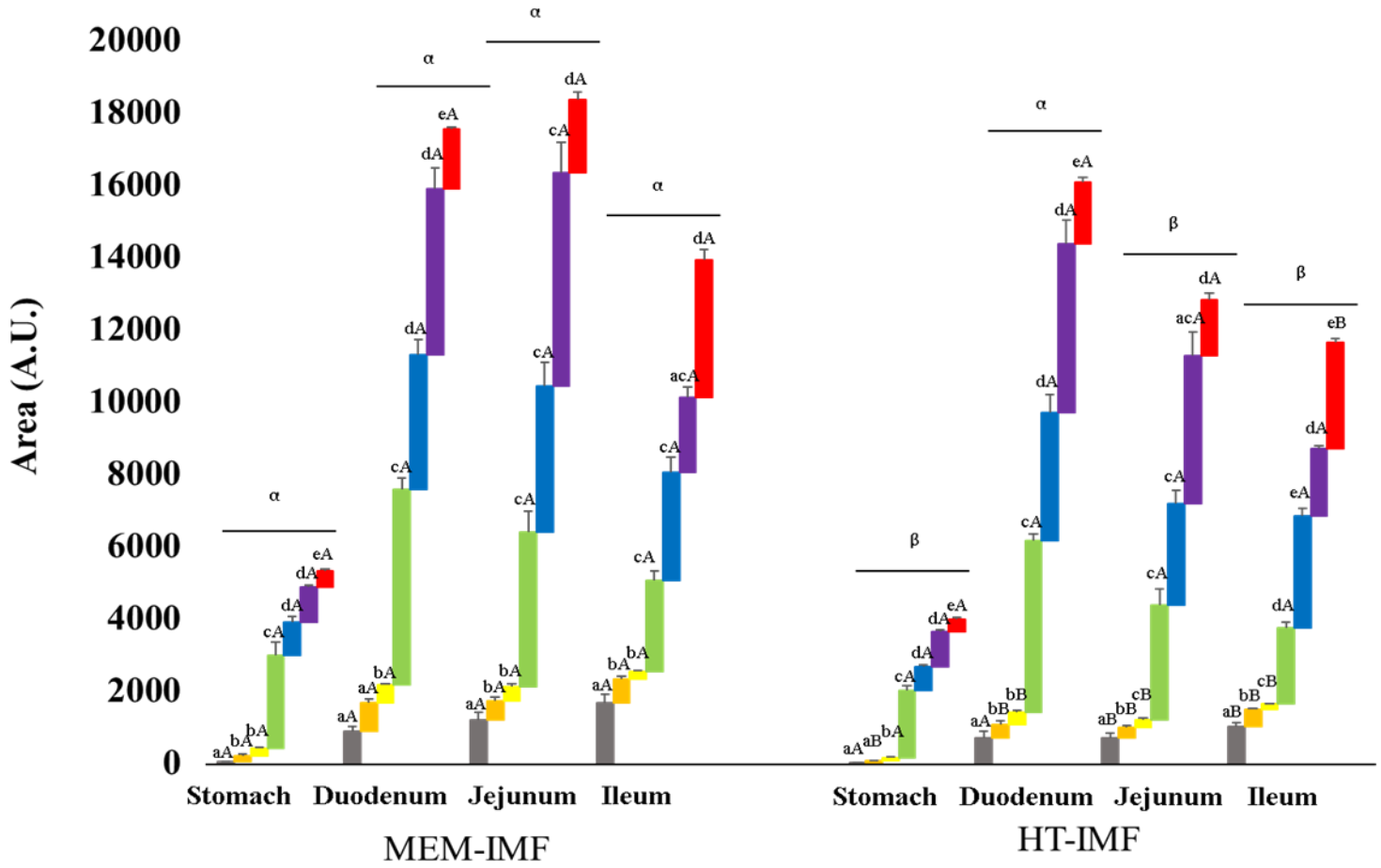


Figure 2.1. Molecular weight distribution as determined by size exclusion chromatography, high performance liquid chromatography (SEC-HPLC) of the water-soluble molecules 28 - 65 kDa (grey), 18 - 28 kDa (orange), and 10 - 18 kDa (yellow) kDa, 0.5 - 10 kDa (green), 0.2 - 0.5 kDa (blue), 0.1 - 0.2 kDa (purple), 0.01- 0.1 kDa (red) in digesta collected from the stomach, duodenum, jejunum and ileum of young pigs fed

infant milk formula produced by membrane filtration (MEM-IMF) or high-temperature infant milk formula (HT-IMF). Values are reported as means ± SEM. Different uppercase letters within a given gastrointestinal location and within the same molecular weight range indicates a significant difference between IMF dietary treatment types ($P < 0.05$). Different lowercase letters within the same IMF dietary treatment types and the same gastrointestinal location indicates a significantly difference between molecules with different molecular weight ranges ($P < 0.05$). Different Greek symbols within a given gastrointestinal location represents a significant difference between IMF dietary treatment type ($P < 0.05$). Bovine serum albumin has a molecular weight of 66 kDa, β-LG has a molecular weight of 18 kDa, α-LA has a molecular weight of 14 kDa.

2.3.4.2. Free amine groups determination by OPA assay

Further evidence of protein hydrolysis in the small intestine of pigs fed MEM-IMF diet was determined by quantifying the amount of free amine groups liberated due to the cleavage of peptide bonds using the OPA assay (Figure 2.2). Consistent with SEC-HPLC results, IMF processing influenced the release of free amine groups during digestion. Overall, in the gastrointestinal tract, MEM-IMF digesta had 1173 ± 46 μmol free amine groups released per gram protein, which was significantly higher than HT-IMF digesta which had 1022 ± 43 μmol free amine groups released per gram protein ($P < 0.001$). More specifically, pigs fed MEM-IMF diet had a significantly higher degree of protein hydrolysis, as determined by free amine groups, in the stomach and duodenum compared to pigs fed HT-IMF diet, 599 ± 45 μmol/g protein *vs* 509 ± 17 μmol/g protein and 1530 ± 136 μmol/g protein *vs* 1174 ± 124 μmol/g protein, respectively ($P < 0.05$). However, free amine groups were at a similar levels in the jejunum and ileum for both dietary groups ($P > 0.05$).

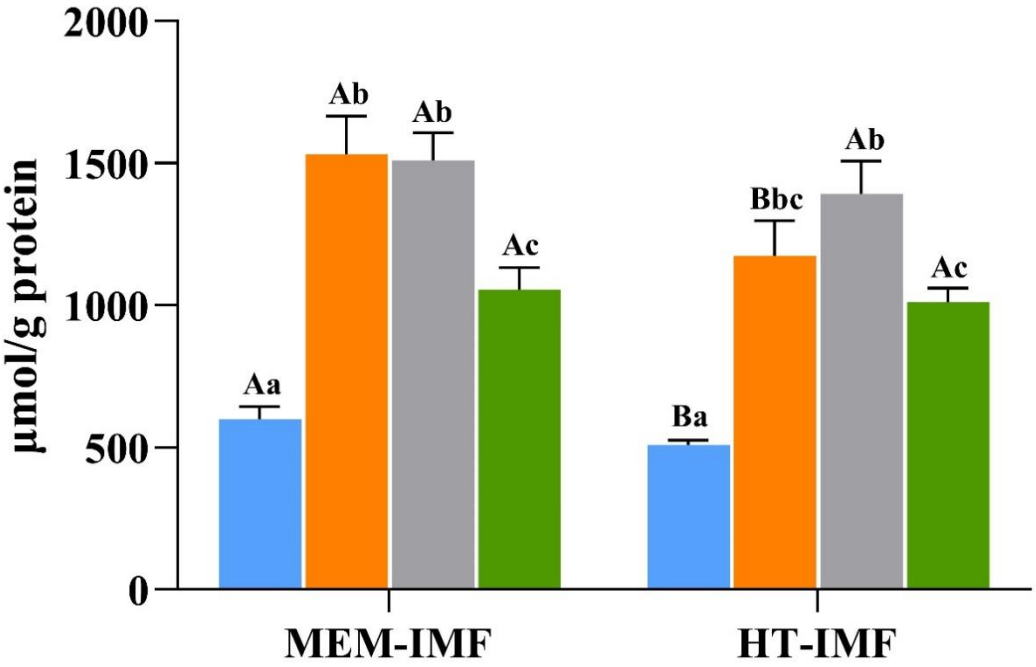


Figure 2.2. Concentration of free amine groups (µmol/g protein) in digesta collected from different gastrointestinal locations of pigs. Values are reported as means ± SEM. Infant milk formula produced by membrane filtration (MEM-IMF) and high-temperature infant milk formula (HT-IMF) digesta collected from the stomach (blue, MEM-IMF n = 10 and HT-IMF n = 10), duodenum (orange, MEM-IMF n = 3 and HT-IMF n = 4), jejunum (grey, MEM-IMF n = 8 and HT-IMF n = 9) and ileum (greem, MEM-IMF n = 4 and HT-IMF n = 7). Different uppercase letters within the same gastrointestinal location indicates significant differences between IMF dietary treatment types ($P < 0.05$). Different lowercase letters within the same IMF dietary treatment types indicates significant differences between gastrointestinal locations ($P < 0.05$).

2.3.4.3. The concentration of free amino acids from diets

The concentration of free amino acids during digestion was investigated to ascertain if processing effects the end point of protein digestion (Table 2.4). The total amount of free amino acids, by combining the data from the stomach, duodenum, and ileum, was unaffected by IMF processing ($P > 0.05$). However, pigs fed MEM-IMF diets tended to have a higher levels of total free amino acids in the jejunum ($P = 0.1$). The concentration of free amino acids was similar at each location along the small intestine of pigs fed MEM-IMF. In contrast, the concentration of free amino acids increased the more distal the small intestine location in pigs fed HT-IMF. For individual amino acids, IMF processing tended to affect the concentrations of cysteine, glycine and taurine ($P = 0.1$) with higher overall amounts found for those pigs who consumed MEM-IMF.

Pigs that consumed MEM-IMF diet had significantly higher concentrations of proline in the duodenum ($P < 0.05$) and taurine in the jejunum ($P < 0.05$) than pigs fed HT-IMF diet. These MEM-IMF fed pigs tended to have higher concentrations of alanine, arginine and taurine in the stomach ($P = 0.1$), alanine in the duodenum ($P = 0.1$), and aspartic**,** cysteine, glycine and threonine in the jejunum ($P = 0.1$) than pigs fed HT-IMF diet. In ileal digesta samples, a treatment difference was found only for tyrosine where it was surprisingly higher in pigs fed HT-IMF diets ($P < 0.05$). The six most abundant amino acids in the duodenum which remained high throughout the small intestine lumen in all pigs were lysine, alanine, glycine, glutamic acid, valine and leucine. For pigs fed HT-IMF diets, the levels of free amino acids were elevated the further down the gastrointestinal tract. However, for pigs fed MEM-IMF diets the concentration of total free amino acids peaked at the jejunum, declining in the ileum.

Table 2.4. Concentration of free amino acids in the stomach, duodenum, jejunum and ileum of young pigs fed MEM-IMF or HT-IMF diets

	Gastrointestinal tract location										
	Stomach		***Duodenum***		***Jejunum***		***Ileum***		***Effect of***		
Amino acid (µmol/g protein)	**MEM-IMF (n=10)**	**HT-IMF (n=10)**	**MEM-IMF (n=5)**	**HT-IMF (n=6)**	**MEM-IMF (n=9)**	**HT-IMF (n=10)**	**MEM-IMF (n=9)**	**HT-IMF (n=9)**	**Diet**	**Location**	**Diet*location**
Alanine	1.9 ± 0.50[a]	1.1 ± 0.13[b]	26.3 ± 5.86[a]	15.8 ± 2.59[b]	20.8 ± 2.39	22.6 ± 2.37	19.9 ± 2.87	22.0 ± 2.17	NS	***	***
Arginine	0.8 ± 0.14[a]	1.1 ± 0.09[b]	17.9 ± 4.48	13.8 ± 3.21	13.3 ± 0.80	11.2 ± 1.51	7.2 ± 1.56	9.4 ± 1.28	NS	***	***
Aspartic	0.6 ± 0.06	0.7 ± 0.06	8.4 ± 2	4.9 ± 0.77	11.3 ± 0.53[a]	8.9 ± 1.14[b]	10.6 ± 2	13.4 ± 1.45	NS	***	***
Cysteic Acid	1.7 ± 0.18	1.7 ± 0.07	3.5 ± 0.29	4.0 ± 0.26	4.1 ± 0.2	3.9 ± 0.25	9.1 ± 0.74	9.2 ± 0.92	NS	***	***
Cysteine	1.5 ± 0.26	1.2 ± 0.15	3.4 ± 0.47	3.0 ± 0.26	2.4 ± 0.13[a]	2.1 ± 0.13[b]	2.4 ± 0.29	2.0 ± 0.29	$P = 0.1$	***	***
GABA	0.7 ± 0.06	0.6 ± 0.05	0.9 ± 0.16	0.7 ± 0.05	1.1 ± 0.32	0.9 ± 0.18	0.4 ± 0.12	0.3 ± 0.06	NS	***	***
Glutamic Acid	1.2 ± 0.14	1.2±0.12	20.5 ± 4.83	13 ± 2.52	27.3 ± 1.24	24.1 ± 2.74	31.6 ± 5.13	39.1 ± 5.02	NS	***	***
Glycine	2.4 ± 0.76	1.5 ± 0.26	21.8 ± 3.98	14.7 ± 2.43	44.7 ± 6.18[a]	31.9 ± 3.64[b]	45.2 ± 8.19	45.5 ± 5.57	$P = 0.1$	***	***

Histidine	2.6 ± 0.35	2.1 ± 0.1	9.9 ± 1.32	9.1 ± 1.34	10.9 ± 0.68	10.2 ± 0.74	6.8 ± 0.72	7.7 ± 0.49	NS	***	***
Isoleucine	0.3 ± 0.04	0.2 ± 0.02	8.7 ± 2.53	4.8 ± 1.38	7.6 ± 0.73	5.8 ± 0.94	10.4 ± 1.76	12.5 ± 1.36	NS	***	***
Leucine	1.2 ± 0.16	1.1 ± 0.14	20.1 ± 5.32	13.1 ± 3.28	14.5 ± 1.26	11.7 ± 1.7	17.2 ± 2.97	20.4 ± 2.32	NS	***	***
Lysine	11 ± 1.63	12.7 ± 0.97	26.7 ± 5.17	24.6 ± 4.8	24.2 ± 2.25	20.1 ± 3.83	12.5 ± 2.2	14.6 ± 1.91	NS	***	***
Methionine	7.1 ± 0.94	8.3 ± 0.55	4.6 ± 1.41	3.3 ± 1.07	4.1 ± 0.54	3 ± 0.96	3.0 ± 0.54	3.3 ± 0.5	NS	***	***
Proline	0 ± 0	0 ± 0	7.8 ± 2.61^{A}	2.2 ± 0.9^{B}	5.9 ± 0.86	5.6 ± 1.25	10.5 ± 1.49	12.7 ± 1.49	NS	***	***
Phenylalanine	1.3 ± 0.21	1.6 ± 0.56	12.5 ±2.62	9.3 ± 2.04	10 ± 0.8	8.2 ± 1.04	9.0 ± 1.43	10.5 ± 1.15	NS	***	***
Serine	0.2 ± 0.05	0.2 ± 0.04	14.1 ± 3.68	7.9 ± 1.78	13.9 ± 0.83	13.1 ± 1.78	14.0 ± 2.13	17 ± 1.67	NS	***	***
Taurine	1.2 ± 0.11^{a}	1.5 ± 0.1^{b}	1.6 ± 0.48	1.5 ± 0.15	4.7 ± 1.55^{A}	1.5 ± 0.37^{B}	9.8 ± 2.6	7.3 ±1.89	*P* = 0.1	***	***
Threonine	8.5 ± 1.15	9.9 ± 0.65	7.4 ± 1.81	5 ± 1.49	8.2 ± 0.48^{a}	6.6 ± 0.77^{b}	7 ± 1.25	7.6 ± 0.83	NS	***	***
Tryptophan	4.3 ± 0.58	4.9 ± 0.33	6.4 ± 1.25	4.8 ± 1.19	4.9±0.37^{A}	3.5 ± 0.48^{B}	4.8 ± 0.85	4.3 ± 0.68	NS	NS	NS
Tyrosine	0 ± 0	0 ± 0	8.3 ± 2.24	5.9 ± 1.66	8.4±0.52	7.3 ± 0.79	8.8 ± 0.98^{A}	11.5 ± 0.86^{B}	NS	***	***

Valine	8 ± 0.8	7.8 ± 0.45	14.7 ± 3.3	10.5 ± 2.05	10.5 ± 1.04	8.9 ± 1.28	14.8 ± 2.35	16.8 ± 1.71	NS	***	***
Total	**56.5 ± 4.60$^{\alpha}$**	**59.4 ± 3.05$^{\alpha}$**	**244.1 ± 49.29$^{\beta}$**	**171.9 ± 29.70$^{\beta}$**	**253.5 ± 15.27$^{a,\beta}$**	**211.4 ± 22.75$^{b,\gamma}$**	**251.5 ± 33.1$^{\beta}$**	**285.0 ± 28.51$^{\delta}$**	**NS**	*******	*******

Statistics were conducted on concentrations of free amino acids in μmol per g protein in collected digesta from stomach, duodenum, jejunum and ileum collected from young pigs fed the infant milk formula produced by membrane filtration (MEM-IMF) and high-temperature infant milk formula (HT-IMF). Values are reported as means ± SEM. Statistically significant differences were referenced with $P < 0.001$ (***), $P < 0.01$ (**), $P < 0.05$ (*) and $P > 0.05$ (NS). When there was a significant interaction, multiple comparisons of means were conducted. For each amino acid, different uppercase letters within the same gastrointestinal location indicates significant differences between IMF dietary treatment types ($P < 0.05$). Different lowercase letters within the same row indicates tendencies of differences between IMF dietary treatment types within the same gastrointestinal location ($0.05 < P < 0.10$). Different Greek symbols within a given gastrointestinal locations indicates significant differences between IMF dietary treatment types within the same gastrointestinal location ($P < 0.05$).

2.4. Discussion

A new process for IMF manufacturing was developed that incorporates a series of membrane filtration steps. At pilot plant scale, MEM-IMF retains a higher content of native whey protein compared to HT-IMF. Two nutrient adequate isocaloric and isonitrogenous diets, containing either 35% of MEM-IMF or HT-IMF, were fed to young pigs from their first day of weaning (28 days old) for 28 days. Protein digestion was influenced by processing method, with higher levels of water-soluble peptides present in the upper digestive tract of pigs fed MEM-IMF. More peptides were released when MEM-IMF diet was digested in the duodenum, jejunum and ileum compared to HT-IMF diet. In addition, there was a higher degree of protein hydrolysis in the stomach, and duodenum of MEM-IMF fed pigs compared to their HT-IMF fed counterparts. This resulted in an increase in free amino acids in the jejunum of these pigs. It should be noted that free amino acids are only temporary in the intestinal lumen and are removed by absorption through the gut wall. However, not all free amino acids are completely absorbed at the end of the small intestine (van der Wielen et al., 2023). The rate of amino acid absorption can differ greatly between amino acids and influenced by the presence of other amino acids (van der Wielen et al., 2023). For highly digestible protein sources, such as whey, the proportion of free amino acids relative to total amino acids had only a minor effect on the true ileal digestibility of whey protein isolate (van der Wielen et al., 2023). In this study, pigs were slaughtered 180 min after their final feeding, while it was sufficient time for digestion and absorption to occur, some amino acids may have remained unabsorbed in the distal portion of the small intestine. Strathe et al. (2008) estimated the maximum velocity of protein hydrolysis and absorption in both upper and lower small intestine, which varies between 0.28 to 0.75 moles of nitrogen per mole (Strathe et al., 2008). It is reasonable to assume a similar rate of absorption between both treatments, hence the differences in the detected amounts of amino acids are due to differences in the digestive kinetics of protein hydrolysis, which is

confirmed by the degree of protein hydrolysis (available amine groups). Hence, it is safe to conclude that MEM-IMF is digested more rapidly than HT-IMF, under the experimental conditions described in this study. However, there may be some innacuracies in instances where a product has previously undergone thermal heat treatment when the degree of protein hydolysis is defined as the concentration of free amine groups released. In such examples a certain percantage of amine groups will not be 'free' (i.e. the Maillard reaction during heat treatment will limit the release of free amine groups, in particular from lysine).

Surprisingly, apparent total tract digestibility of dry matter and crude protein was significantly reduced in the pigs fed MEM-IMF diets compared to those fed HT-IMF diets. However, apparent total tract digestibility is based on the total digestive tract (i.e. including the large intestine) with its nitrogen-containing gut microbiota, rather than the true ileal digestibility (digestible indispensable amino acid score (DIAAS)) recommended by the Food and Agriculture Organisation (Mathai et al., 2017). Overall, growth and feed intake parameters were unaffected by IMF processing, albeit some transitory differences were observed. It is worth highlighting that the pig's diets contained both dairy and plant (maize, soy and barley) proteins. It is important to understand that these protein-protein interactions can influence the functional and nutritional properties of dairy products (Hinderink et al., 2021). These proteins can form complexes that may hinder digestibility.

As an alternative processing technique to conventional thermal treatment, membrane filtration produced an IMF with significantly more native whey protein. In our previous study, laboratory-scale processing of MEM-IMF resulted in only 18.3% native whey as a percentage of total whey protein (Chen et al., 2021), whereas upscaling to pilot plant scale resulted in an increase to 59.9% native whey protein as a percentage of total whey protein. The higher content of native whey protein in the pilot plant process is attributed to the larger size of the microfiltration instrumentation used, which reduced the proportion of retentate volume and

dead volume among the total feed volume (Skrzypek & Burger, 2010). Hence, the volumetric concentration factor reached a much higher value of 9.5 for the 0.2 μm microfiltration in pilot scale compared to a 3.2 volumetric concentration factor using the laboratory scale size 0.2 μm microfiltration. These results are in agreement with previously published work where a semi-industrial scale production of IMF treated by 0.8 μm microfiltration resulted in 6 ± 4% denatured protein compared to 58 ± 0% denatured protein in an extensively, heat-treated IMF (Yu et al., 2021). Transitioning from laboratory scale to pilot plant scale resulted in increased native whey protein in IMF powders. Therefore, it is possible that further upscaling to industrial scale may result in an additional increase in the percentage of native whey protein in the final IMF product.

This study provides *in vivo* evidence that protein digestion in the intestinal tract is influenced by IMF processing history. Firstly, we observed more water-soluble peptides in MEM-IMF digesta in the upper small intestine compared to that of digesta collected from pigs fed HT-IMF. The stomach contents of MEM-IMF fed pigs had significantly more soluble material due to the significant difference in the 18 - 28 kDa molecular weight group. This implies that proteins in MEM-IMF were degraded faster than that of HT-IMF. In addition, β-LG, with a molecular weight of 18 kDa, was largely retained in its native form. Previously, we performed an *in vitro* semi-dynamic digestion of laboratory scale MEM-IMF and HT-IMF using a protocol that models the infant stomach (Chen et al., 2022). Within 125 min of digestion, MEM-IMF had yielded twice the amount of water-soluble materials as HT-IMF *in vitro* (Chen et al., 2022). Typically, casein coagulates under gastric conditions (Halabi et al., 2020) while native whey proteins remain water-soluble. This results in a faster gastric emptying rate for native whey protein compared to casein (Wang et al., 2018). The higher degree of protein hydrolysis in the small intestine could simply be explained by the higher amount of native water-soluble whey protein in MEM-IMF. It may be desirable to preserve the native structure

of milk proteins (i.e. lactoferrin) during processing to ensure effective delivery to the small intestine, where they can have a bioactive affect (Wang et al., 2019).

The observations of greater levels of protein hydrolysis of MEM-IMF in the stomach and duodenum agree with our *in vitro* semi-dynamic digestion data (Chen et al., 2022). At 156 min of *in vitro* gastric digestion the concentration of free amine groups was 1338 ± 253 μmol/g protein for MEM-IMF compared to 845 ± 78 μmol/g protein for HT-IMF. However, the pig trial revealed that in the jejunum and ileum, protein hydrolysis was similar for both MEM-IMF and HT-IMF. This was consistent with protein hydrolysis results we obtained for the end of the intestinal phase using an *in vitro* static infant digestion method (Bavaro et al., 2021). Additional work is required to quantify the concentration of free amine groups released from both the IMF powder and the pig's diets.

Our *in vitro* gastric model observed that smaller coagulates were formed during MEM-IMF digestion compared to HT-IMF (Chen et al., 2022). Native whey proteins in IMF do not contribute to protein coagulation. The higher concentration of native whey protein in MEM-IMF may have resulted in the formation of smaller gastric curds in the stomach of pigs fed MEM-IMF, whereas pigs fed HT-IMF with the higher concentration of denatured proteins had larger curds formed in their stomachs. Smaller coagulates will facilitate enzyme accessibility and result in accelerated disintegration of gastric curd (Mulet-Cabero et al., 2019; Ye, 2021). This increases free amine group release during digestion, with a faster gastrointestinal transit (Mulet-Cabero et al., 2019; Ye, 2021). In line with our *in vitro* and *in vivo* results, Navis, Schwebel, et al. (2020) noticed that gastric chyme was retained in the stomach of young pigs fed an IMF with extensively heated whey protein concentrate (WPC) (30% native whey protein), in contrast to young pigs fed an IMF with mildly pasteurised WPC (99% native whey protein) (Navis, Schwebel, et al., 2020). This suggests delayed and/or incomplete gastric emptying in young pigs fed an IMF with extensively heated WPC. Furthermore, denatured

whey protein resulting from heat treatment and gastric digestion will form aggregates on their own and on the surface of casein micelles. This aggregation produces large protein complexes, capable of forming large protein gels and curds in the presence of gastric acid, leading to higher viscosity in the stomach, which delays gastric emptying and enzymolysis. In addition, Bouzerzour et al (2012) fed young pigs for 28 days with model IMF (60:40 whey to casein ratio) or formula milk with 20:80 whey to casein ratio (Bouzerzour et al., 2012). They observed that the higher proportion of whey protein in formula accelerated gastric emptying, which led to reduced casein digestion in the small intestine. Our previous *in vitro* digestion model revealed the mechanism by which processing affects IMF digestion. Peptides are derived from the surface of the gelled curd and coagulated proteins rather than from the soluble proteins as there is less protein in the serum of HT-IMF. In contrast, MEM-IMF provides both abundant serum water-soluble protein and coagulated protein gels for enzymatic hydrolysis (Chen et al., 2022).

Our present study determined that IMF processing tended to affect the concentration of total free amino acids at the jejunum. A constant level of total free amino acids was observed throughout the length of the small intestine in pigs fed MEM-IMF. In contrast, the amount of total free amino acids rose distally along the small intestine of pigs fed HT-IMF. This suggests that MEM-IMF is digested faster than HT-IMF. However, the appearance of free amino acids in the gut lumen is followed by the removal of amino acids by absorption. A future dietary intervention study should include a parallel analysis of the blood, postprandial to gain insight into amino acid absorption. In addition, the recovery of some individual free amino acids, in certain regions of the digestive tract, differed or tended to differ depending on the type of IMF in the diet. MEM-IMF tended to have a higher concentration of cysteine and its derivative, taurine, in the overall gastrointestinal tract compared to HT-IMF. There was a significantly higher level of proline in MEM-IMF digesta than HT-IMF from the duodenum. The higher

amount of free proline indicates that peptides in MEM-IMF are more accessible to aminopeptidases (Santos-Hernández et al., 2018). According to these findings, we can deduce that MEM-IMF was digested faster than HT-IMF. Interestingly, the most abundant free amino acid released from IMF was glycine which agrees with our previous *in vitro* static digestion results (Bavaro et al., 2021). Previous research demonstrated that the bioavailability of the amino acid, lysine, could be reduced by heat treatment which resulted in the formation of advanced glycation end products (Birlouez-Aragon et al., 1998). Moreover, Rérat et al. (2002) demonstrated that the loss in nutritional quality due to pasteurisation of cows' milk for skim milk powder preparation is mainly driven by the reduction in bioavailability of lysine, and to a lesser extent by the reduced bioavailability of other essential amino acids such as phenylalanine, cysteine, aspartic acid and glycine (Rérat et al., 2002). In the present study, lysine was not affected by either processing method, whereas there was significantly higher concentration of cysteine overall in the gut lumen, and in particular in the jejunum, in pigs fed MEM-IMF diet compared to those fed HT-IMF diet. This result is in line with Sarwar et al. (1989) who demonstrated that heat treatment reduced the true digestibility of cysteine in IMF (Sarwar et al., 1989).

In contrast with gut lumen protein digestion data, the apparent total tract digestibility of crude protein was significantly reduced in pigs fed MEM-IMF diets compared to those fed HT-IMF. Although apparent total tract digestibility is a parameter widely used to determine overall nutrient digestibility in pigs (Rooney et al., 2020; Torres-Pitarch et al., 2020), it has several important limitations. One major limitation is that it includes the protein content of intestinal microbiota (Zhang & Adeola, 2017).

In our study, the overall growth and feed intake of young pigs was similar between the two treatment groups over the 28 days of intervention. This is not surprising since the experimental diets were formulated to be isocaloric and isonitrogenous. Similar to our results, Navis,

Muncan, et al. (2020) and Navis, Schwebel, et al. (2020) fed an IMF with mildly pasteurised WPC (73 °C for 30 s) to preterm (106 days gestation) and near-term piglets (113 days gestation) for five days and compared postnatal growth to piglets fed an IMF with extensively heated WPC (73 °C for 30 s and 80 °C for 6 min) (Navis, Muncan, et al., 2020; Navis, Schwebel, et al., 2020). Postnatal growth of piglets was not significantly different between the mildly pasteurised WPC and the extensively heated WPC based diet groups (Preterm piglets: 10 ± 7.3 g/kg day *vs* 11 ± 9.8 g/kg day; near-term piglets: 15 ± 3.8 g/kg day *vs* 18 ± 7.5 g/kg day) (Navis, Muncan, et al., 2020). Similarly, Li et al. (2013) fed three isocaloric IMF exclusive diets to preterm piglets for five days with formula containing conventional WPC (filtration, multi-pasteurisation, standard spray-drying); formula containing gently treated WPC (reduced filtration and pasteurisation, gentle spray-drying); and formula containing minimally treated WPC (rennet precipitation, reduced filtration, heat treatment < 40°C, freeze-drying) (Li et al., 2013). The study found no treatment difference in weight gain (Li et al., 2013). Rafiee-Tari et al. (2019) fed exclusively liquid IMF with a 60:40 whey protein to casein ratio to newborn piglets for 18 days (Rafiee-Tari et al., 2019). Increasing the β-casein ratio from 12.5 to 17.1% of total protein had no effect on the growth performance or feed intake of piglets. In our study, young pigs on the MEM-IMF diet had significantly higher ADG and ADFI from day 0 to day 7 ($P < 0.05$). This period is critical for the maturation of the digestive performance of young pigs (Moughan et al., 1991) and may imply that MEM-IMF is more easily digested by the immature gut. However, results from the first week of the trial should be treated with some degree of caution as this is a period of transition where pigs have to adapt to solid food.

IMF fed infants after two months of age have higher growth trajectories to breast fed babies, gaining more body weight than breastfed infants (Hester et al., 2012; Ziegler, 2006). This can be attributed to different dairy protein profiles and energy content in IMF compared to breast milk and different feeding patterns between IMF fed and breast fed babies (Hester et al., 2012;

Koletzko et al., 2013; Koletzko et al., 2009). In our current study, we also observed a tendency of lower ADG and lower ADFI in the final week (day 21 to day 27) for MEM-IMF compared to HT-IMF fed pigs. This difference might have been greater had the IMF intervention been extended. In addition, at day 28, we observed higher protein hydrolysis in the stomach contents of pigs fed MEM-IMF compared to HT-IMF. During our *in vitro* gastric digestion of MEM-IMF, we observed a phase separation in the modelled stomach, which consisted of a serum phase with water-soluble materials at the bottom, and a floated gelling curd phase (fat/protein network) (Chen et al., 2022). The aqueous serum phase with higher levels of water-soluble whey protein probably resulted in faster gastric emptying as observed by Mulet-Cabero (2018). This may trigger the intestinal brake due to the rapid absorption of whey protein derived peptides and a higher protein quality (Boirie et al., 1997; Fleddermann et al., 2014). If this intestinal brake occurred in the pigs as they consumed MEM-IMF, then these pigs may have higher satiety which would explain the reduced food intake (Santos-Hernández et al., 2018). In contrast, HT-IMF did not display separated gastric emptying. Chyme was continuously emptied into the small intestine, resulting in a delayed or weakened intestinal brake. Nutrient bioavailability may therefore differ with processing. A future acute study should include measuring satiety hormone responses every 15 min postprandial to determine if satiety was influenced by membrane filtration processing. In addition, to explore growth trajectories further, the dietary intervention study should include younger pigs and for a longer period of time.

2.5. Conclusion

The current study demonstrated that introducing membrane filtration steps into IMF processing to reduce thermal load significantly increased the native whey protein content in the final powder. The higher native whey protein content enhanced protein hydrolysis, peptides and free amino acid concentration. Finally, this pig study provides *in vivo* evidence that feeding IMF processed by membrane filtration to babies should have minimal effects on overall growth trajectories and food conversion efficiencies compared to IMF processed by traditional thermal processing.

2.6. References

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Chapter 3

Infant milk formula, produced by membrane filtration, promotes mucus production in the upper small intestine of young pigs

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Published version of this chapter is presented in Appendix B.

Abstract

Human breast milk promotes maturation of the infant gastrointestinal barrier, including the promotion of mucus production. In the quest to produce next generation infant milk formula (IMF), we have produced IMF by membrane filtration (MEM-IMF). With a higher quantity of native whey protein, MEM-IMF more closely mimics breast milk than IMF produced using conventional heat treatment (HT-IMF). After a 4-week dietary intervention in young pigs, animals fed a MEM-IMF diet had a higher number of goblet cells, acidic mucus and mucin-2 in the jejunum compared to pigs fed HT-IMF ($P < 0.05$). In the duodenum, MEM-IMF fed pigs had higher trypsin activity in the gut lumen, higher mRNA transcript levels of *claudin-1* in the mucosal scrapings and higher lactase activity in brush border membrane vesicles than those pigs fed HT-IMF ($P < 0.05$). In conclusion, MEM-IMF is superior to HT-IMF in the promotion of mucus production in the young gut.

Keywords: Infant milk formula; membrane filtration; goblet cells; digestive enzymes; gut barrier.

3.1. Introduction

At birth, the human gastrointestinal tract is underdeveloped with lower levels of digestive enzymes and less uniform mucus in the gut barrier than the adult (Demers-Mathieu, 2022; Neu, 2007). The undeveloped functionality of the infant gastrointestinal tract at birth increases the risk of infections which may result in necrotising enterocolitis (Renz et al., 2011). Nutritional strategies implemented with new-born infants have a major influence on gastrointestinal development and maturity (Neu, 2007). Human breast milk is proven to be easier to digest and, promotes gastrointestinal maturity, gut microbiota diversity and production of mucus from goblet cells (Dimitroglou et al., 2022). When breast milk is unavailable, infant formula is the alternative (Bookhart et al., 2021; Phosanam et al., 2021). Infant milk formula (IMF) recipes are traditionally bovine milk based and must have a minimum protein quantity of 1.8 g/100 kcal to be classified as an IMF (Codex-Alimentarius, 1981).

Unlike breast milk, IMF and it's ingredients routinely undergo high-thermal processing to ensure microbial safety (Wada & Lönnerdal, 2015). Regardless of processing systems, a heating step is often introduced where the IMF liquid is spray dried to produce powders for extended shelf life and worldwide distribution. Thermal loads imposed throughout production results in native protein denaturation, protein aggregation and formation of advanced glycation end products (Lund et al., 2021).

Thermal processing of IMF influences digestion kinetics. When examined *in vitro*, Ye et al. (2021) found higher protein aggregation and lower protein digestibility in ultra-high temperature (UHT) IMF compared to lower heat-treated IMF (Ye et al., 2021). Indeed, high temperature processing has been shown to reduce digestive and brush border membrane enzyme activities in rat (Wang & Zhao, 2021) and pig studies (Li et al., 2018; Sun et al., 2022). Heat treatment of IMF also negatively impacts on intestinal morphology, integrity and health

in vitro and *in vivo* (Bavaro et al., 2021; Li et al., 2018; Li et al., 2013; Navis, Muncan, et al., 2020; Navis, Schwebel, et al., 2020; Sun et al., 2022).

Alternative membrane filtration processing systems have been utilised in recent studies to produce IMF powders with a reduced thermal load (Chen et al., 2021; Yu et al., 2021). Yu et al. (2021) produced IMF powders using membrane filtration. This IMF powder had significantly lower whey protein denaturation compared to an IMF produced using multiple heating steps (6 ± 4% *vs* 58 ± 0%) ($P < 0.05$) (Yu et al., 2021). Recently, we produced an IMF powder by membrane filtration (MEM-IMF) using 1.4 and 0.2 µm membranes (Chen et al., 2023). The native whey protein quantity in MEM-IMF was 59.9% (Chen et al., 2023). There was a significantly lower amount of native whey protein (4.5%) present in an IMF produced by conventional heat treatment (HT-IMF) ($P < 0.001$) (Chen et al., 2023). In both studies, after spray drying, there was no significant differences in N(6)-carboxymethyllysine (CML) levels (3.65 ± 0.74 ng/mg IMF powder in MEM-IMF and 3.97 ± 0.70 ng/mg IMF powder in HT-IMF) in IMF powders produced by membrane filtration compared to high temperature processing (Chen et al., 2021; Yu et al., 2021).

In vitro, using a semi dynamic model of the infant gut, the digestive behaviour in the gastric phase differed between MEM-IMF and HT-IMF (Chen et al., 2022). MEM-IMF had a fragmented curd and a quicker (93 min) dissociation of protein aggregates from fat droplets compared to HT-IMF, which had a firmer, larger curd with protein aggregates still visible on fat droplets at 156 min (Chen et al., 2022). In young pigs, an MEM-IMF diet resulted in significantly higher levels of protein hydrolysis in the duodenum compared to the duodenum of pigs fed HT-IMF ($P < 0.05$) (Chen et al., 2023). There was a significantly higher concentration of water-soluble peptide/protein content in the jejunum of pigs fed a MEM-IMF diet compared to pigs fed a HT-IMF diet ($P < 0.001$), due to higher levels of 10 - 18 kDa, 18 - 28 kDa and 28 - 65 kDa ($P < 0.05$) molecular mass groups (Chen et al., 2023). Interestingly,

using an *in vitro* gut barrier model, simulated gastro-intestinal digested MEM-IMF was also found to increase barrier integrity and levels of tight junction protein claudin-1 (CLDN-1) (Bavaro et al., 2021).

The objective of this follow-up study was to investigate if a diet formulated with MEM-IMF resulted in digestive enzyme activity differences in the duodenum and jejunum of these young pigs that coincided with our observations of faster protein digestion kinetics. In addition, the study investigated whether or not membrane filtration processing altered gut physiology, for example mucus secretion or gut barrier structure in the young pigs. The hypothesis was that MEM-IMF will increase digestive capability and support gut barrier health in the upper small intestine.

3.2. Materials and Methods

3.2.1. Materials

Xylose Lysine Deoxycholate (XLD) Agar, Listeria Selective Oxford Agar and Violet Red Bile + Glucose Agar (VRBGA) were purchased from Oxoid (Ireland). The RNeasy Mini RNA extraction kit and DNase digestion kit were obtained from Qiagen (Germany). Oligonucleotide primers for reverse transcription quantitative real-time PCR (RT-qPCR) analysis were synthesised by Eurofins Genomics (Germany). The LightCycler 480 SYBR Green I Master was purchased from Roche Diagnostics (Germany). RIPA buffer, 1% EDTA and 1% Halt Protease Inhibitor Cocktail were purchased from Thermo Fisher Scientific (USA). The primary mucin-2 (MUC-2) polyclonal antibody (Cat. No. PA5-119292) was purchased from Invitrogen (USA). The housekeeping β-Actin polyclonal antibody (Cat. No. bs-0061R) was obtained from Bioss Antibodies (USA). The Trans-Blot Turbo Transfer System containing PVDF membranes was purchased from Bio-Rad (USA). All other reagents were purchased from Sigma Aldrich (Ireland).

3.2.2. Infant milk formula preparation and manufacturing

A detailed description and schematic diagram of IMF processing was previously reported (Chen et al., 2021). Briefly, the IMFs used in this trial were manufactured at pilot-plant scale based on the recommendations for first stage IMF (infant's aged < six months). Raw bovine whole milk was collected from the dairy farm at the Teagasc, Animal and Grassland Research and Innovation Centre Moorepark (Ireland). Whey protein isolate (protein content of 91.6 %) was obtained from Agropur (USA). Both IMFs were manufactured in Moorepark Technology Limited (Ireland). Both IMFs had identical ingredients with protein: lactose: fat content of 1.29: 7: 3.49. The IMF had a whey:casein protein ratio of 60:40. MEM-IMF and HT-IMF powders were manufactured independently in a single batch (250 kg). To reduce heat treatment, milk

was not pasteurised prior to processing of MEM-IMF. The formulation was filtered using 1.4 and 0.2 µm filtration membranes. Only the microfiltration retentate from the 0.2 µm filtration membrane was heated at 125 °C for 5 s. HT-IMF was heated at 125 °C for 5 s. Both IMFs were homogenised at 15 MPa and spray dried at inlet and outlet temperatures of 185 °C and 80 °C, respectively. A total volume of 250 kg of each IMF powder was produced.

3.2.3. Microbial analysis of infant milk formula powders

Before inclusion in pig diets, microbial analysis of IMF powders was performed in accordance with Commission Regulation (EC) No 2073/2005 (Commission Regulation, 2005). In brief, 1 g of IMF powder was added to 9 ml of Ringer's solution and serially diluted. Dilutions were plated (100 µl) on Tryptic Soy Agar (TSA), XLD Agar, Listeria Selective Oxford Agar, VRBGA and *Cronobacter spp*. ChromoSelect Agar, modified. Plates were incubated for 48 h at 37 °C prior to plate counting. Microbial analysis of both IMF powders was performed in triplicate on different days.

3.2.4. Pig trial

The pig trial was performed between June and August 2020, at the Teagasc Pig Development Department Moorepark (Ireland) (Chen et al., 2023). Ethical approval for this study was provided by the Teagasc Animal Ethics Committee (approval no. TAEC2020-248), and the project was sanctioned by the Health Products Regulatory Authority (project authorisation no. AE19132/P111). The experiment was performed in accordance with Irish legislation (SI no. 543/2012) and the EU Directive 2010/63/EU on the protection of animals used for scientific purposes. The health and welfare status of all animals was monitored daily throughout the duration of this study. Twenty (10 male and 10 female) recently weaned pigs [(Large White x Landrace; PIC Hermitage, Ireland) x Tempo; Topigs Norsvin; Premier Pig Genetics Ltd., Ireland] were chosen for this study. At weaning (27 ± 0.7 days of age) pigs were blocked on the basis of sex, body weight (8.3 ± 0.59 kg) and litter origin. Within each block, pigs were

randomly assigned to one of two dietary treatment groups (n = 10) until 28 days post-weaning; (1) a starter diet containing 35% of MEM-IMF or (2) a starter diet containing 35% of HT-IMF. To meet the National Research Council nutrient requirements for pigs at weaning (National Research Council, 2012), a maximum of 35% inclusion of IMF powders was permitted. The ingredient and chemical composition of the starter diets were previously published (Chen et al., 2023). The experimental diets were prepared to be both isonitrogenous and isocaloric. The pigs were individually penned in fully slatted pens (pen dimensions: 2.4 m x 1.8 m) and each pen was equipped with a single-spaced stainless steel feeder (30 cm wide; O'Donovan Engineering, Ireland) and a drinker bowl (BALP, France). From day 0 to 28 post-weaning, pigs were fed twice daily and access to water was on an *ad libitum* basis. All pigs remained healthy throughout the course of the trial.

3.2.5. Slaughter and sample collection

On day 28 post-weaning, 3 h after the pigs were given their final feed, they were humanely euthanized by captive bolt stunning and immediately exsanguinated. Following slaughter, gut lumen content was immediately collected from the duodenum (50 cm caudal to the pylorus) and jejunum (2.5 m caudal to the pylorus). The gut lumen content was snap frozen immediately in liquid nitrogen and stored at -80 °C until further analysis. The entire digestive tract was isolated and tissue from the duodenum (10 cm caudal to the pylorus) and jejunum (1.5 m caudal to the pylorus) were aseptically removed and gently rinsed with sterile phosphate buffered saline (PBS). For biochemical analysis, tissue samples were immediately snap frozen in liquid nitrogen and stored at -80 °C. For histological analysis, duodenal and jejunal tissue sections (2 cm) were rinsed in PBS, placed in a No-Tox fixative solution (Cruinn Diagnostics Ltd., Ireland), stored at room temperature and then shipped on ice to NationWide Laboratories (UK) for mounting on glass microscope slides. To collect the mucosal scrapings, intestinal tissue sections (5 cm) were rinsed in PBS, cut longitudinally and the mucosa gently blotted dry with

tissue paper. Using a glass microscopic slide, the muscularis layer was then removed and mucosal scrapings were immediately snap-frozen in liquid nitrogen and stored at -80 °C until analysis.

3.2.6. Intestinal histology measurement and analysis

Mounting of duodenal and jejunal tissue was performed as previously described (Crespo-Piazuelo et al., 2022). In brief, fixed duodenal and jejunal tissue sections (2 cm) were removed from the No-Tox fixative solution and dehydrated through a graded alcohol series and cleared with xylene and embedded in paraffin wax. Tissue samples were cut finely into 5 µm sections using a microtome, mounted on glass microscope slides and then stained with haematoxylin and eosin. Slides were coded to ensure measurements and analysis were performed without prior knowledge of treatment group.

For histological analysis, an Olympus BX51 Light Microscope (Olympus Corporation, Japan) equipped with a ProgRes CT3 camera (Jenoptik AG, Germany) was used in conjunction with ProgRes Capture Pro 2.10.00 software (Jenoptik AG, Germany). Mucosal thickness, villus height and crypt depth were measured using 10× objective magnification. For each pig, ten randomly selected, clearly identifiable villi and crypts were measured. Mucosal thickness was measured from the base of the mucosa to the tip of the villus. Villus height was then measured from the tip of the villus to its base. An associated crypt was then measured from the base of the villus to the base of the mucosa. For each villus measured, accompanying goblet cell numbers were counted using 40× objective magnification.

3.2.7. Measurement of acidic mucins in mucus layer of pig small intestine using Alcian blue dye

To quantify acidic mucins, the procedure described previously (Smirnov et al., 2004) was adopted, with slight modifications. Briefly, 50 mg tissue was defrosted on ice and submerged

in 1% Alcian blue dye (1 ml) prepared in 160 mM sucrose, 50 mM sodium acetate, pH 5.8. Following a 2 h incubation at 4 °C, excess dye was removed by rinsing the tissue in 250 mM sucrose. Subsequently, the absorbed Alcian blue dye was extracted from the tissue by incubation in 1 ml 500 mM magnesium chloride for a further 2 h at 4 °C. Samples were immediately centrifuged at 16000× *g* for 20 min at 4 °C. The supernatant was retained for absorbance reading at 620 nm using a Synergy plate reader (BioTek, USA). The quantity of Alcian blue was determined using a standard curve of Alcian blue (0 - 250 µg/ml). The results are reported as µg Alcian blue dye absorbed per mg tissue. Each measurement was performed with technical triplicates on two different days (n = 10 pigs).

3.2.8. Analysis of mucin-2 expression by Western blot

Mucosal scrapings were removed from -80 °C storage and thawed on ice. RIPA buffer (1 ml) in the presence of 1% EDTA and 1% Halt Protease Inhibitor Cocktail was added to 50 mg mucosal scraping. Total protein was then extracted from the mucosal scraping via homogenisation on ice with a T 25 ULTRA-TURRAX (IKA Works, Inc., USA). The homogenate was left agitating on ice for 2 h prior to centrifugation at 16000 × *g* for 20 min at 4 °C to pellet excess debris. The supernatant was then collected, total protein content determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, USA) and samples were stored at -80 °C for future analysis. Mucosal scraping protein extracts containing equal amounts of protein (25 µg) were separated by SDS-PAGE under reducing conditions using 4 - 15% stain-free polyacrylamide gels at a constant voltage (100 V). SDS-PAGE gel was then transferred onto a PVDF membrane using the Trans-Blot Turbo Transfer System (Bio-Rad, USA).

The Western blotting protocol outlined in the WesternBreeze Chemiluminescent Western Blot Immunodection Kit (Thermo Fisher Scientific, USA) was followed, with slight modifications.

Separate, side-by-side, PVDF membranes were used for the target MUC-2 antibody and the β-actin housekeeping antibody. The membrane was initially blocked in blocking solution for 2 h at 4 °C to decrease non-specific binding. The membrane was then rinsed 3 times for 10 min with distilled water before being incubated with the primary antibody for MUC-2 (1:1500) overnight at 4 °C or the housekeeping antibody anti-β-actin (1:10000) diluted directly in blocking solution. The membrane was then washed 3 times for 10 min with antibody wash solution and then incubated for 2 h at 4 °C with the secondary IgG antibody. The membrane was exposed to the CDP-Star® chemiluminescent substrate (Thermo Fisher Scientific, USA) for 5 min. Immunoreactive proteins were visualised using the ChemiDoc XRS+ Gel Imaging System (Bio-Rad, USA). Densitometry analysis was undertaken with normalisation against the β-actin housekeeping antibody using Image Lab 6.1 software (Bio-Rad, USA). Western blots were performed in triplicate on two different days (n = 3 pigs).

3.2.9. RNA extraction and complementary DNA synthesis

Mucosal scrapings were removed from -80 °C storage and thawed on ice. RNA extraction was performed on 30 mg intestinal mucosal scraping using the RNeasy Mini Kit (Qiagen, Germany), according to the manufacturer's instructions, including the on-column DNase digestion step. Total RNA yields were quantified spectrophotometrically using the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, USA). A quality control check to determine 260/280 absorbance ratios ensured only RNA samples with 1.8 - 2.1 ratios were used. RNA integrity was analysed by gel electrophoresis on a 1% agarose gel (Bio-Rad, USA) in 1X TAE buffer at 130 V for 30 min. Reverse transcription from 700 ng RNA to complementary DNA (cDNA) was performed using the SensiFAST cDNA Synthesis Kit (Meridian Bioscience, USA) according to the manufacturers instructions. A sample containing no reverse transcription enzyme was also included as a negative control to track any genomic

DNA contamination. cDNA synthesis was performed to a final volume of 20 µl and stored at -20 °C.

3.2.10. Quantification of mRNA transcript by reverse transcription quantitative real-time PCR

All tight junction and mucus secreting primers were designed to span intron-exon boundaries, where possible, using the Primer-BLAST software at the NCBI website (http://www.ncbi.nlm.nih.gov/tools/primer-blast) (Supplementary Table 3.1). Primers for *interleukin 6* (*IL-6*), *interleukin 8* (*IL-8*) and *tumor necrosis factor alpha* (*TNF-α*) were obtained from Navis, Muncan, et al. (2020). RT-qPCR analyses was performed adhering to the MIQE guidelines (Bustin et al., 2009). For RT-qPCR, cDNA samples were removed from -20 °C storage and thawed on ice. The annealing temperature for each individual primer pair was determined by running a real time quantitative temperature gradient from 55 to 65 °C. Primer amplification efficiencies were in the range 90 - 110% determined by running a RT-qPCR with four different dilutions (1:1, 1:10, 1:100 and 1:1000) of cDNA. RT-qPCR was performed with a LightCycler® 96 instrument (Roche Diagnostics, Germany) and LightCycler 480 SYBR Green I Master kit. For each RT-qPCR run, 1 µl cDNA (1:10 dilution of cDNA synthesis reaction) and 0.5 µl of both forward and reverse primer (10 µM) was added to 5 µl SYBR Green. The final reaction volume was brought to 10 µl using RNase free water. The thermal cycling protocol was performed as follows; 10 min pre-incubation at 95 °C, followed by 45 cycles of three step RT-qPCR with 10 s denaturation at 95 °C, 10 s annealing at primer specific annealing temperature (55 - 63 °C) and extension at 72 °C for 30 s. A melting curve analysis was included with each RT-qPCR cycle to indicate single product melting and ensure no primer-dimers were present. A no-reverse transcription control sample and RNase free water sample were run with all RT-qPCR plates. The relative amount of target mRNA was determined using the reference gene, RPLP0. The relative mRNA expression was calculated

using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). Data is presented as the fold changes compared to the HT-IMF (control) diet. Each RT-qPCR cycle was performed with technical duplicates on two different days (n = 7 pigs).

3.2.11. Measurement of trypsin activity in the small intestine

Trypsin activity (EC 3.4.21.4) in the gut lumen was determined using a commercial trypsin activity colorimetric assay kit (Trypsin Activity Colorimetric Assay Kit MAK290, Sigma-Aldrich, USA) using p-nitroaniline as a substrate, according to the manufacturer's instructions, with some slight modifications. Briefly, the gut lumen samples were defrosted on ice and centrifuged at 14000 × *g* for 10 min at 4°C to obtain a clear supernatant. Total protein content was determined spectrophotometrically at 562 nm using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, USA) on the collected supernatant, according to the manufacturers protocol (Supplementary Figure 3.1). Aliquots (200 µl) of the supernatant were prepared, to limit freezing and thawing of samples, and diluted (1:200) in trypsin assay buffer for the enzyme assay. To 50 µl of this diluted gut lumen sample, 50 µl p-nitroaniline in trypsin assay buffer was added. The initial (T = 0 min) absorbance of the reaction was measured using a Synergy plate reader (BioTek, USA) at 405 nm. The final (T = 20 min) absorbance was again measured at 405 nm while still in the linear range of the reaction. A standard curve of p-nitroaniline (0 - 40 nmol) was also prepared. One unit of trypsin activity was defined as the amount of enzyme causing the formation of one µmol of p-nitroaniline per min at 25°C. Trypsin activity was expressed as mU/mg total protein. Trypsin activity was measured with technical triplicates on two different days (n = 9 pigs).

3.2.12. Isolation and purification of brush border membrane vesicles from pig small intestine

Intestinal brush border membrane vesicles were isolated and purified from duodenal and jejunal mucosal scrapings according to the protocol described by Mamone and Picariello (2023). Briefly, 500 mg mucosal scrapings were thawed in 10 ml ice-cold 50 mM mannitol, 2 mM Tris-HCl, pH 7.1. The mucosal scraping was then homogenized on ice with a T 25 ULTRA-TURRAX (IKA Works, Inc., USA). $MgCl_2$ (500 mM) was then added to the homogenate to reach a final concentration of 10 mM. The suspension was agitated for 20 min at 4°C. Centrifugation at 2500 × *g* for 15 min at 4°C was performed to remove excess cell debris, basolateral, membranes, nuclei and mitochondria. The supernatant was then collected and ultra-centrifuged at 26000 × *g* for 30 min at 4°C using an ultracentrifuge (Beckman Coulter, USA). The supernatant was discarded and the pellet was retained. To further purify the brush border membrane vesicles, the pellet was again resuspended in 10 ml ice-cold 50 mM mannitol, 2 mM Tris-HCl, pH 7.1 and both centrifugation steps were repeated. The final pellet was resuspended in 6 ml of 300 mM mannitol, 0.1 mM MgSO4, and 2 mM Tris, pH 7.4. To obtain a clear protein solution, the final suspension was passed several times through a 27-gauge needle. The isolated brush border membrane vesicles were snap-frozen in liquid nitrogen and stored in aliquots at -80 °C for future analysis. The Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, USA) was used to determine the total protein content in the purified brush border membrane vesicles (Supplementary Figure 3.2).

3.2.13. Determination of brush border membrane enzymatic activity in purified brush border membrane vesicles from the pig small intestine

Brush border membrane vesicles were removed from -80 °C storage and defrosted on ice. Brush border membrane enzyme activities were determined spectrophotometrically using enzyme-specific substrates in a Synergy plate reader (BioTek, USA) at the respective absorbance. A standard curve of known concentrations of each respective substrate was also prepared and absorbance measured at specific wavelength. All brush border membrane enzyme

activities were expressed per mg cellular protein content of brush border membrane vesicles. Aminopeptidase N (EC 3.4.11.2) activity was determined using l-leucine p-nitroaniline substrate in 50 mM sodium phosphate buffer, pH 7.2 at 37 °C. Undiluted brush border membrane vesicle (10 µl) was added to each well and mixed with 190 µl sodium phosphate buffer (50 mM), pH 7.2 and l-leucine p-nitroaniline substrate. The liberated p-nitroaniline was measured at 405 nm. One unit of aminopeptidase N activity was defined as the amount of enzyme that catalyses the conversion of one µmol of p-nitroaniline per minute at pH 7.2 at 37 °C. Aminopeptidase N activity was expressed as U/mg protein. Aminopeptidase A (EC 3.4.11.7) activity was assayed using L-glutamic acid 1-(4-nitroanilide) as substrate in 50 mM sodium phosphate buffer, pH 8 at 37 °C. Undiluted brush border membrane vesicle (10 µl) was added to each well and mixed with 190 µl sodium phosphate buffer (50 mM), pH 8. The amount of p-nitroanailine released was measured at 405 nm. One unit of aminopeptidase A activity was expressed as the amount of enzyme that catalyses the formation of one µmol of p-nitroanailine per minute at pH 8 at 37°C. Aminopeptidase A activity was expressed as mU/mg protein. Intestinal alkaline phosphatase activity (EC 3.1.3.1) was determined using p-nitrophenyl phosphate as a substrate using a colorimetric assay kit (Alkaline Phosphatase Activity Colorimetric Assay Kit BN00665, AssayGenie, Ireland), according to the manufacturer's protocol. In brief, 80 µl diluted (1:100) brush border membrane vesicle was added to each well and mixed with 50 µl assay buffer and p-nitrophenyl phosphate substrate. The liberated p-nitrophenol was measured at 405 nm. One unit of intestinal alkaline phosphatase activity was defined as the amount of enzyme that causes the formation of one µmol of p-nitrophenyl phosphate per minute at pH 9.6 and 25 °C. Intestinal alkaline phosphatase activity was expressed as U/mg protein. Lactase (EC 3.2.1.108) activity was analysed according to the protocol described previously (Dahlqvist, 1984), with minor adjustments. Briefly, 50 µl brush border membrane vesicle extracts were incubated for 60 min at 37 °C at a ratio of 1:1 with 50

µl lactose (56 mM) in 100 mM Na-maleate, pH 6. The liberated glucose was then measured at 510 nm using the glucose oxidase assay kit (Glucose Oxidase Assay Kit K-GLOX, Megazyme, Ireland), according to manufacturer's instructions. Lactase activity was expressed as nmol of glucose/mg protein. One unit of lactase activity was defined as the amount of enzyme causing the formation of one µmol of glucose per minute at 37 °C. Each brush border membrane enzyme activity was measured with technical triplicates on two different days (n = 7 pigs).

3.2.14. Statistical Analysis

All data are reported as means ± standard error of the mean (SEM) of at least three or more biological replicates. Statistical analysis was performed using IBM SPSS Statistics Version 27 (IBM Statistics, USA). Results were regarded as statistically significant when $P \leq 0.05$ and considered as tendencies at $0.05 < P < 0.10$.

3.3. Results

Microbial analysis of IMF powders produced at pilot plant scale, revealed that both IMFs met the microbiological safety requirements for IMFs outlined under Commission Regulation (EC) No 2073/2005 (Commission Regulation, 2005). Neither *Cronobacter sakazakii* nor *Salmonella* spp were detected in either powder. Although both powders had low levels of total bacterial counts, it was noted that MEM-IMF powder had significantly higher total bacterial counts compared to HT-IMF powder (1.8×10^7 *vs* 8.3×10^2 CFU/g powder) ($P < 0.05$). To meet the National Research Council nutrient requirements for pigs at weaning (National Research Council, 2012), a maximum inclusion of 35% of IMF powders was permitted in the starter diet. The post-weaning growth performance of pigs fed either MEM-IMF diet or HT-IMF diet has been previously described by Chen et al. (2023). In brief, over the 28 day trial, pigs in both treatment groups had similar average daily gain, average daily feed intake and feed conversion efficiency ($P > 0.05$). Body weight of pigs was not affected by IMF treatment ($P > 0.05$).

3.3.1. Measurement of trypsin activity in the small intestine

As protein digestion kinetics in the upper gastrointestinal tract were faster in MEM-IMF fed pigs compared to pigs fed HT-IMF diets (Chen et al., 2023), trypsin activities were measured in the gut lumen collected from the duodenum and jejunum of pigs 3 h after their final feed (Figure 3.1). Pigs fed MEM-IMF diet had significantly higher trypsin activity in the duodenum than pigs fed HT-IMF diet (1.36 ± 0.19 mU/mg protein *vs* 0.91 ± 0.20 mU/mg protein) ($P < 0.05$). There were no differences in trypsin activity in the jejunum of pigs fed either IMF ($P > 0.05$). Pigs fed MEM-IMF diet tended to have higher trypsin activity in the duodenum (1.36 ± 0.19 mU/mg protein) compared to the jejunum (1.01 ± 0.09 mU/mg protein) ($P < 0.10$). There was no difference in trypsin activity in the duodenum and jejunum in pigs fed HT-IMF diet ($P > 0.05$).

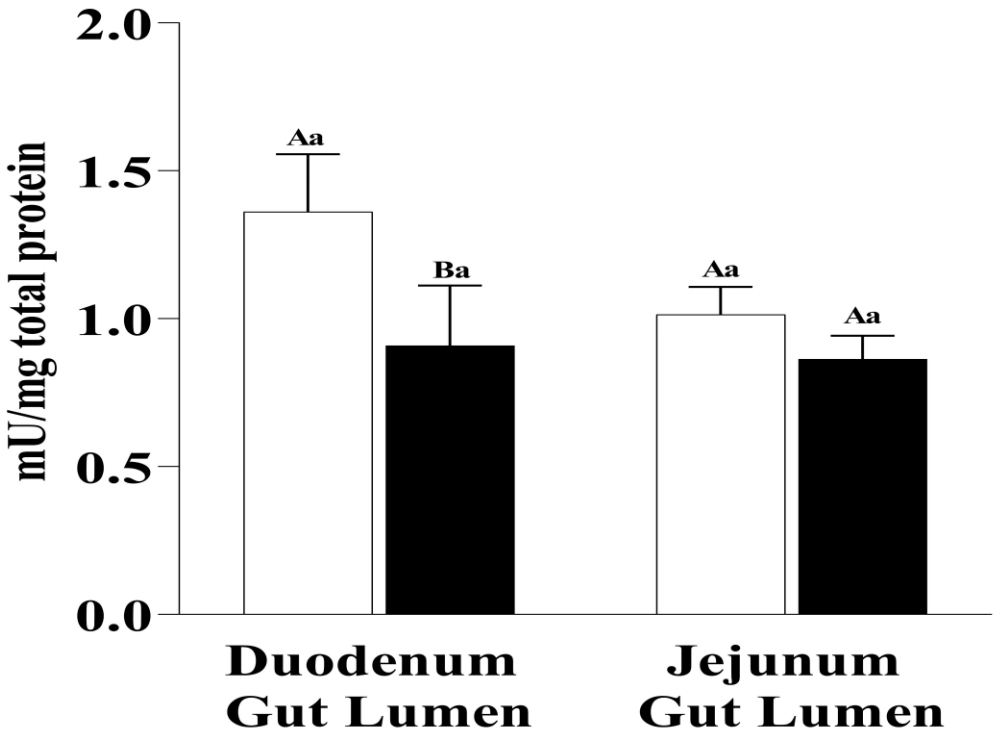


Figure 3.1. Influence of infant milk formula produced by membrane filtration (MEM-IMF) (white) or high-temperature infant milk formula (HT-IMF) (black) diets on trypsin activity (mU/mg total protein) in gut lumen collected from pigs. Values are reported as means ± SEM (n = 9 pigs per treatment). Different uppercase letters within the same intestinal location indicates significant differences between treatment types ($P < 0.05$). Different lowercase letters within the same treatment type indicates a significant difference between intestinal locations ($P < 0.05$).

3.3.2. Influence of processing on brush border membrane enzymatic activity

To investigate if IMF processing altered brush border membrane enzymatic activities, aminopeptidase N, aminopeptidase A, intestinal alkaline phosphatase and lactase activity were determined in the isolated brush border membrane vesicles (Table 3.1). These vesicles were extracted from mucosal scrapings collected from the pigs' upper small intestine. In the duodenal brush border membrane, a significantly lower aminopeptidase N activity was detected in pigs fed MEM-IMF diet versus HT-IMF fed pigs (5.23 ± 0.26 U/mg protein *vs* 7.44 ± 0.69 U/mg protein) ($P < 0.05$). Overall, the total aminopeptidase N activity in the brush border membrane of the duodenum and jejunum had a tendency to be lower in MEM-IMF fed pigs compared to HT-IMF fed pigs ($P < 0.10$). There was a significant increase in lactase activity in the brush border membrane of the duodenum of MEM-IMF fed pigs compared to HT-IMF fed pigs (166.59 ± 22.09 nmol of glucose/mg protein *vs* 110.75 ± 7.21 nmol of glucose/mg protein) ($P < 0.05$). No significant differences were observed in lactase activity between treatments in the jejunum. Processing did not influence aminopeptidase A and intestinal alkaline phosphatase activity in the small intestine brush border membrane, with no significant differences detected between treatment groups ($P > 0.05$). Interestingly, the aminopeptidase N activity in the brush border membrane of MEM-IMF pigs increased the further down the intestinal tract (5.23 ± 0.26 U/mg protein in the duodenum *vs* 6.59 ± 0.47 U/mg protein in the jejunum) ($P < 0.05$). There was no significant differences in aminopeptidase N activity in brush border membranes between duodenum and jejunum for HT-IMF fed pigs ($P > 0.05$). Duodenal brush border membrane aminopeptidase A activity was significantly higher than the jejunum for both MEM-IMF and HT-IMF fed pigs ($P < 0.05$). Higher brush border membrane lactase activity was observed in the HT-IMF group in the jejunum (168.45 ± 18.04 nmol of glucose/mg protein) compared to the duodenum (110.75 ± 7.21 nmol of glucose/mg protein) ($P < 0.05$).

Table 3.1. Brush border membrane enzymatic activity in pigs fed infant milk formula produced by membrane filtration (MEM-IMF) or high-temperature infant milk formula (HT-IMF) diets for 28 days

	MEM-IMF	**HT-IMF**
Duodenum brush border membrane		
Aminopeptidase N (U/mg cellular protein)	5.23 ± 0.26^{Aa}	7.44 ± 0.69^{Ba}
Aminopeptidase A (mU/mg cellular protein)	176.52 ± 13.83^{Aa}	155.17 ± 22.83^{Aa}
Intestinal Alkaline Phosphatase (U/mg cellular protein)	185.53 ± 29.68^{Aa}	167.44 ± 30.49^{Aa}
Lactase (nmol glucose/mg cellular protein)	166.59 ± 22.09^{Aa}	110.75 ± 7.21^{Bb}
Jejunum brush border membrane		
Aminopeptidase N (U/mg cellular protein)	6.59 ± 0.47^{Ab}	6.95 ± 1.13^{Aa}
Aminopeptidase A (mU/mg cellular protein)	29.82 ± 3.46^{Ab}	57.44 ± 18.17^{Ab}
Intestinal Alkaline Phosphatase (U/mg cellular protein)	135.07 ± 10.82^{Aa}	154.27 ± 27.13^{Aa}
Lactase (nmol glucose/mg cellular protein)	154.16 ± 10.30^{Aa}	168.45 ± 18.04^{Aa}

Enzymatic activities were measured in isolated brush border membrane vesicles (n = 7 pigs per treatment) 3 h after final feeding. Values are reported as means ± SEM. Different uppercase letters within the same intestinal location indicates significant differences between IMF dietary treatment types ($P < 0.05$). Different lowercase letters within the same IMF dietary treatment types indicates significant differences between intestinal locations ($P < 0.05$).

3.3.3. Effects of IMF processing on small intestinal/gut physiology of pigs

To investigate if IMF processing altered gut barrier physiology, mucosa thickness, villus height, crypt depth and goblet cell numbers, histological analysis was performed on the duodenum and jejunum of pigs fed MEM-IMF or HT-IMF diets (Table 3.2, Supplementary Figures 3.3 and 3.4). Pigs fed MEM-IMF diet had a significantly higher numbers of goblet cells in the jejunum (17.39 ± 1.43) compared to pigs on the HT-IMF diet (11.7 ± 1.27) ($P < 0.05$). No significant difference in goblet cells numbers were observed between treatment types in the duodenum. Processing type had no significant effect on duodenal or jejunal villus height, crypt depth or villus height:crypt depth ratio. However, significant differences were noted within treatment type between intestinal regions. There were significantly higher numbers of goblet cells in the duodenum compared to the jejunum in both IMF treatments (MEM-IMF (27.98 ± 3.56 vs 17.39 ± 1.43 ($P < 0.05$) and HT -IMF (30.81 ± 1.67 *vs* 11.7 ± 1.27) ($P < 0.05$)). A decrease in crypt depth from duodenum to jejunum was also observed in both treatments (MEM-IMF (381 ± 24.08 µm *vs* 293.46 ± 13.73) and HT-IMF group (366.78 ± 23.63 *vs* 281.87 ± 7.6 µm) ($P < 0.05$)). Subsequently, there was a significant increase in villus height:crypt depth ratio for both IMF treatments in the pig jejunum compared to the duodenum ($P < 0.05$). There was a significant increase in villus height distally in MEM-IMF fed pigs ($P < 0.05$). Mucosal thickness was significantly higher in the duodenum (816.40 ± 18.21 µm) compared to the jejunum (748.84 ± 24.63 µm) of pigs fed HT-IMF diets ($P < 0.05$). As previously noted by others (Verdile et al., 2019), it was difficult to observe Paneth cells in the histology slides of these young pigs. IL-6 stimulates Paneth cell proliferation and is critical in maintaining crypt health via Paneth cells and the Wnt signalling pathway (Andrews et al., 2018; Jeffery et al., 2017). Hence, *IL-6* mRNA transcript levels were measured in both the duodenum and jejunum mucosal scrapings of pigs fed MEM-IMF or HT-IMF diets (Supplementary Figure 3.5). Interestingly, *IL-6* mRNA transcript levels were significantly higher (3.7 fold) in the duodenum

of pigs fed MEM-IMF diets compared to those fed HT-IMF diets ($P < 0.01$). No differences were detected in *IL-6* mRNA levels in the jejunum ($P > 0.05$).

Table 3.2. Histological analysis of duodenum and jejunum of pigs fed infant milk formula produced by membrane filtration (MEM-IMF) or high-temperature infant milk formula (HT-IMF) diets for 28 days

	MEM-IMF	HT-IMF
Number of goblet cells		
Duodenum	27.98 ± 3.56^{Aa}	30.81 ± 1.67^{Aa}
Jejunum	17.39 ± 1.43^{Ab}	11.7 ± 1.27^{Bb}
Mucosa thickness (µm)		
Duodenum	785.68 ± 29.38^{Aa}	816.40 ± 18.21^{Aa}
Jejunum	770.51 ± 31.85^{Aa}	748.84 ± 24.63^{Ab}
Villus height (µm)		
Duodenum	404.66 ± 22.34^{Ab}	449.60 ± 24.72^{Aa}
Jejunum	477.06 ± 29.54^{Aa}	466.96 ± 26.19^{Aa}
Crypt depth (µm)		
Duodenum	381 ± 24.08^{Aa}	366.78 ± 23.63^{Aa}
Jejunum	293.46 ± 13.73^{Ab}	281.87 ± 7.6^{Ab}
Villus height:crypt depth ratio		
Duodenum	1.19 ± 0.11^{Ab}	1.33 ± 0.15^{Ab}
Jejunum	1.75 ± 0.14^{Aa}	1.74 ± 0.12^{Aa}

Values are reported as means ± SEM (MEM-IMF, n = 8 pigs, HT-IMF, n = 6 pigs). Different uppercase letters within the same intestinal location indicates significant differences between IMF dietary treatment type ($P < 0.05$). Different lowercase letters within the same IMF dietary treatment indicates significant differences between intestinal locations ($P < 0.05$).

3.3.4. Measurement of acidic mucins in mucus layer of pig's small intestine

As there was a higher number of goblet cells in the jejunum of MEM-IMF fed pigs and the primary function of goblet cells is to secrete mucus, the amount of acidic mucus in the duodenum and jejunum tissue was quantified by Alcian blue dye (Figure 3.2). In the jejunum tissue, the amount of acidic mucus was significantly higher in pigs fed MEM-IMF (3 ± 0.24 µg Alcian blue/mg tissue) in comparison with those fed HT-IMF (2.48 ± 0.23 µg Alcian blue/mg tissue) ($P < 0.05$). IMF processing did not influence the amount of acidic mucus in the duodenum tissue. Levels of mucus increased distally with higher amounts of acidic mucus recorded in the jejunum tissue than the duodenum tissue of pigs for both IMF treatments (MEM-IMF (3 ± 0.24 µg/mg *vs* 1.76 ± 0.18 µg/mg) and HT-IMF (2.48 ± 0.23 µg /mg *vs* 1.75 ± 0.12 µg /mg) $P < 0.05$).

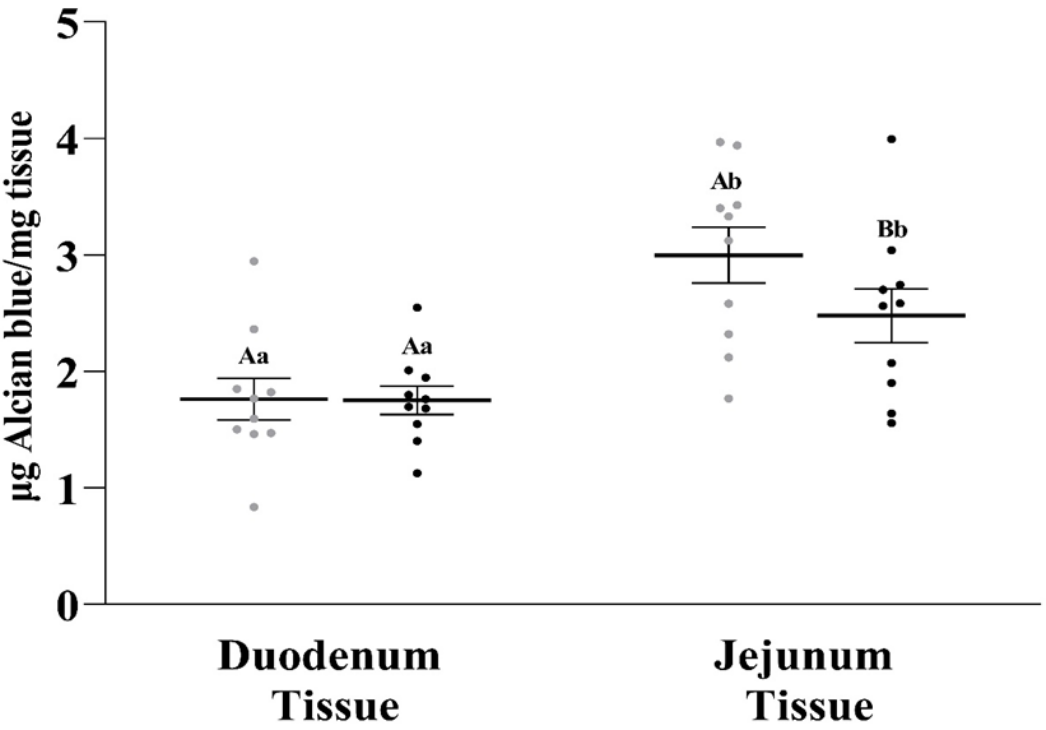
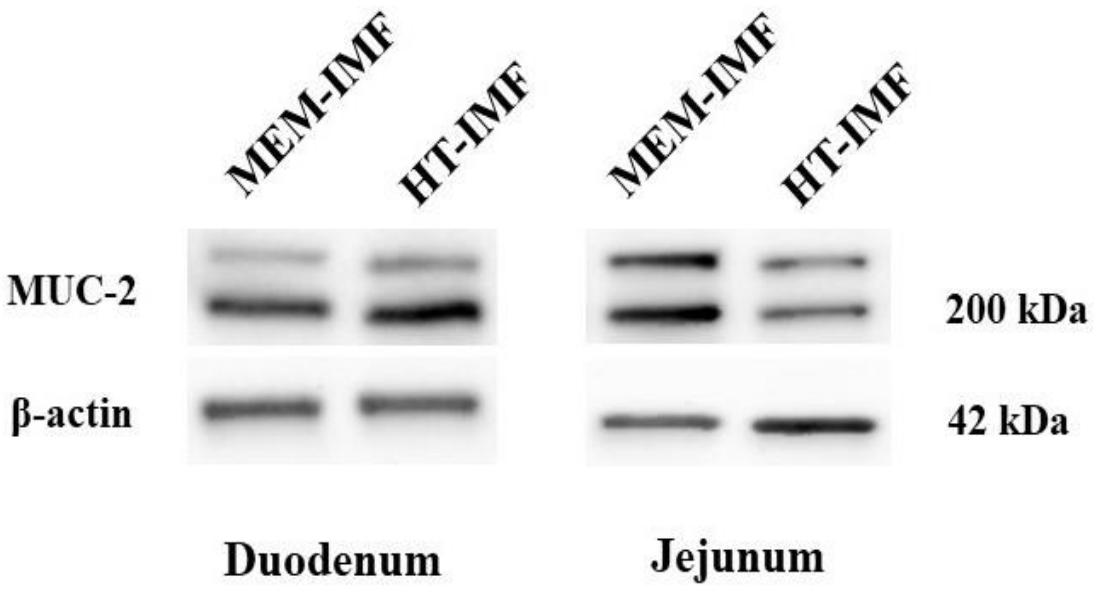


Figure 3.2. Measurement of acidic mucins by Alcian blue dye (µg Alcian blue/mg tissue) in the tissue of pigs fed infant milk formula produced by membrane filtration (MEM-IMF) (grey dots) or high-temperature infant milk formula (HT-IMF) (black dots) diets for 28 days. Samples from the pig duodenum and jejunum were collected at slaughter 3 h after final feeding. Values are reported as means ± SEM (n = 10 pigs per treatment). Different uppercase letters within the same intestinal location indicates significant differences between IMF dietary treatment types ($P < 0.05$). Different lowercase letters within the same IMF dietary treatment types indicates significant differences between intestinal locations ($P < 0.05$).

3.3.5. Western blot analysis of mucin-2

Next, levels of MUC-2 protein, the primary gel-forming mucin in the small intestine, were quantified by immunoblotting in the small intestinal mucosal scrapings (Figure 3.3). As MUC-2 is a highly glycosylated protein (Arike & Hansson, 2016), two distinct bands were detected at approximately 200 kDa. In line with Nishida et al. (2009) both bands were included for quantification purposes. Densitometric analysis, employing β-actin as a housekeeping control, showed that protein levels of MUC-2 were significantly higher in the jejunum of pigs fed MEM-IMF diets compared to those fed HT-IMF diets ($P < 0.05$). However, protein levels of MUC-2 in the duodenum were similar in both IMF treatment groups. For the HT-IMF fed pigs, higher levels of MUC-2 were observed in the duodenum compared to the jejunum ($P < 0.05$).

A)



B)

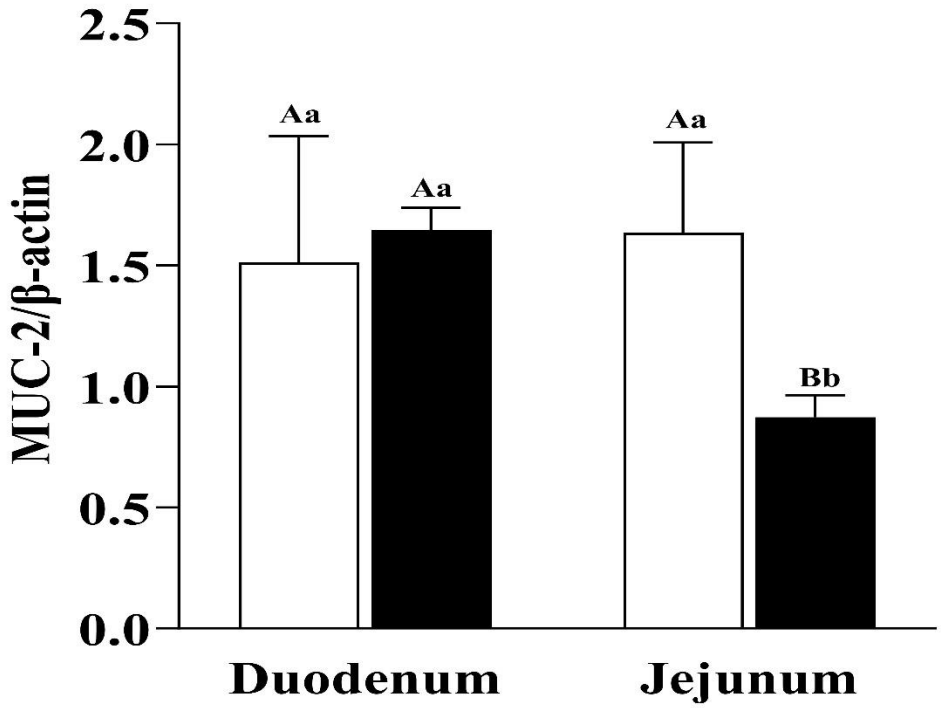


Figure 3.3. Western blot analysis of mucin-2 (MUC-2) in the mucosal scrapings of pigs fed infant milk formula produced by membrane filtration (MEM-IMF) (white) or high-temperature infant milk formula (HT-IMF) (black) diets for 28 days. Mucosal scrapings were collected at

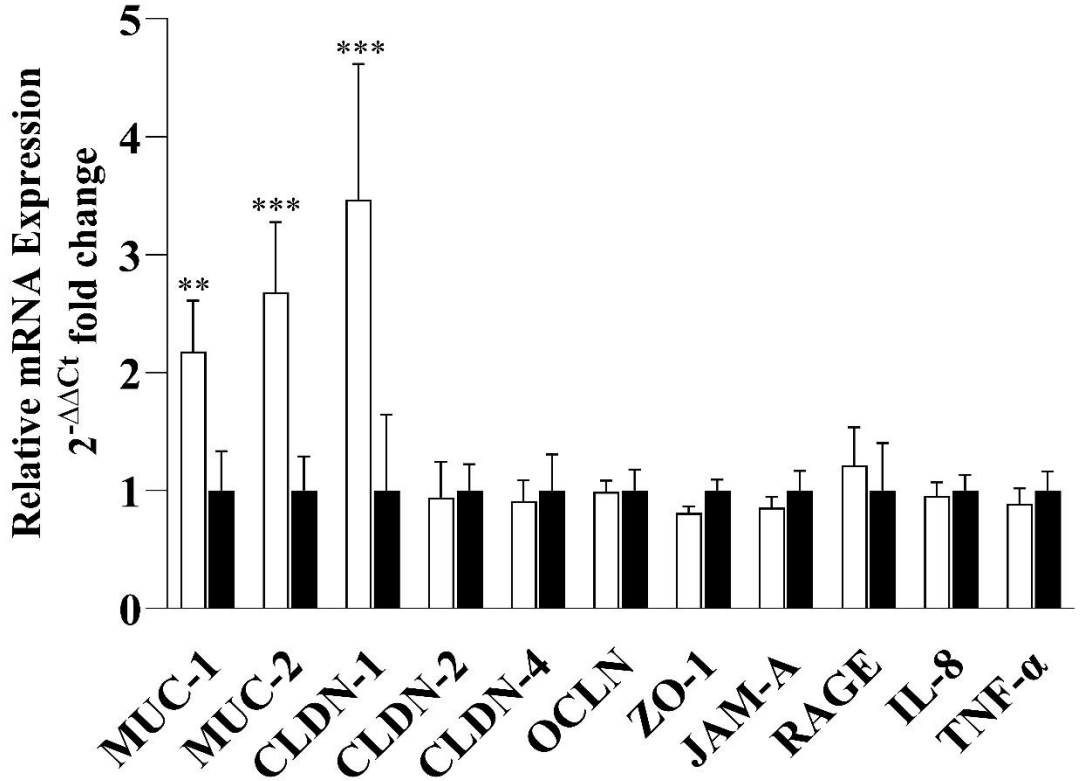
slaughter 3 h after final feeding. (**A**) Immunoreactive bands were quantified using Image Lab 6.1 software. (**B**) Quantification of MUC-2 bands were calibrated against the intensity of the housekeeping β-actin band. Values are reported as means ± SEM (n = 3 pigs per treatment). Different uppercase letters within the same intestinal location indicates significant differences between IMF dietary treatment types ($P < 0.05$). Different lowercase letters within the same IMF dietary treatment types indicates significant differences between intestinal locations ($P < 0.05$).

3.3.6. Effect of IMF processing on biomarkers of gut barrier health and advanced glycation end products

To further examine the influence of IMF processing on gut barrier health, mRNA transcript levels of a range of biomarkers were measured in the duodenal and jejunal mucosal scrapings (Figure 3.4). In the duodenal mucosal scrapings, the MEM-IMF diet significantly increased mRNA transcript levels of mucus biomarkers, *mucin-1* (*MUC-1*) ($P < 0.01$), *MUC-2* ($P < 0.001$) and tight junction biomarker, *CLDN-1* ($P < 0.001$) compared to the HT-IMF fed pigs (Figure 3.4A). IMF processing did not alter mRNA transcript levels of other tight junction biomarkers (*CLDN-2*, *CLDN-4*, *OCLN*, *ZO-1* and *JAM-A*) in the duodenum ($P > 0.05$). In the jejunum, mRNA transcript levels of the receptor for advanced glycation end products (*RAGE*), was significantly lower in the jejunum (Figure 3.4B, $P < 0.05$) of pigs fed MEM-IMF diet compared to those fed HT-IMF diet. No significant differences in mRNA transcript levels were observed in the jejunum between IMF treatment groups for *MUC-1*, *MUC-2*, *CLDN-1*, *CLDN-2*, *CLDN-4*, *OCLN*, *ZO-1* and *JAM-A* ($P > 0.05$).As an indicator of intestinal inflammation (Tixier et al., 2005), *IL-8* mRNA transcript levels were measured in the duodenum (Figure 3.4A) or jejunum (Figure 3.4B). No significant differences in *IL-8* mRNA transcript levels were observed in either gut locations with IMF treatment ($P > 0.05$).

Previously, Bjornvad et al. (2008) linked higher localised TNF-α levels to increases in intestinal permeability (Bjornvad et al., 2008). As such *TNF-α* mRNA transcript levels were tracked in the duodenum and jejunum mucosal scrapings, revealing no significant difference in *TNF-α* mRNA expression in either location for pigs fed MEM-IMF compared to those pigs fed HT-IMF ($P > 0.05$) (Figure 3.4).

A)


Relative mRNA Expression
$2^{-\Delta\Delta Ct}$ fold change
5
4
3
2
1
0
**
***
***
MUC-1
MUC-2
CLDN-1
CLDN-2
CLDN-4
OCLN
ZO-1
JAM-A
RAGE
IL-8
TNF-α

B)

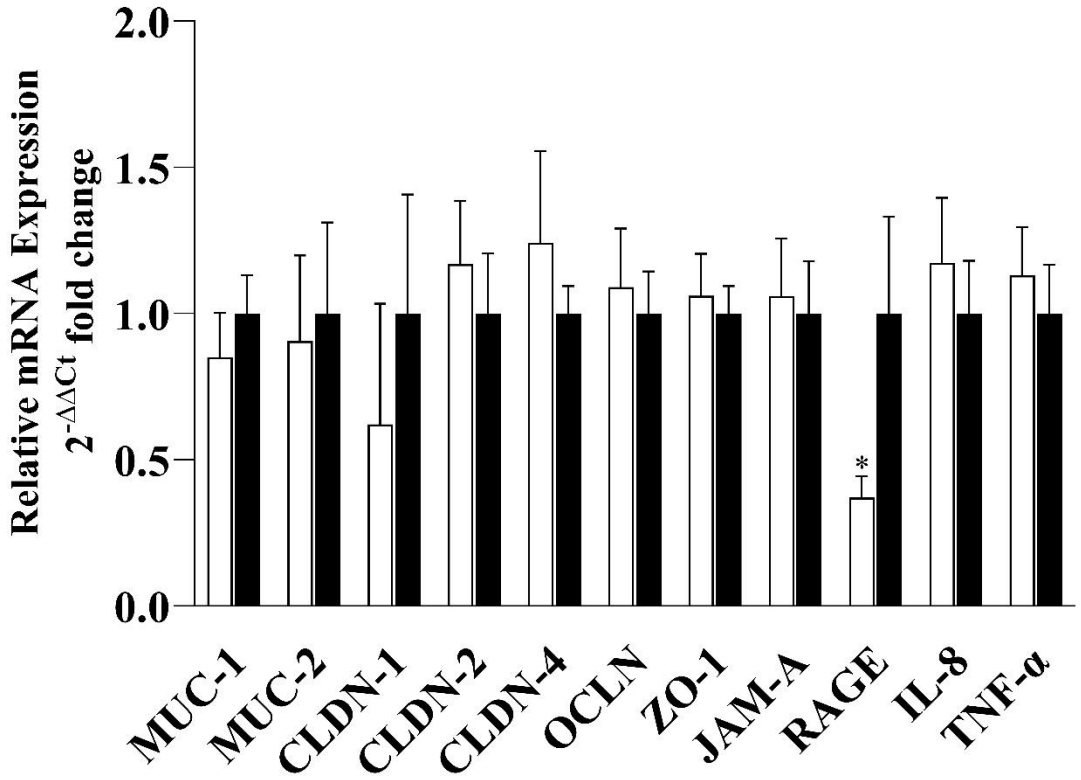

Relative mRNA Expression
$2^{-\Delta\Delta Ct}$ fold change
2.0
1.5
1.0
0.5
0.0
*
MUC-1
MUC-2
CLDN-1
CLDN-2
CLDN-4
OCLN
ZO-1
JAM-A
RAGE
IL-8
TNF-α

Figure 3.4. mRNA transcript levels in (**A**) duodenum and (**B**) jejunum mucosal scrapings of pigs fed infant milk formula produced by membrane filtration (MEM-IMF) (white) or high-temperature infant milk formula (HT-IMF) (black) diets for 28 days. Mucosal scrapings were collected at slaughter 3 h after final feeding. Values are reported as means ± SEM (n = 8 pigs per treatment). The relative quantification of target mRNA was calculated using the reference gene RPLP0 following the $2^{-\Delta\Delta Ct}$ method. Transcript levels are expressed as the fold change relative to the mean value of the HT-IMF (control) diet. Significant differences between diets for each individual gene are indicated by * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. *MUC-1, mucin-1; MUC-2, mucin-2; CLDN-1, claudin-1; CLDN-2, claudin-2; CLDN-4, claudin-4; OCLN, occludin; ZO-1, zona occludens-1; JAM-A, junctional adhesion molecule-A; RAGE, receptor for advanced glycation end products; IL-8, interleukin 8*; *TNF-α, tumor necrosis factor alpha.*

3.4. Discussion

Feeding MEM-IMF diets to pigs for 28 days resulted in higher goblet cell numbers, mucus levels, trypsin activity and brush border membrane lactase activity in the upper small intestinal tract of pigs compared to pigs on HT-IMF diets. The significant increases observed in jejunum goblet cell numbers and acidic mucins was accompanied with increases in MUC-2 protein levels in pigs fed MEM-IMF. In addition, *MUC-1* and *MUC-2* mRNA transcript levels were significantly higher in the duodenum of these pigs compared to HT-IMF fed pigs. Goblet cells are scattered, at different densities, throughout the intestinal tract and function to secrete mucus which lubricates and protects the epithelium (Kim & Khan, 2013). Newborn infants have inadequate levels of mucus and an underdeveloped epithelium architecture (Wells et al., 2022). Breast milk is known to promote gut barrier maturity, including increasing mucus production (Dimitroglou et al., 2022). Evidence is now emerging that reducing thermal processing of IMF or its protein ingredients can increase mucus secretion and support the intestinal barrier architecture with some differences based on gut location (Li et al., 2018; Li et al., 2013; Navis, Muncan, et al., 2020; Sun et al., 2022; Wang & Zhao, 2021). Previously, Navis, Muncan, et al. (2020) observed an increase in goblet cells, higher alkaline phosphatase and lower lesion scores in the colon of near-term piglets, fed mildly pasteurised IMF (Navis, Muncan, et al., 2020). A five-day dietary intervention study was performed with preterm (n = 34 at 106 days gestation) and near-term (n = 18 at 113 days of gestation) piglets (Navis, Muncan, et al., 2020). Piglets received increasing doses (6 ml/kg/3 h to 10 ml/kg/3 h) of IMF containing 80 g/L of either; (1) mildly pasteurised whey protein concentrate (WPC) (73°C for 30 s) or (2) extensively heated WPC (73°C for 30 s and 80°C for 6 min) powder by orogastric feeding tube (Navis, Muncan, et al., 2020). However, this mildly processed IMF did not significantly change mRNA transcript levels of *MUC-1* and *MUC-2* in the colon or *MUC-2* in the distal small intestine (Navis, Muncan, et al., 2020). In our study, the colon was not sampled as evidence indicates

that WPC is completely digested in the small intestine with its reported digestible indispensable amino acid score of 107% at the ileum (Mathai et al., 2017). The orogastric administration used by Navis, Muncan, et al. (2020) may have delayed whey protein digestion, resulting in a more distal goblet cell response (Navis, Muncan, et al., 2020).

Although no differences in villus height or crypt depth after our 28 day intervention were observed, significant increases in duodenal *CLDN-1* mRNA transcript levels was observed in pigs fed MEM-IMF diets compared to pigs fed HT-IMF diets. A significant increase in CLDN-1 and a tendency to increase OCLN protein concentration was also observed, previously by us in an *in vitro* study using polarised Caco-2 monolayers exposed to laboratory scale MEM-IMF (Bavaro et al., 2021). In other studies, higher CLDN-1 protein levels and *CLDN-1* mRNA levels have been observed in the jejunum and ileum of pigs where villus height has increased (Dowley et al., 2021; Ma et al., 2022; Xiao et al., 2016). This CLDN-1 and villus height association was not observed in our study. In contrast, orogastric intervention studies with dairy proteins have observed increases in villus height and villus height:crypt depth ratio in the duodenum, jejunum and ileum (Li et al., 2018; Li et al., 2013; Sun et al., 2022; Wang & Zhao, 2021). Sun et al. (2022) reported significant increases in intestinal villus height in the proximal, mid and distal small intestine of preterm pigs (n = 56 at 106 days gestation) who received pasteurised liquid IMF by orogastric feeding tube compared to animals who received liquid UHT IMF (Sun et al., 2022). Increasing volumes of IMF were administered over the 3 days test period (40 ml/kg/day to 120 ml/kg/day). Similarly Li et al. (2013) treated preterm pigs (n = 55 at 105 days gestation) with IMF powder (24 ml/kg/day to 120 ml/kg/day), by orogastric feeding, for 5 days (Li et al., 2013). Li et al. (2013) found that villus height in the proximal, but not the distal, small intestine was significantly higher in pigs that received IMF with gently processed WPC (582 ± 46 μm) compared to pigs that were fed IMF containing extensively, processed WPC (440 ± 47 μm) (Li et al., 2013). In a follow-up study, administration of WPC

alone, resulted in similar villus height alterations (Li et al., 2018). Wang and Zhao (2021) fed unglycated caseinate (liquid) (with no lactose or heat) and a lactose-glycated caseinate with a high thermal load (100°C for 180 min) to Wistar rats (n = 6) by intra-gastric administration for 7 days (Wang & Zhao, 2021). Rats fed the unglycated caseinate (400 mg/kg body weight/day) had significantly higher villus height and villus height:crypt depth ratio in the duodenum, jejunum and ileum compared to rats fed the glycated caseinate with high thermal load (Wang & Zhao, 2021). In these acute studies, the test food was the sole source of food administered to the animals for the duration of the study, albeit additional parenteral nutrition (e.g. carbohydrates, protein, fat, vitamins, minerals and electrolytes) was supplied. It is worth noting that in our study, IMF was included in the diet at 35%. This may explain why villus height increases were not observed with an MEM-IMF dietary intervention. In this study, we did not perform a lactulose: mannitol test to assess intestinal permeability. However, other markers of higher intestinal permeability are a reduction in villus height (Li et al., 2014; Li et al., 2013) and increases in localised TNF-α (Bjornvad et al., 2008). Neither of these responses were observed in our study, indicating intestinal permeability was unlikely to be affected by IMF processing, similar to that reported previously (Rasmussen et al., 2016). Although Paneth cells were not observed, mRNA transcript levels of *IL-6*, were significantly higher in the duodenum of pigs fed MEM-IMF compared to HT-IMF fed pigs. This increase may suggest higher numbers of Paneth cells or a healthy crypt (Andrews et al., 2018)

Digestive enzymes were also impacted by IMF diet. There were significant increases in trypsin activity in the duodenal gut lumen and lactase but lower aminopeptidase N activity in the duodenal brush border membrane of pigs fed MEM-IMF compared to pigs fed HT-IMF diet. This significant increase in trypsin activity coincided with higher digestion kinetics of MEM-IMF (Chen et al., 2023). Pigs receiving MEM-IMF diet had significantly higher protein hydrolysis in the stomach and duodenum compared to pigs fed HT-IMF (599 ± 45 and 1530 ±

136 µmol/g protein *vs* 509 ± 17 and 1174 ± 124 µmol/g protein) (Chen et al., 2023). Although digestion kinetics were not tracked, Wang and Zhao (2021) also reported significantly higher trypsin and lactase activity in all three small intestinal locations of rats fed unglycated caseinate (Wang & Zhao, 2021). Lactase activity was also significantly higher in the proximal and mid small intestine of preterm pigs fed pasteurised liquid IMF in comparison with UHT processed IMF (Sun et al., 2022). In addition, a significantly higher lactase activity in the proximal small intestine was observed in piglets fed low heat treated WPC versus a standard heat treated WPC (Li et al., 2018). Lactose is sensitive to high temperature processing and can reduce available substrate for lactase. Interestingly, gastric emptying of lactose was faster in pigs fed pasteurised milk compared to those fed UHT milk (Montoya et al., 2024). In our study, significantly lower levels of aminopeptidase N activity may be correlated with the higher concentrations of duodenal proline in pigs fed MEM-IMF diet (Chen et al., 2023). A higher concentration of free proline suggests that aminopeptidases are able to hydrolyse MEM-IMF peptides more freely. In contrast, curd formation and protein aggregation of HT-IMF during the gastric phase of digestion (Chen et al., 2022) may hamper enzyme access in the lumen requiring higher levels of intestinal brush border aminopeptidase N activity prior to amino acid absorption. Interestingly, Li et al. (2013) and Navis, Schwebel, et al. (2020) found no effect of processing on aminopeptidase N, aminopeptidase A and lactase activities in the small intestine (Li et al., 2013; Navis, Schwebel, et al., 2020). However, digestion kinetics assays were not performed in these studies so whether the different WPCs were digested at different rates could not be ascertained. Many of these studies have attributed the beneficial gut responses to significantly lower advanced glycation end products, or more specifically, CML levels in the IMFs produced by lower temperatures (Navis, Schwebel, et al., 2020; Sun et al., 2022). Sun et al. (2022) reported that administrating IMF liquid with lower levels of advanced glycation end products results in a lower accumulation of Maillard reaction products in the ileal mucosa of pigs (Sun

et al., 2022). It is widely accepted that high levels of advanced glycation end products in the gut lumen are harmful to the gut barrier (Phuong-Nguyen et al., 2023). Previously, we and others have reported no differences in CML levels in spray dried IMF powders produced by membrane filtration or high-temperature processing (Chen et al., 2021; Yu et al., 2021). However, a significant decrease in *RAGE* mRNA transcript levels were observed in the jejunum of pigs fed MEM-IMF diet compared to HT-IMF diet. In the intestine, RAGE serves as a receptor for CML but also other ligands including other advanced glycation end products (Oshima et al., 2022). Ligand binding to RAGE activates intestinal inflammatory responses (Body-Malapel et al., 2019). CML is widely considered the primary advanced glycation end product biomarker. CML exists in its free state (glycated to amino acids) and in the bound state (conjugated to proteins) *in vivo* (Yuan et al., 2022). Free CML is likely to be absorbed into intestinal epithelial cells by simple diffusion and can accumulate in different tissues (Yuan et al., 2022). In our study, mRNA transcript levels of the inflammatory marker *IL-8* were similar in the duodenum and jejunum of MEM-IMF fed pigs and HT-IMF fed pigs, indicating our IMF processing was unlikely to result in an inflammatory response via the IL-8 pathway. It has been observed that more extensive processing of WPC (73°C for 30 s and 80°C for 6 min) will result in significant increases in colonic IL-8 levels in piglets (Navis, Muncan, et al., 2020). Further investigations are required to understand better how a decrease in *RAGE* mRNA transcript levels is physiologically relevant. However, it is also likely that reducing thermal processing in IMF products has additional benefits apart from reducing advanced glycation end products, such as preserving whey protein bioactivities (Li et al., 2018). Bavaro et al. (2021) observed that *in vitro* digestion of MEM-IMF released different peptides, with potentially different bioactivities, than HT-IMF (Bavaro et al., 2021).

Unsurprisingly, our study observed physiological and enzymatic differences between different small intestinal locations in young pigs. Irrespective of treatment type, the number of goblet

cells were significantly higher in the duodenum versus the jejunum. Święch et al. (2019) observed a similar trend in young pigs, with the duodenum having a higher number of goblet cells compared to the jejunum (Święch et al., 2019). These findings disagree with the accepted belief that number of goblet cells increases distally (Specian & Oliver, 1991). There was also some notable treatment by location interactions. MEM-IMF fed pigs had higher crypt depth, aminopeptidase A activity and trypsin activity but decreased villus height in the duodenum compared to the jejunum. HT-IMF fed pigs had higher crypt depth, mucosa thickness and aminopeptidase A activity, but decreased lactase activity in the duodenum versus the jejunum. In agreement, Li et al. (2018) reported higher lactase activity from the proximal to distal small intestine of preterm piglets (Li et al., 2018). However, Sun et al. (2022) observed that brush border lactase activity decreased along the preterm pig's small intestinal tract (Sun et al., 2022). Regardless of dietary treatment, reporting on physiological and enzymatic differences between locations in the young gut is important for nutrition specific recommendations for this life stage.

MEM-IMF can protect the intestinal barrier from pathogenic attack. Future work should investigate whether higher mucus production alters bacterial adhesion in the jejunum and what impact IMF processing had on colonic physiology. In addition, *in vitro* models of the gut barrier should be employed for mechanistic studies on mucus production. Ultimately, intervention studies must be performed in babies to determine if MEM-IMF is easier to digest, promotes gut barrier health and, to evaluate how it compares to breast milk.

3.5. Conclusion

An IMF produced by membrane filtration has beneficial effects on gut health. A 35% inclusion of MEM-IMF in the diet of young pigs for 28 days resulted in higher goblet cell numbers, mucus levels, trypsin activity and lactase activity in the upper small intestine. This promotion of mucus production in the young gut is superior to IMF produced by high temperature processing. Next generation IMF should therefore seek to reduce thermal processing in its quest to mimic breast milk bioactivity.

3.6. References

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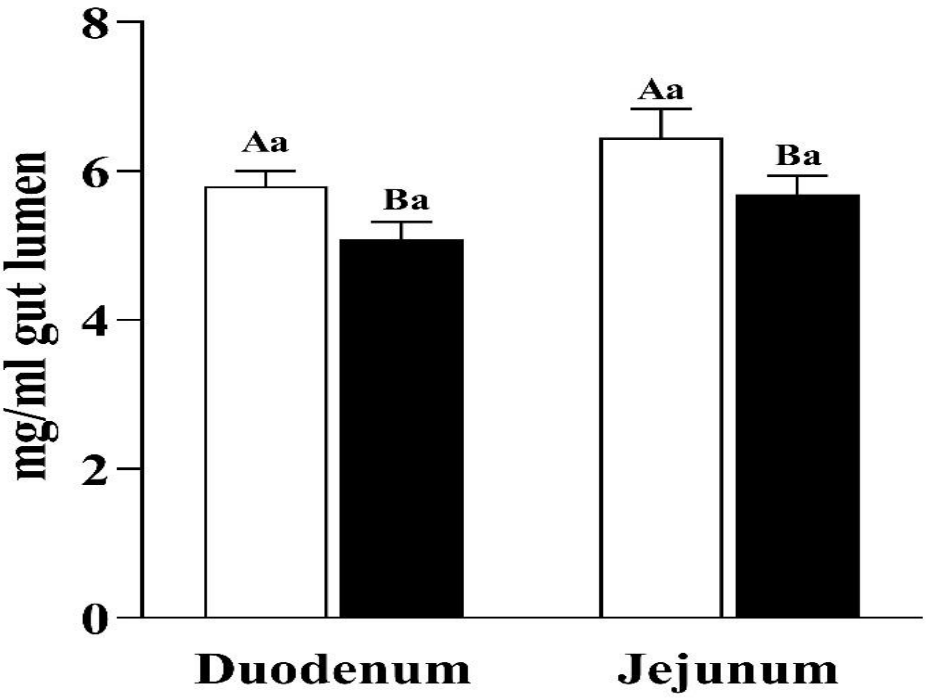
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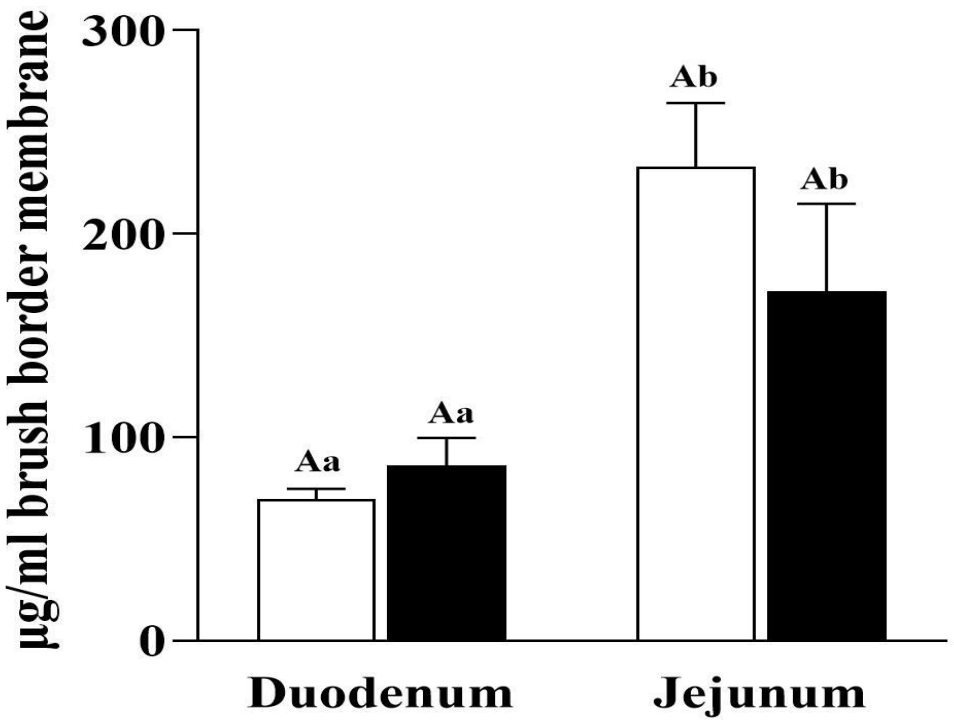
Supplementary Table 3.1. Gene name, GenBank accession number, primer sequences, annealing temperature and amplicon size for RT-qPCR analysis

Gene	GenBank Accession Number	Primer sequences (5'-3')	T_a (°C)	Amplicon size (bp)
RPLP0	NM_001098598.1	F: GACAAAGTGGGAGCCAGTGA R: CAGGGTTGTAGATGCTGCCA	56	112
MUC-1	XM_021089729.1	F: AGTCCATGTTGGCACCTCC R: GGAGTACCTTTGCTGACTGG	55	117
MUC-2	XM_013989745.1	F: TCTGGATCCGCAGCTCTCTGG R: CACTGGGCTGGGAGACAGGT	60	157
CLDN-1	NM_001244539.1	F: ACCGTGTGGGAACAACCAGA R: ACACATGAAAATGGCTTCCCTCC	62	195
CLDN-2	NM_001161638.1	F: GCATCATTTCCTCCCTGTT R: TCTTGGCTTTGGGTGGTT	60	156
CLDN-4	NM_001161637.1	F: CAACTGCGTGGATGATGAGA R: CCAGGGGATTGTAGAAGTCG	60	140
OCLN	NM_001163647.2	F: TCCTGGGTGTGATGGTGTTC R: CGTAGAGTCCAGTCACCGCA	63	144
ZO-1	XM_003353439.2	F: ATGGCGGAAAGTGAACCTCG R: TCACACCCTGCTTAGAATCCG	55	199
JAM-A	NM_001128444.1	F: CCAGGAAAGACACAGGGACG R: GGTTCCCGATGGTAGCAGAG	58	144
RAGE	NM_001123218.1	F: AGGTGGAGCTGTACCTCCTG R: TCCTTGGTCCAGTGGATTTG	60	87
IL-6	NM_214399.1	F: TGGCTACTGCCTTCCCTACC R: CAGAGATTTTGCCGAGGATG	60	132
IL-8	NM_213867.1	F: GAAGAGAACTGAGAAGCAACAACA R: TTGTGTTGGCATCTTTACTGAGA	60	99
TNF-α	NM_214022.1	F: CACGTTGTAGCCAATGTCAAAG R: GAGGTACAGCCCATCTGTCG	60	129

T_a, annealing temperature; bp, base pairs; *RPLP0, ribosomal protein lateral stalk subunit P0; MUC-1, mucin-1; MUC-2, mucin-2; CLDN-1, claudin-1; CLDN-2, claudin-2; CLDN-4, claudin-4; OCLN, occludin; ZO-1, zona occludens-1; JAM-A, junctional adhesion molecule-A*; *RAGE, receptor for advanced glycation end products*. *IL-6, interleukin 6*; *IL-8, interleukin 8*; *TNF-α, tumor necrosis factor alpha*.

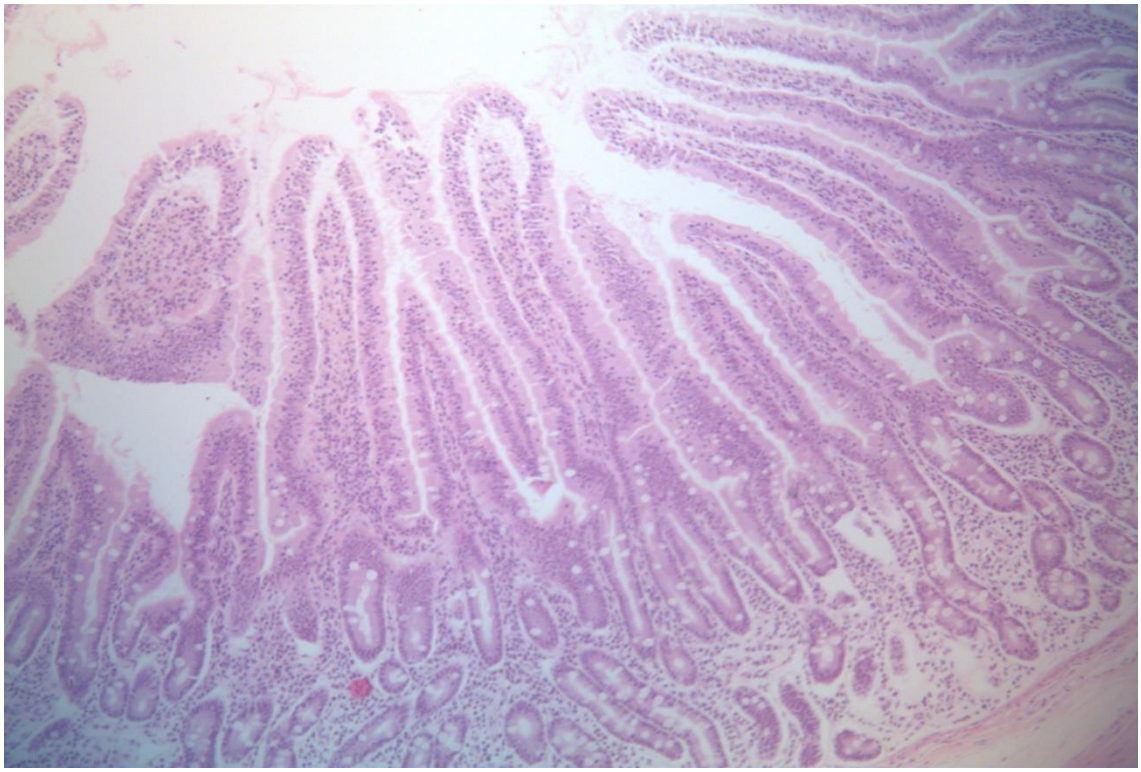


Supplementary Figure 3.1. Total protein concentration (mg/ml gut lumen) in gut lumen collected from the pigs following slaughter. MEM-IMF, infant milk formula produced by membrane filtration (white); HT-IMF, high-temperature infant milk formula (black). Values are reported as means ± SEM (n = 9). Different uppercase letters within the same intestinal location indicates significant differences between treatment types ($P < 0.05$). Different lowercase letters within the same treatment type indicates significant differences between intestinal locations ($P < 0.05$).

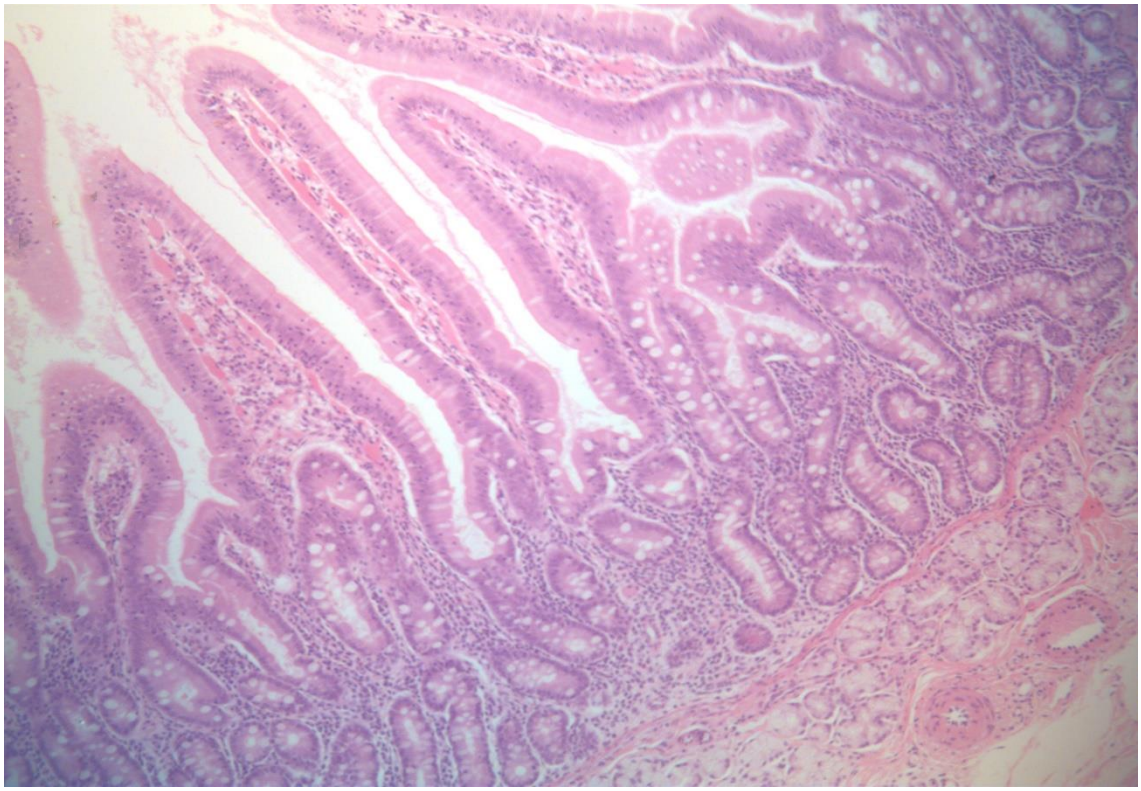


Supplementary Figure 3.2. Cellular protein concentration (µg/ml brush border membrane) in brush border membrane vesicles isolated from the pigs small intestinal mucosal scrapings. MEM-IMF, infant milk formula produced by membrane filtration (white); HT-IMF, high-temperature infant milk formula (black). Values are reported as means ± SEM (n = 7). Different uppercase letters within the same intestinal location indicates significant differences between treatment types ($P < 0.05$). Different lowercase letters within the same treatment type indicates significant differences between intestinal locations ($P < 0.05$).

A)



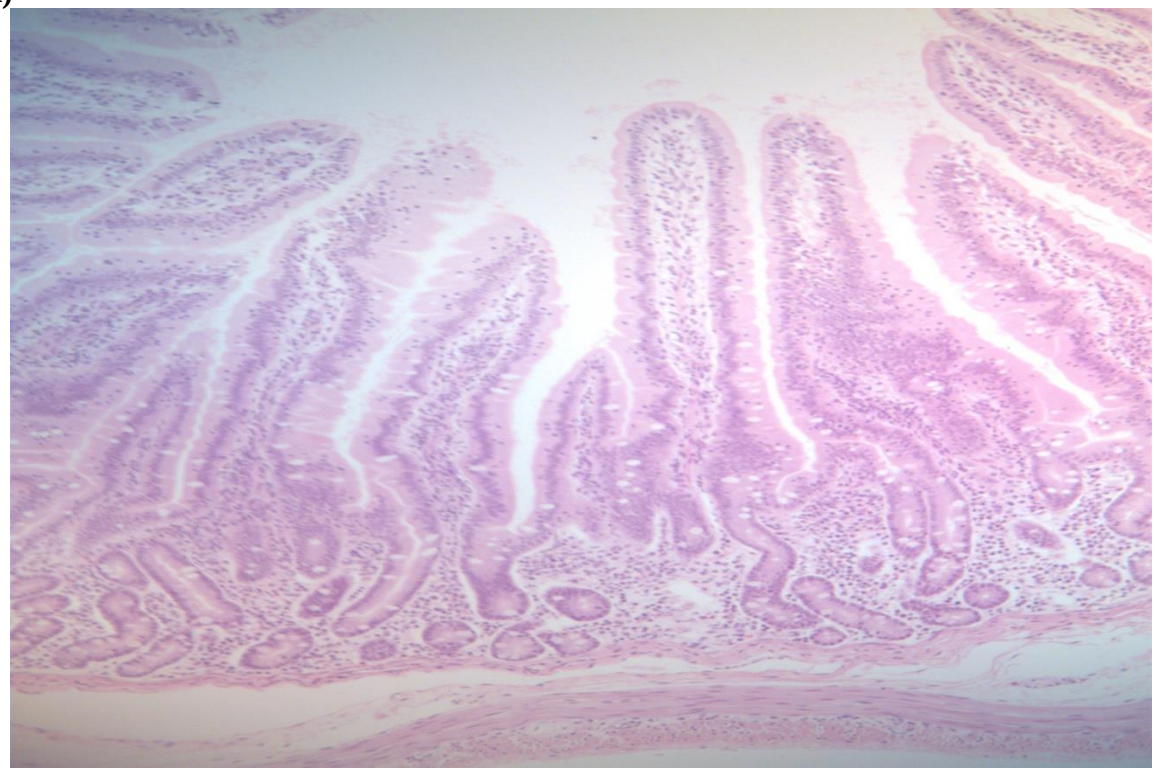
B)



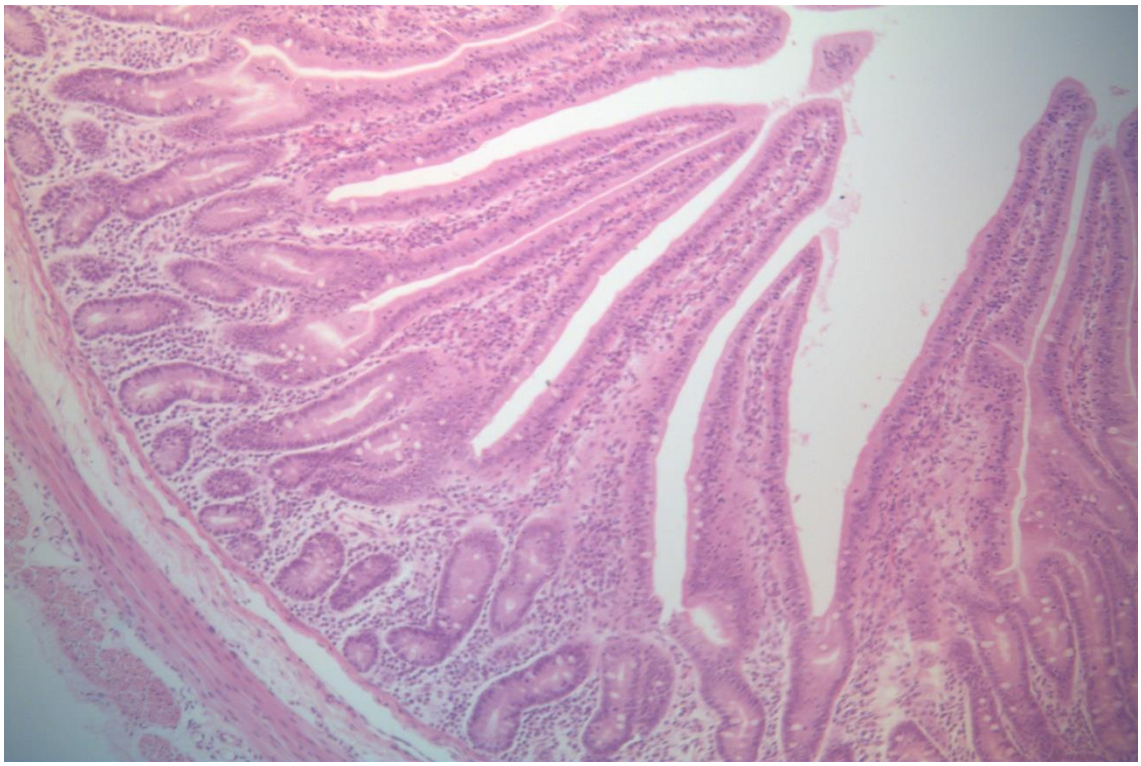
Supplementary Figure 3.3. Histology images from the duodenum of pigs fed (**A**) infant milk formula produced by membrane filtration (MEM-IMF) or (**B**) high-temperature infant milk

formula (HT-IMF) diets for 28 days. Tissue samples from the duodenum were cut into 5 μm sections, mounted on glass microscope slides and stained with haematoxylin and eosin.

A)

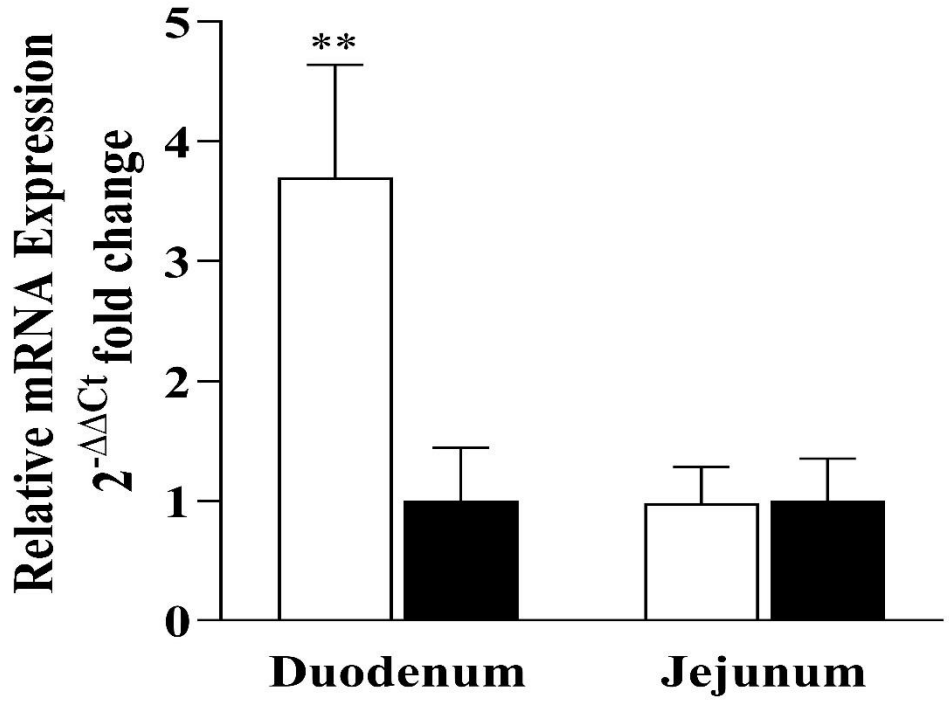


B)



Supplementary Figure 3.4. Histology images from the jejunum of pigs fed (**A**) infant milk formula produced by membrane filtration (MEM-IMF) or (**B**) high-temperature infant milk

formula (HT-IMF) diets for 28 days. Tissue samples from the jejunum were cut into 5 µm sections, mounted on glass microscope slides and stained with haematoxylin and eosin.



Supplementary Figure 3.5. mRNA transcript levels of *IL-6* in the duodenum and jejunum mucosal scrapings of pigs fed infant milk formula produced by membrane filtration (MEM-IMF) (white) or high-temperature infant milk formula (HT-IMF) (black) diets for 28 days. Mucosal scrapings were collected at slaughter 3 h after final feeding. Values are reported as means ± SEM (n = 8 pigs per treatment). The relative quantification of target mRNA was calculated using the reference gene RPLP0 following the $2^{-\Delta\Delta Ct}$ method. Transcript levels are expressed as the fold change relative to the mean value of the HT-IMF (control) diet. Significant differences between diets for *IL-6* at each gut location are indicated by * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. *IL-6, interleukin 6*.

Chapter 4

Intestinal mucus *in vitro* response to membrane filtration processing of infant milk formula

A version of this chapter was submitted to the International Dairy Journal as:

Dold, C. A., Sahin, A. W., Lawlor, P. G., Hickey, R. M., & Giblin, L. (2025). Employing membrane filtration technology during Infant Milk Formula processing, will improve intestinal barrier function *in vitro*. *International Dairy Journal*, 106283. https://doi.org/10.1016/j.idairyj.2025.106283

Published version of this chapter is presented in Appendix B.

Abstract

Several preclinical studies observed that lowering thermal loads during infant milk formula (IMF) manufacturing has beneficial effects on the immature gut of the infant. To further unravel this benefit, the objective of this study was to determine if IMF produced by membrane filtration (MEM-IMF) can provide health benefits to an *in vitro* model of the gut barrier (Caco-2/HT29-MTX). *Ex vivo* and *in vitro* gastrointestinal digested samples of MEM-IMF, protected monolayers from *Salmonella enterica* adhesion and increased mucin-2 protein, tight junction proteins claudin-1 and occludin. The bioaccessible MEM-IMF peptide, α_{s1}-casein ^{23}FFVAP^{27}, increased mucin-1 and mucin-2 protein and mRNA transcripts of *mucin-5AC, Krüppel-like factor 4* and *atonal BHLH transcription factor 1*. To conclude, reducing thermal loads during IMF processing offers beneficial effects to mucus in the small intestine.

Keywords: Infant milk formula, membrane filtration, gut barrier health, mucus and bioactive peptides.

4.1. Introduction

The human small intestine has a total absorptive surface area of 30 m^2 (Helander & Fändriks, 2014), due to its villi and crypt configuration. The intestinal barrier consists primarily of enterocytes, goblet cells, enteroendocrine cells and Paneth cells (Yao et al., 2024). The barrier not only absorbs nutrients, but serves as a defensive barrier against pathogens (Yao et al., 2024). Tight junction integrity between neighboring cells ensures barrier integrity, with zonula occludens-1 (*ZO-1*) involved in connecting claudin-1 (CLDN-1) to occludin (OCLN) (Niessen, 2007). Enterocytes are absorptive cells and predominate in the adult small intestine (Demers-Mathieu, 2022). Goblet cells increase distally from 4% in the duodenum to 12% in the ileum (Karam, 1999), enteroendocrine cells make up 2% and Paneth cells account for 3 - 8% of total epithelial cells (Demers-Mathieu, 2022). Paneth cells are secretory cells located in the crypt and play a role in barrier maintenance (Demers-Mathieu, 2022). Enteroendocrine cells (K cells, L cells, I cells) respond to food intake by secreting incretin and satiety hormones (such as glucagon-like peptide 1 (GLP-1), cholecystokinin, peptide YY and gastric inhibitory polypeptide) (Gribble & Reimann, 2019). Goblet cells produce a single layer of mucus in the small intestine (Herath et al., 2020) that acts as a barrier to limit pathogenic contact with the intestinal barrier (Zarepour et al., 2013). Mucins, mucin-2 (MUC-2), mucin-5AC (MUC-5AC), mucin-5B and MUC-6 are key gel forming glycoproteins found within the mucus layer, with MUC-2 predominating in the small intestine and MUC-5AC in the stomach (Bäckström et al., 2013). Mucin-1 (MUC-1), mucin-3 and mucin-4 are transmembrane mucins, with MUC-1 located on the apical surface of both enterocytes and goblet cells (Li et al., 2019). Krüppel-like factor 4 (KLF4) and atonal BHLH transcription factor 1 (ATOH1) transcription factors regulate goblet cell maturation and differentiation (Katz et al., 2002; Shroyer et al., 2007).

The infant small intestine is immature with lower numbers of epithelial cells (Demers-Mathieu, 2022), lower villus height, reduced tight junction expression and higher intestinal permeability

(Gleeson et al., 2021) compared to the adult. In newborn infants, the intestinal barrier is considered 'leaky' allowing enterocytes to absorb nutrients, but also larger compounds (i.e. lactulose) to cross the barrier (Camilleri, 2019). Therefore, promoting faster gut closure of the infant intestinal barrier could reduce intestinal permeability. The mucosal layer strengthens with age, with 19 times less goblet cells observed in 12 day old rats compared to 24 day old rats (Cummins et al., 1988). Fasting plasma GLP-1 concentrations are higher in newborn infants (79.1 pmol/l) compared to adults (19 pmol/l) (Padidela et al., 2008).

Maturity of the infant gut barrier is promoted by human breast milk. Preterm infants fed breast milk had significantly lower intestinal permeability compared to infants fed infant milk formula (IMF) at two weeks postpartum ($P = 0.02$) (Taylor et al., 2009). Breast milk reduces pathogenic adhesion and invasion to epithelial cells *in vitro* (Hall et al., 2013). At 28 days old, breastfed piglets had significantly lower postprandial GLP-1 levels compared to piglets fed an infant formula ($P < 0.05$) (Le Huërou-Luron et al., 2010). Many babies consume IMF as an alternative or in addition to breast milk. Thermal processing is essential to produce a safe product but leads to denaturation and aggregation of native milk proteins, reducing enzyme accessibility and protein digestibility (van Lieshout et al., 2020). Recently, attention has switched to protecting native milk protein structure in IMF by reducing thermal loads (Lund et al., 2021) and using alternative, membrane filtration technology (Chen et al., 2021; Yu et al., 2021). In animal intervention studies, lowering heat treatment during IMF manufacturing led to significant increases in small intestinal villus height (Li et al., 2013), decreases in crypt depth (Sun et al., 2022), increases in goblet cells and mucus biomarkers (Dold et al., 2024; Navis, Muncan, et al., 2020) and reductions in intestinal permeability (Li et al., 2013; Navis, Schwebel, et al., 2020).

Previously, in our work, young pigs fed a diet with IMF produced by membrane filtration (MEM-IMF) had significantly higher goblet cell numbers, MUC-2 protein and acidic mucus

levels in the jejunum compared to pigs fed a diet with heat-treated IMF (HT-IMF) ($P < 0.05$) (Dold et al., 2024). MEM-IMF powder had 59.9% native whey protein compared to 4.5% in HT-IMF, which resulted in significantly higher concentrations of water-soluble proteins and peptides in the stomach, jejunum and ileum, suggesting faster protein digestion in the young pig ($P < 0.001$) (Chen et al., 2023). Other studies have attributed physiological gut benefits to IMF produced with reduced thermal loads to a reduction in Maillard reaction compounds (Aasmul-Olsen et al., 2024; Sun et al., 2022). However, MEM-IMF and HT-IMF powders had similar levels of N(6)-carboxymethyllysine (Chen et al., 2021) suggesting differences observed in mucus biomarkers may be due to differences in peptide profiles or bioactive capacity. Liquid chromatography-mass spectrometry (LC-MS)/MS analysis identified 112 unique peptides released from MEM-IMF, and 97 unique peptides released from HT-IMF at the end of the intestinal phase *in vitro* (Bavaro et al., 2021). Wada and Lönnerdal (2015) also reported peptidomic differences post *in vitro* gastrointestinal digestion of a hydrolysed IMF powder (9 bioactive peptides) versus a standard IMF powder (39 bioactive peptides) (Wada & Lönnerdal, 2015). Other studies have demonstrated that individual milk peptides can alter gut mucus. β-casomorphin-7 (BCM-7) ^{60}YPFPGPI^{66}, a milk opioid peptide from β-casein (β-CN), released during gastrointestinal digestion of IMF (De Noni, 2008) can increase mucus secretion via the μ-opioid receptor pathway but loss of mucus bioactivity was observed in the presence of opioid antagonists (Claustre et al., 2002; Trompette et al., 2003; Zoghbi et al., 2006).

The objective of this study was to investigate if jejunal lumen samples from young pigs fed MEM-IMF could modulate mucus and other health biomarkers in the *in vitro* gut barrier (Caco-2/HT29-MTX model), in line with our pre-clinical observations. Comparison of *in vitro* with *in vivo* data is critical in the optimisation and validation of *in vitro* models to reduce dependency on animal trials. Our hypothesis is that *ex vivo* jejunal MEM-IMF and *in vitro* MEM-IMF

digesta, will increase mucus and promote tight junction integrity, protecting the *in vitro* gut barrier against pathogenic bacteria adhesion.

4.2. Materials and Methods

4.2.1. Materials

Promega CellTiter 96® AQueous One Solution Cell Proliferation Assay reagent (Cat. No. G3580) was purchased from MyBio (Ireland). Syringe filters (0.22 µm) (Cat. No. 83.1826.001) and 12-well Transwell polyethylene terephthalate membrane inserts (Cat. No. 83.3931.041) were purchased from Sarstedt (Ireland). RIPA Lysis and Extraction Buffer (Cat. No. 89900), Halt Protease and Phosphatase Inhibitor Cocktail (100X) (Cat. No. 78440) and Pierce™ bicinchoninic acid (BCA) Protein Assay Kit (Cat. No. 23225) were purchased from Thermo Fisher Scientific (USA). RNeasy Mini Kit (Cat. No. 74104) and RNase-Free DNase Set (Cat. No. 79254) were obtained from Qiagen (Germany). SensiFAST cDNA Synthesis Kit (Cat. No. BIO-65053) was from Meridian Bioscience (USA). Oligonucleotide primers were synthesised by Eurofins Genomics (Germany). LightCycler 480 SYBR Green I Master (Cat. No. 04707516001) was from Roche Diagnostics (Germany). Trans-Blot Turbo Transfer System, with polyvinylidene difluoride membranes, was obtained from Bio-Rad (USA). Target primary antibodies for western blot analysis were all purchased from Invitrogen (USA); rabbit claudin-1 (CLDN-1) polyclonal antibody (Cat. No. 51-9000), rabbit OCLN polyclonal antibody (Cat. No. 71-1500), mouse ZO-1 monoclonal antibody (ZO1-1A12) (Cat. No. 33-9100), Armenian hamster mucin-1 (MUC-1) monoclonal antibody (MH1 (CT2)) (Cat. No. MA5-11202) and rabbit MUC-2 polyclonal antibody (Cat. No. PA5-103083). Housekeeping mouse monoclonal β-actin antibody (8H10D10) (Cat. No. 3700) was from Cell Signalling Technology (USA). Goat anti-Armenian hamster IgG Secondary Antibody, horseradish peroxidase (Cat. No. PA1-32045) was purchased from Invitrogen (USA). CDP-Star® Chemiluminescent Substrate (Cat. No. WP20002) and SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Cat. No. 34580) were from Thermo Fisher Scientific (USA). Human MUC-5AC ELISA Kit (Cat. No. HUEB0842) was from AssayGenie (Ireland). MILLIPLEX Mouse Metabolic Hormone

Expanded Panel for active GLP-1 (Cat. No. MMHE-44K) was purchased from Merck Millipore (Ireland). All other reagents were supplied by Sigma-Aldrich (Ireland) unless stated otherwise.

4.2.2. Jejunum lumen samples

Jejunal lumen samples were collected during a dietary intervention study in young pigs described by Chen et al. (2023). In summary, 250 kg IMF was produced at pilot plant scale by membrane filtration (Chen et al., 2021; Chen et al., 2023). MEM-IMF and HT-IMF powders had identical ingredients and were categorised as 'stage one' formulae with protein, lactose and fat ratios of 1.29:7:3.49 and a whey to casein ratio of 60:40. MEM-IMF and HT-IMF powders were manufactured independently in a single batch (250 kg). For dietary intervention, 20 pigs at weaning (28 day old) were fed a starter diet containing (1) MEM-IMF or (2) HT-IMF at 35% inclusion for 28 days (n = 10 pigs per treatment). Jejunal lumen samples were collected 3 h after final feeding (2.5 m caudal from pyloric junction). Samples were freeze dried for 7 days at -50 °C and were then ground using a pestle and mortar. Samples were stored at 4 °C until further analysis. Samples were reconstituted at 10% (*w/v*) in distilled H_2O and agitated overnight at 4 °C. Total protein content in jejunal lumen samples was determined using the LECO FP-528 instrument (LECO Corporation, USA) applying a nitrogen-to-protein conversion factor of 6.25. Each measurement was performed in triplicate on different days. Samples were kept at -20 °C until further analysis.

4.2.3. Infant static *in vitro* simulated gastrointestinal digestion

Static *in vitro* simulated gastrointestinal digestion (SGID) mimicking that in the infant was performed as described previously (Bavaro et al., 2021; Ménard et al., 2018). In brief, 2.25 g MEM-IMF or HT-IMF powder was mixed with 5 ml distilled H_2O. Reconstituted IMF was mixed with simulated gastric fluid at a ratio (*v/v*) of 63 to 37. Simulated gastric fluid was made

up of 94 mM NaCl, 13 mM KCl, pH 5.1. Gastric digestion was performed using pepsin and lipase from rabbit gastric extract (Lipolytech, France) based on a pepsin activity of 268 U/ml. Gastric digestion was performed for 60 min at 37 °C on a Stuart SB3 rotator (VWR International, USA) at 15 rpm. For intestinal digestion, pH was raised to 7 with addition of simulated intestinal fluid to gastric digesta at a ratio (*v/v*) of 61 to 39. Simulated intestinal fluid was composed of 164 mM NaCl, 10 mM KCl, 85 mM $NaHCO_3$, pH 7. $CaCl_2$ (3 mM) was added separately at the start of intestinal phase. Intestinal digestion was performed using pancreatin with a trypsin activity of 16 U/ml and bovine bile at a final concentration of 3.1 mM. Digestion in the intestinal phase was continued for a further 60 min at 37 °C on the Stuart SB3 rotator (VWR International, USA) at 15 rpm. To cease intestinal digestion, 20 µl protease (Pefabloc, Roche Diagnostics, Germany) and lipase (Orlistat) inhibitors, prepared directly in ethanol, were added at a concentration ratio of 100 mM:10 mM per ml digesta. Infant SGID was performed in triplicate on different days. An SGID control was included, using H_2O instead of IMF. Protein content in I60 MEM-IMF and I60 HT-IMF were determined using the Pierce™ BCA Protein Assay Kit. Samples were stored at -20 °C until further analysis.

4.2.4. Synthesised peptides

Bavaro et al. (2021) reported on individual peptides, identified by LC-MS/MS, in the apical chambers of Caco-2 monolayers treated with *in vitro* gastrointestinal digested MEM-IMF (4 h, 200 µg protein/cm^2) (Bavaro et al., 2021). Peptide Ranker Scores were calculated to identify potentially bioactive peptides (Mooney et al., 2012). The milk bioactive peptide database (available at https://mbpdb.nws.oregonstate.edu/) was employed to identify peptides with proven bioactivity (Nielsen et al., 2017). SwissTargetPrediction web tool was then used to identify peptides that potentially interacted with µ-opioid receptors (Daina et al., 2019). From this *in silico* analysis, five peptides were selected for synthesis. Peptides were synthesised using microwave-assisted solid phase peptide synthesis performed on a Liberty Blue microwave

peptide synthesiser (CEM Corporation, USA). Peptides α_{s2}-casein (α_{s2}-CN) ^{100}YQGPI^{105}, BCM-7 and β-CN ^{62}FPGPI^{66} were synthesised using Fmoc-L-Ile Wang resin (Matrix Innovation, Canada). Peptide α_{s1}-casein (α_{s1}-CN) ^{143}AYFYPE^{148} was synthesised with a Fmoc-Glu(OtBu)-Wang resin (CEM Microwave Technology Ltd., UK) and α_{s1}-CN ^{23}FFVAP^{27} was synthesised with a Novobiochem® H-Pro-2-ClTrt resin (Merck Millipore, Ireland). Crude peptides were then purified using reverse-phase HPLC on a semi preparative Jupiter C18 (10 × 250mm, 5µ, 300Å) column (Phenomenex, UK) running gradients suited to individual peptides where mobile phase A was 0.1% TFA and mobile phase B was 100% acetonitrile containing 0.1% TFA. HPLC fractions containing the desired molecular mass were identified using Matrix Assisted Laser Deionisation Time of Flight mass spectrometry (MALDI-TOF MS) on an iD^{plus} Performance MALDI-TOF MS (Shimadzu Europa GmbH, Germany) and were pooled and lyophilized on a Genevac HT 4X lyophilizer (Genevac Ltd., UK).

For cell culture assays, a stock solution (10 mM in Hanks' Balanced Salt Solution (HBSS)) of each individual peptide was prepared. Stock solutions were stored at -20 °C. Prior to cell culture experiments, stock solutions were thawed at 37 °C, before dilution in HBSS to required concentrations (pH 7).

4.2.5. Eukaryotic cell and bacterial strain culturing conditions

The human intestinal epithelial cell line, Caco-2 (Cat. No. HTB-37), human colorectal adenocarcinoma cell line, HT29-MTX (Cat. No. HTB-38) and murine intestinal neuroendocrine cell line, STC-1 (Cat. No. CRL-3254) were obtained from the American Type Culture Collection (ATCC) (USA). Cells were grown in 75 cm^2 cell culture flasks in a humidified incubator maintained at 37 °C and 5% CO_2. HT29-MTX or Caco-2/HT29-MTX co-cultures were routinely cultured in 'complete Dulbecco's Modified Eagle Medium (DMEM) media' (4.5 g/L glucose, 2 mM L-glutamine) (Cat. No. D5796) supplemented with

10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin, 100 mg/ml streptomycin and 1% non-essential amino acids. Where Caco-2 cells were cultured on their own, non-essential amino acids were not included as a supplement. STC-1 cells were also cultured in complete DMEM without non-essential amino acids. At ≥ 80% confluence, cells were passaged by trypsinisation with 0.25% Trypsin-EDTA. All experiments were performed with passage numbers 19 - 30 for Caco-2, 55 - 70 for HT29-MTX and 15 - 25 for STC-1.

*S. enteric*a subsp. *enterica* serovar Typhimurium (ATCC BAA185) was obtained from ATCC (USA). *S enterica* stock was removed from -80 °C storage, streaked on brain heart infusion (BHI) agar plates and incubated overnight at 37 °C under aerobic conditions. Single colonies were isolated and inoculated directly in 10 ml BHI broth.

4.2.6. Cell viability assays

For cell viability assays, Caco-2/HT29-MTX (90:10) (for jejunum lumen and I60 IMF) or HT29-MTX (for synthetic peptides) were seeded in 96-well plates at a seeding density of 5×10^4 cells/well (100 µl) for 24 h at 37 °C and 5% CO_2. The following day, spent media was aspirated and cells were washed twice with phosphate buffered saline (PBS) (100 µl). Jejunum lumen and I60 IMF samples were prepared by filter sterilisation, using 0.22 µm filters, and dilution in complete DMEM media. Cells were treated for 4 h at 37 °C and 5% CO_2 with diluted jejunum lumen or I60 IMF (100 µl) to give a final protein concentration of 10 to 200 µg protein/cm^2. HT29-MTX cells were exposed to synthetic MEM-IMF peptides diluted in HBSS (0.1 - 2.5 mM) for 2 h at 37 °C and 5% CO_2. Following incubation, 20 µl CellTiter 96® AQueous One Solution Cell Proliferation Assay reagent was directly added to wells and incubated for an additional 1 h at 37 °C and 5% CO_2. Absorbance was measured at 490 nm using a BioTek Synergy HT microplate reader (BioTek, USA). Cell viability was calculated as

percentage of untreated cells (complete DMEM media or HBSS, assigned 100% viability). Cells with a viability ≤ 80% were considered cytotoxic.

4.2.7. Differentiated Caco-2/HT29-MTX co-culture

Caco-2/HT29-MTX co-culture was seeded at a ratio of 90:10 on 12-well Transwell polyethylene terephthalate membrane inserts at a seeding density of 6×10^4 cells/insert. Apical insert received 500 µl complete DMEM media and the basolateral chamber received 1.5 ml. To avoid multilayer formation, the day after initial seeding, spent media was removed and replaced with fresh complete DMEM media (Hubatsch et al., 2007). Co-culture was incubated for 21 days at 37 °C and 5% CO_2 to form a fully differentiated, polarised monolayer. Media was routinely replaced every two days. Monolayer integrity was monitored on day 7, day 14 and day 21 using a Millicell® ERS-2 Voltohmmeter (Merck Millipore, Ireland) to measure transepithelial electrical resistance (TEER). A blank (no cells, complete DMEM media only) membrane insert was included with all plates to account for background resistance.

On experimental day (day 21), only monolayers with TEER values ≥ 800 $\Omega.cm^2$ were used. Monolayers were gently washed with PBS and incubated for 30 min at 37 °C and 5% CO_2 in complete DMEM media to acclimatise monolayers. Jejunal lumen samples and I60 IMF were thawed at 37 °C and diluted to 200 µg protein/cm^2 in complete DMEM media. Test samples (500 µl, 200 µg protein/cm^2) were added to the apical compartment and 1.5 ml complete DMEM was added to the basolateral compartment. TEER was recorded at 0 h. Following a 4 h incubation, TEER values were again recorded. Caco-2/HT29-MTX monolayers were collected for either total RNA or cellular protein extraction. For total RNA extraction, monolayers were washed twice with ice-cold PBS (500 µl). RLT lysis buffer (1% β-mercaptoethanol) (350 µl) from the RNeasy Mini Kit was added and monolayers were agitated on ice for 10 min. Cell lysates were scraped from the membrane using a pipette tip and

transferred to sterile Eppendorf tubes. Lysates were snap-frozen in liquid nitrogen and transferred to -80 °C storage. For cellular protein extractions, monolayers were washed twice with 500 µl ice-cold PBS buffer. RIPA buffer (250 µl), with 1% Halt Protease and Phosphatase Inhibitor Cocktail, was then added to lyse monolayers. Cell lysates were removed from Transwell membrane inserts using a pipette tip and transferred to sterile Eppendorf tubes for storage at -80 °C until analysis by western blot or ELISA.

4.2.8. Mucosal barrier of HT29-MTX cells

HT29-MTX cells were seeded on 12-well Transwell polyethylene terephthalate membrane inserts at a final seeding density of 6×10^4 cells/insert in complete DMEM media. Apical insert was supplemented with 500 µl complete DMEM media with the basolateral chamber receiving 1.5 ml. Cells were incubated for at least 12 h at 37 °C and 5% CO_2 before spent media was removed and replaced with fresh complete DMEM media. Cells were allowed differentiate into monolayers for 21 days at 37 °C and 5% CO_2. Complete DMEM media was routinely replaced every 2-3 days over this 21-day period. On day 20, fresh complete DMEM media was added to monolayers.

Monolayer integrity was monitored on experimental day (day 21) and only monolayers with TEER values $\geq 200\ \Omega.cm^2$ were utilised for ensuing experiments (Martínez-Maqueda et al., 2015). Monolayers were gently rinsed once with PBS and incubated in HBSS for 30 min at 37 °C and 5% CO_2 to allow acclimatisation. Synthetic MEM-IMF peptides were thawed and diluted in pre-warmed HBSS to 0.5 mM. Peptides (0.5 mM, 500 µl) were added to the apical chamber and HBSS (1.5 ml) was added to the basolateral chamber. TEER values were recorded before (0 h) and after (2 h) incubation at 37 °C and 5% CO_2. HT29-MTX monolayers were collected for either total RNA (using RLT lysis buffer) or cellular protein extraction (using RIPA buffer).

4.2.9. RNA extraction and cDNA synthesis from cell lysate

Cell lysates for RNA extraction were thawed on ice and vortexed vigorously using a Vortex-Genie 2T (Scientific Industries Inc., USA) for 1 min. Total RNA extraction was performed using the RNeasy Mini Kit (Qiagen, Germany), including a DNase digestion step. Total RNA concentrations were quantified spectrophotometrically using the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, USA). A quality control check of 260/280 absorbance ratio of 1.8 - 2.1 was imposed. Reverse transcription from 1 µg RNA to cDNA was performed in a final reaction volume of 20 µl, using the SensiFAST cDNA Synthesis Kit (Meridian Bioscience, USA).

4.2.10. Quantification of mRNA transcript levels by reverse transcription quantitative real-time PCR (RT-qPCR)

Primers used are listed in Supplementary Table 4.1. Primer amplification efficiencies were between 95 and 110% determined using 10-fold dilutions of cDNA. RT-qPCR analyses was performed according to the MIQE guidelines (Bustin et al., 2009). For RT-qPCR, cDNA samples were thawed on ice. RT-qPCR was performed with a LightCycler® 96 instrument (Roche Diagnostics, Germany) and the LightCycler 480 SYBR Green I Master kit. RT-qPCR was performed to a final reaction volume of 10 µl; 1 µl cDNA (10-fold dilution), 0.5 µl forward primer, 0.5 µl reverse primer, 5 µl SYBR Green and 3 µl RNase free H_2O. Thermal cycling protocol was 10 min pre-incubation at 95 °C, followed by 45 cycles of three step RT-qPCR with 10 s denaturation at 95 °C, 10 s annealing at primer specific annealing temperature (57 °C or 60 °C) and extension for 30 s at 72 °C. A melting curve analysis was included with all plates, to indicate single product melting. Homo sapiens ribosomal protein lateral stalk subunit P0 (RPLP0) was selected as the reference gene. The relative amount of target mRNA transcripts

was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). Data is presented as fold changes relative to untreated monolayers (complete DMEM media or HBSS).

4.2.11. Quantification of tight junction and mucin proteins by western blot or ELISA

Cell lysates were thawed at room temperature, vortexed using a Vortex-Genie 2T (Scientific Industries Inc., USA) and sonicated twice for 2 min on ice using a Clifton SW3H ultrasonic bath (Nickel-Electro Ltd., UK). Homogenised cell lysates were centrifuged at 16000 × *g* for 20 min at 4 °C to pellet cell debris. Supernatant was collected and protein concentration was quantified using the Pierce™ BCA Protein Assay Kit. Samples (25 µg protein) were mixed with an equal volume of 2 X Laemmli buffer (50 mM dithiothreitol), heated for 10 min at 95 °C and loaded on 4 - 15% w/v stain-free polyacrylamide gels (Bio-Rad, USA). NuPAGE™ Antioxidant (Invitrogen, USA) (500 µl) was added to SDS-PAGE running buffer to keep proteins under reducing conditions. SDS-PAGE was performed with an initial voltage of 50 V for 5 min followed by a constant voltage at 150 V. Separated proteins were transferred onto a polyvinylidene difluoride membrane using the Trans-Blot Turbo Transfer System (Bio-Rad, USA).

Washing and incubation steps were performed at 4 °C under gentle agitation at 50 rpm using an orbital plate shaker (Allsheng, China). The membrane was washed with 20 ml distilled H_2O and blocked for 2 h in 5% bovine serum albumin (BSA). Prior to antibody addition, the membrane was washed three times with 20 ml distilled H_2O for 10 min. The membrane was then incubated overnight with 10 ml primary antibody diluted in 2% BSA for either CLDN-1 (1:5000), OCLN (1:5000), ZO-1 (1:2000), MUC-1 (1:1000) or MUC-2 (1:5000). Membrane was then washed three times with 20 ml Tris-buffered saline, 0.1% Tween 20 and incubated for a further 2 h with 10 ml anti-rabbit or anti-mouse IgG secondary antibody, alkaline phosphatase (1:10000) or anti-Armenian hamster IgG secondary antibody, horseradish

peroxidase (1:10000) in 2% BSA. Membrane was washed with 20 ml Tris-buffered saline, 0.1% Tween 20 and rinsed with distilled H_2O. CDP-Star® Chemiluminescent Substrate (3ml) was added to the membrane for chemiluminescence detection of alkaline phosphatase conjugated anti-rabbit or anti-mouse IgG secondary antibody. SuperSignal™ West Pico PLUS Chemiluminescent Substrate (3 ml) was added to membranes for the detection of the horseradish peroxidase conjugated anti-Armenian hamster IgG secondary antibody. Immunoreactive proteins were visualised using the Vilber FUSION Solo S western blot imaging system (Vilber, France). Separate membranes were used for each individual antibody. Each membrane was stripped for follow-up probing with the housekeeping mouse monoclonal β-actin antibody. Stripping occurred at room temperature using mild stripping buffer; 1.5% (w/v) glycine, 0.1% (w/v) SDS, 1% (v/v) Tween 20, pH 2.2. Membrane was immersed twice in 20 ml stripping buffer for 10 min. The membrane was then washed twice with PBS and twice with Tris-buffered saline, 0.1% Tween 20 for 10 min. Finally, the membrane was blocked before proceeding to overnight incubation with housekeeping mouse monoclonal β-actin antibody (1:5000). In this case, anti-mouse IgG secondary antibody, alkaline phosphatase (1:10000) was used. Densitometric analysis was performed using the GelAnalyzer 19.1 software (available at http://www.gelanalyzer.com by Istvan Lazar Jr., PhD and Istvan Lazar Sr., PhD, CSc) with normalisation against β-actin signal.

MUC-5AC protein concentrations were determined using an ELISA kit (AssayGenie, Ireland). Absorbance at 450 nm was measured using a BioTek Synergy HT microplate reader (BioTek, USA). MUC-5AC concentrations (ng/ml) were calculated using a 4-parameter logistic standard curve generated with MyAssays online software (MyAssays Ltd., UK). This ELISA kit detects MUC-5AC at a minimum detection limit of 0.78 ng/ml.

4.2.12. Bacteria adhesion assays

Bacteria adhesion assays were performed as described previously by Morrin et al. (2019), with slight modifications. HT29-MTX cells were seeded in 12-well Corning CellBIND plates (Corning, USA) at a seeding density of 2×10^5 cells/well in a final volume of 1 ml complete DMEM media (without antibiotics) and grown for 10 days at 37 °C and 5% CO_2. Every second day, monolayers were washed with PBS and media was replenished.

To prepare the bacterial inoculum, a single pure colony of *S. enterica* was inoculated overnight in 10 ml BHI broth at 37 °C under aerobic conditions. *S. enterica* was sub-cultured at least once in 10 ml BHI broth before use. Bacterial inoculum (1 ml) was centrifuged at 16000 × *g* for 2 min. The pellet was washed with 1 ml PBS and centrifuged again at 16000 × *g* for 2 min. The pellet was resuspended in 1 ml complete DMEM media (without antibiotics). Colony counting for the bacterial inoculum was performed by serial dilution and spread plating (100 µl) on BHI agar plates overnight at 37 °C under aerobic conditions. On experimental day (day 10), media was removed from HT29-MTX monolayers. HT29-MTX monolayers were washed three times with PBS (500 µl) and then treated with 500 µl jejunal lumen or I60 IMF (200 µg protein/cm^2) in complete DMEM media (without antibiotics) for 4 h at 37 °C and 5% CO_2. Treatment was removed and HT29-MTX monolayers were washed three times with PBS (500 µl). *S. enterica* (600 µl) (1×10^6 colony forming units (CFU)) in complete DMEM media (without antibiotics) was added to HT29-MTX monolayers and incubated for 45 min at 37 °C and 5% CO_2 under aerobic conditions. The bacterial sample was removed, and HT29-MTX monolayers were then washed three times with PBS. Triton X100 (0.1%) was then added (500 µl) for 20 min at room temperature, to lyse HT29-MTX monolayers, releasing adherent *S. enterica*. HT29-MTX cell lysate was collected, serially diluted and spread plated (100 µl) on BHI agar overnight at 37 °C under aerobic conditions. Results were expressed as the number of adherent *S.enterica* (CFU/ml) as a percentage of the number of *S.enterica* (CFU/ml) added to HT29-MTX monolayers.

4.2.13. Active GLP-1 secretion from enteroendocrine STC-1 cells

To ensure biocompatibility of jejunal lumen and I60 IMF samples for STC-1 cell experiments, trypsin (23.3 kDa) was removed from samples using 10 kDa molecular weight cut-off Vivaspin® Turbo 4 Ultrafiltration Units (Sartorius, Germany) at 4000 × *g* for 20 min at 4 °C. Filtrate test samples were collected and immediately snap-frozen in liquid nitrogen for storage at -80 °C. STC-1 cells were seeded in 12-well plates at a seeding density of 5×10^5 cells/well in a final volume of 1 ml complete DMEM media (without non-essential amino acids). Cells were incubated for 24 h at 37 °C and 5% CO_2 before depleted media was removed and cells were washed twice with Krebs-Ringer buffer (11 mM glucose). STC-1 cells were then acclimatized with 500 µl Krebs-Ringer buffer for 30 min at 37 °C and 5% CO_2. Filtrate test samples were thawed at room temperature and protein concentrations were quantified using the Pierce™ BCA Protein Assay Kit. These test samples were diluted in Krebs-Ringer buffer to a protein concentration of 500 µg protein/ml. Test samples (500 µl) were added to STC-1 cells for 4 h at 37 °C and 5% CO_2. Immediately following treatment, 1% Halt Protease and Phosphatase Inhibitor Cocktail was added, and cell supernatants were collected. Cell supernatants were centrifuged at 900 × *g* for 5 min at 4 °C to pellet cell debris. Supernatants were snap-frozen in liquid nitrogen and stored at -80 °C. Active GLP-1 content was quantified in the supernatants using the MILLIPLEX Mouse Metabolic Hormone Expanded Panel kit (Merck Millipore, Ireland) and MagPix fluorescent detection system (Luminex, Netherlands). MILLIPLEX Kit detects active GLP-1 concentrations in the range 41 - 30,000 pg/ml.

4.2.14. Statistical analysis

All data are reported as means ± standard deviation (SD). Cell treatments and experiments were performed at least in duplicate on at least two different days. Statistical analysis was performed using IBM SPSS Statistics Version 29 (IBM Statistics, USA). Statistically significant

differences between controls, jejunal lumen and I60 IMF samples was determined using one-way analysis of variance (ANOVA) followed by post-hoc Bonferroni multiple comparison test ($P \leq 0.05$). For cell viability assays and experiments with synthetic peptides, statistically significant differences between controls and test samples were determined using one-way ANOVA followed by post-hoc Dunnett's multiple comparisons test ($P \leq 0.05$).

4.3. Results

Lumen samples were collected from the jejunum of young pigs fed MEM-IMF and HT-IMF diets, 3 h after their final meal on day 28 of an intervention trial (Chen et al., 2023). As IMF was included at 35% in the pig's diet, jejunal samples contained other dietary nutrients. Therefore, for comparison purposes and to provide 'cleaner' digesta samples, a static *in vitro* gastrointestinal digestion was performed on MEM-IMF and HT-IMF powders. The degree of protein hydrolysis in jejunal MEM-IMF and jejunal HT-IMF was 1508 ± 99 µmol free amines/g protein and 1393 ± 114 µmol free amines/g protein), respectively ($P > 0.05$) (Chen et al., 2023). The degree of protein hydrolysis at the end of the *in vitro* intestinal phase (I60) was 4673 ± 14 µmol free amines/g protein in I60 MEM-IMF and 4239 ± 98 µmol free amines/g protein in I60 HT-IMF ($P > 0.05$) (Supplementary Figure 4.1).

Initially, cytotoxicity assays were performed to assess biocompatibility of jejunal MEM-IMF, jejunal HT-IMF, I60 MEM-IMF and I60 HT-IMF with co-cultures of enterocytes (Caco-2) and goblet cells (HT29-MTX) (Supplementary Figure 4.2). Following a 4 h incubation, none of the concentrations were found to be cytotoxic to cells. Indeed, some sample concentrations, jejunal MEM-IMF (25, 50 and 100 µg protein/cm^2), jejunal HT-IMF (25, 50 and 100 µg protein/cm^2), I60 MEM-IMF (100 and 200 µg protein/cm^2) and I60 HT-IMF (100 and 200 µg protein/cm^2) had a slight, yet significant proliferative effect on co-cultures compared to media alone ($P < 0.05$). To evaluate the effect of jejunal MEM-IMF, jejunal HT-IMF, I60 MEM-IMF and I60 HT-IMF on gut barrier integrity, Caco-2/HT29-MTX co-cultures at 90:10 ratio were differentiated into polarised monolayers. A 90:10 co-culture best represented the small intestine (Karam, 1999; Pan et al., 2015). Barrier integrity, as determined by TEER, was undisturbed after 4 h incubation with jejunal MEM-IMF, jejunal HT-IMF, I60 MEM-IMF and I60 HT-IMF ($P > 0.05$) (Supplementary Table 4.2).

4.3.1. Effect of MEM-IMF on mucus biomarkers in an *in vitro* Caco-2/HT29-MTX gut barrier model

To investigate the ability of jejunal MEM-IMF and I60 MEM-IMF to promote mucus in Caco-2/HT29-MTX monolayers, mRNA transcript levels of *MUC-1*, *MUC-2* and *MUC-5AC* were measured following a 4 h treatment with jejunal MEM-IMF, jejunal HT-IMF, I60 MEM-IMF or I60 HT-IMF (Figure 4.1A). *MUC-2* and *MUC-5AC* mRNA transcript levels were significantly higher by 0.95-fold and 0.63-fold, respectively, with jejunal MEM-IMF compared to treatment with jejunal HT-IMF ($P < 0.05$). *MUC-1* transcripts were similar in monolayers treated with jejunal samples and media alone ($P > 0.05$). Interestingly, adding I60 MEM-IMF to monolayers significantly increased *MUC-1* (0.41-fold) and *MUC-2* (1.29-fold) mRNA transcript levels compared to I60 HT-IMF ($P < 0.05$). No significant differences were observed in *MUC-5AC* mRNA levels in monolayers treated with either I60 MEM-IMF or I60 HT-IMF ($P > 0.05$).

As *MUC-2* mRNA transcripts were upregulated with both jejunal MEM-IMF samples and I60 MEM-IMF samples, MUC-2 protein levels were quantified by western blotting in Caco-2/HT29-MTX monolayers (Figure 4.1B). MUC-2 protein was significantly higher in monolayers treated with I60 MEM-IMF compared to I60 HT-IMF, jejunal MEM-IMF, jejunal HT-IMF and media control ($P < 0.05$). Regardless of processing, I60 MEM-IMF and I60 HT-IMF significantly increased MUC-2 protein levels compared to media control ($P < 0.05$).

To investigate whether or not the increase in *MUC-1* mRNA transcript levels by I60 MEM-IMF, resulted in higher MUC-1 protein, MUC-1 protein was quantified in monolayer lysates by western blot (Figure 4.1C). Treatment with I60 MEM-IMF did not significantly increase MUC-1 protein compared to monolayers treated with I60 HT-IMF ($P > 0.05$), but levels were significantly higher compared to media control ($P < 0.05$).

To understand whether or not the increase in *MUC-5AC* mRNA transcripts with jejunal MEM-IMF resulted in an increase in MUC-5AC protein, MUC-5AC protein concentrations were quantified by ELISA (Figure 4.1D). There was no significant difference in MUC-5AC protein concentration in Caco-2/HT29-MTX monolayers exposed to jejunal MEM-IMF or jejunal HT-IMF (100.98 ± 2.82 ng/ml *vs* 100.33 ± 4.54 ng/ml) ($P > 0.05$). However, both jejunal IMF samples had significantly higher MUC-5AC protein levels compared to media control (88.59 ± 3.49 ng/ml) ($P < 0.05$).

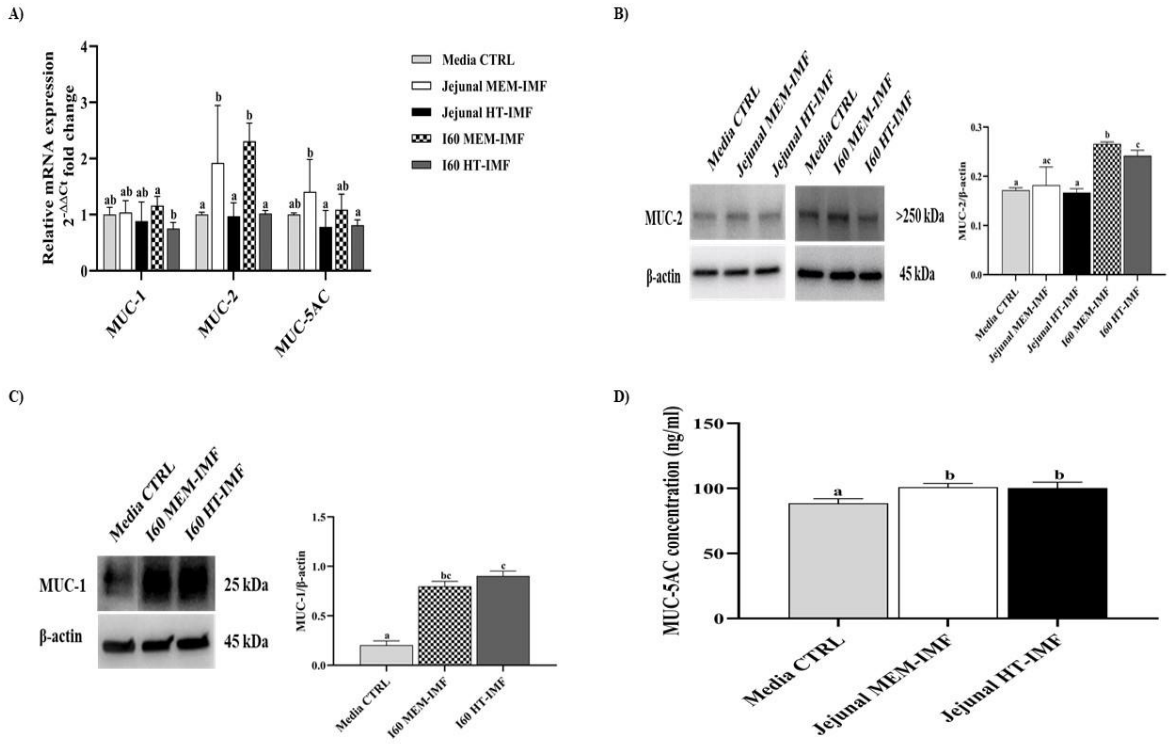


Figure 4.1. Mucus biomarkers measured in Caco-2/HT29-MTX monolayers after 4 h treatment with jejunal IMF or I60 IMF (200 µg protein/cm^2). (**A**) Relative quantification of mRNA transcript levels of mucin genes; *MUC-1*, *MUC-2* and *MUC-5AC*. Western blot analysis of (**B**) MUC-2 and (**C**) MUC-1 protein levels. (**D**) ELISA quantification of MUC-5AC protein concentrations (ng/ml). Media CTRL, complete DMEM media control. Jejunal lumen collected from at least n = 3 pigs per treatment was used. For assays with I60 IMF, n = 3 samples from different days were used. RT-qPCR analysis was performed in triplicate with technical duplicates on two different days. Values are reported as means ± SD (n = 12). Western blot analysis and ELISA was performed in triplicate on two different days. Values are reported as means ± SD (n = 6). Different letters indicate significant differences between treatment types ($P < 0.05$). MEM-IMF, infant milk formula produced by membrane filtration and HT-IMF, high-temperature infant milk formula.

4.3.2. Adhesion of *S. enterica* subsp. *enterica* serovar Typhimurium to HT29-MTX monolayers following treatment with jejunum lumen and IMF digesta

Previously, jejunal goblet cell numbers were significantly increased by MEM-IMF dietary intervention (Dold et al., 2024), so to assess whether or not jejunal-MEM-IMF or I60 MEM-IMF prevents adhesion of pathogenic bacteria to the mucosal barrier, bacterial adhesion assays were performed with HT29-MTX monolayers using *S. enterica* subsp. *enterica* serovar Typhimurium (Figure 4.2). Prior to pathogen exposure, differentiated HT29-MTX monolayers were treated with jejunal MEM-IMF, jejunal HT-IMF I60 MEM-IMF or I60 HT-IMF (4 h, 200 μg protein/cm^2). Pre-treatment of HT29-MTX monolayers with jejunal MEM-IMF led to significant reductions in *S. enterica* adhesion compared to monolayers treated with jejunal HT-IMF (16.71 ± 3.42% *vs* 26.73 ± 5.76%) ($P < 0.001$). Similarly, preconditioning of HT29-MTX monolayers with I60 MEM-IMF significantly reduced adhesion of *S. enterica* compared to HT29-MTX monolayers treated with I60 HT-IMF (9.62 ± 2.21% *vs* 14.06 ± 1.70%) ($P < 0.05$). Monolayers pre-treated with I60 IMF samples had a significant reduction in *S. enterica* adhesion compared to monolayers treated with jejunal HT-IMF ($P < 0.05$).

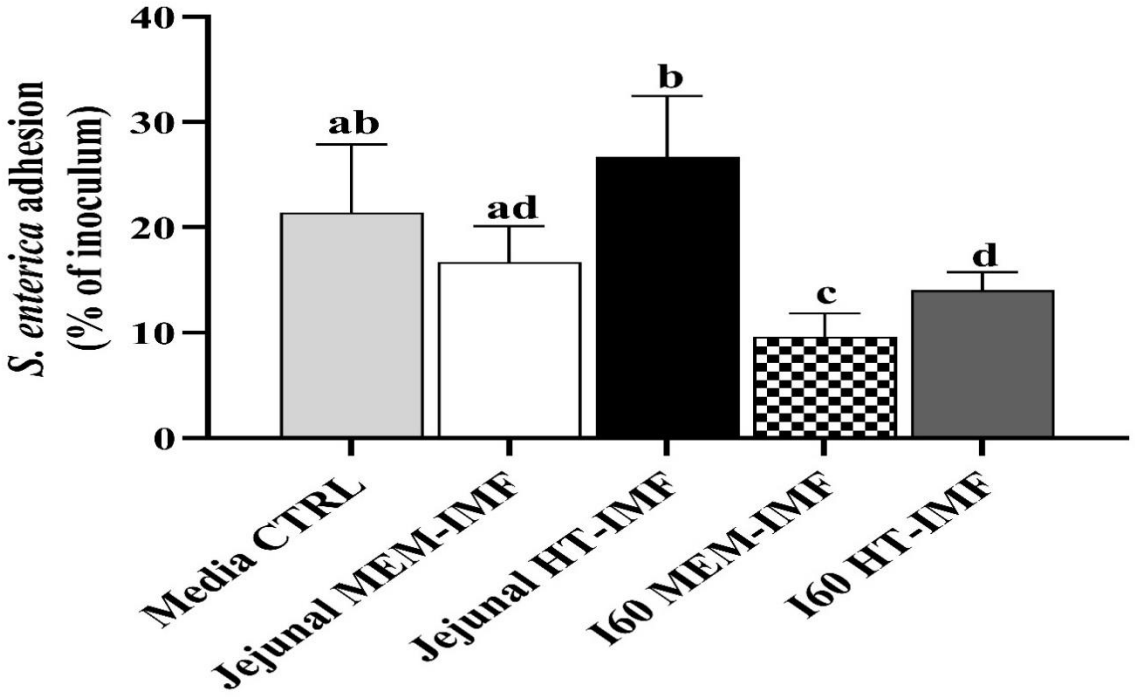


Figure 4.2. Adherence of *S. enterica* subsp. *enterica* serovar Typhimurium to HT29-MTX monolayers after pre-treatment with jejunal IMF or I60 IMF (4 h, 200 µg protein/cm^2). Results are expressed as the number of adherent bacteria as a percentage of the number of bacteria in the inoculum added. Bacterial adhesion assays were performed with technical duplicates on at least three different days. For assays with jejunal IMF at least n = 6 pigs per treatment were included. For assays with I60 IMF, n = 3 samples from different days were used. Values are reported as means ± SD. Different letters indicate significant differences between treatment types (P < 0.05). Media CTRL, complete DMEM media control, MEM-IMF, infant milk formula produced by membrane filtration and HT-IMF, high-temperature infant milk formula.

4.3.3. Tight junction integrity of Caco-2/HT29-MTX gut barrier

As our pig trial demonstrated an increase in *CLDN-1* mRNA transcripts in the pig duodenum in response to MEM-IMF diet (Dold et al., 2024), a range of tight junction biomarkers were tracked in Caco-2/HT29-MTX monolayers (Figure 4.3A). Jejunal MEM-IMF significantly increased mRNA transcript levels of *CLDN-1* (0.47-fold), *OCLN* (2.36-fold) and *ZO-1* (0.36-fold) in monolayers compared to jejunal HT-IMF ($P < 0.05$). *OCLN* and *ZO-1* mRNA levels in monolayers treated with jejunal MEM-IMF were significantly higher than untreated monolayers and all other samples ($P < 0.05$). *CLDN-1* and *OCLN* mRNA transcript levels were significantly higher by 0.54-fold and 0.38-fold, respectively, in monolayers treated with I60 MEM-IMF compared to I60 HT-IMF ($P < 0.05$). Monolayer mRNA transcript levels of *CLDN-1* and *OCLN* were significantly higher when treated with I60 MEM-IMF compared to levels in monolayers treated with media alone ($P < 0.05$).

Where differences in mRNA transcripts were noted, CLDN-1, OCLN and ZO-1 proteins were quantified by western blotting (Figures 4.3B - 4.3D). CLDN-1 and OCLN protein were significantly higher in monolayers treated with jejunal MEM-IMF compared to monolayers treated with jejunal HT-IMF ($P < 0.05$). Jejunal MEM-IMF also significantly increased OCLN protein levels compared to media control ($P < 0.05$). Interestingly, ZO-1 protein levels tended to be lower in monolayers treated with jejunal MEM-IMF compared to jejunal HT-IMF ($P = 0.07$) while no differences were observed between jejunal IMF samples and media ($P > 0.05$). Protein concentrations of CLDN-1 and OCLN in Caco-2/HT29-MTX monolayers were unaffected by treatment with media, I60 MEM-IMF or I60 HT-IMF ($P > 0.05$). There were significantly lower CLDN-1 protein in monolayers treated with jejunal HT-IMF compared to I60 HT-IMF ($P < 0.05$).

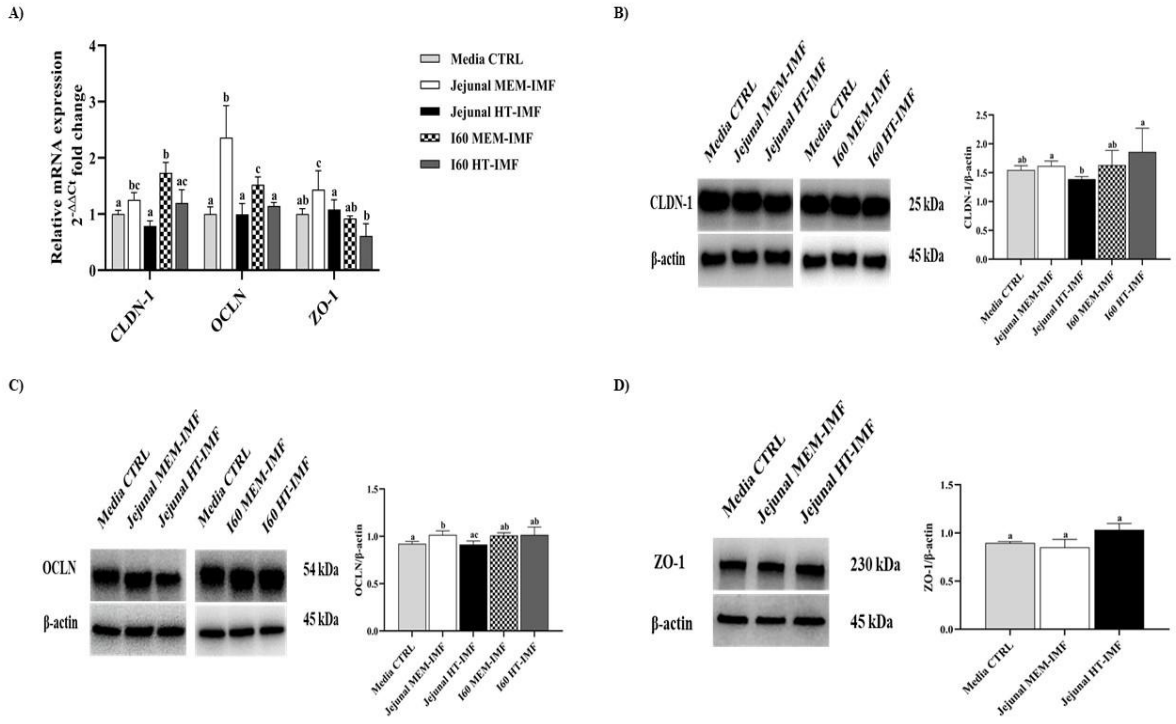


Figure 4.3. Tight junction integrity of Caco-2/HT29-MTX monolayers after treatment with jejunal IMF or I60 IMF (4 h, 200 µg protein/cm^2). (**A**) Relative quantification of mRNA transcript levels of tight junction genes; *CLDN-1*, *OCLN* and *ZO-1*. Western blot analysis of (**B**) CLDN-1, (**C**) OCLN and (**D**) ZO-1 protein levels. Media CTRL, complete DMEM media control. For assays with jejunal IMF at least n = 3 pigs per treatment were included. For assays with I60 IMF, n = 3 samples from different days were used. RT-qPCR analysis was performed in triplicate with technical duplicates on two different days. Values are reported as means ± SD (n = 12). Western blot analysis was performed in triplicate on two different days. Values are reported as means ± SD (n = 6). Different letters indicate significant differences between treatment types ($P < 0.05$). MEM-IMF, infant milk formula produced by membrane filtration and HT-IMF, high-temperature infant milk formula.

4.3.4. MEM-IMF and HT-IMF influence on active GLP-1 secretion from enteroendocrine cells

In the intestinal barrier, enteroendocrine cells respond to food, particularly dietary protein (Geraedts et al., 2011) so their response to test samples was investigated by monitoring secreted levels of active GLP-1. Any residual trypsin was removed from jejunal IMF and I60 IMF samples using a 10 kDa molecular weight cut-off as STC-1 cells are particularly sensitive to detachment (Corrochano et al., 2018). STC-1 cells treated with 10 kDa jejunal MEM-IMF resulted in secretion of 96.45 ± 35.54 pg/ml active GLP-1, similar to levels from STC-1 cells treated with 10 kDa jejunal HT-IMF (179.47 ± 139.28 pg/ml) ($P > 0.05$) (Figure 4.4). Treatment of STC-1 cells with 10 kDa I60 MEM-IMF resulted in secretion of 150.60 ± 6.94 pg/ml active GLP-1 significantly lower than 566.35 ± 150.21 pg/ml from STC-1 cells treated with 10 kDa I60 HT-IMF ($P < 0.01$) (Figure 4.4). Active GLP-1 was significantly lower from STC-1 cells treated with 10 kDa I60 MEM-IMF and 10 kDa jejunal IMF samples compared to 10 kDa I60 HT-IMF ($P < 0.05$). Active GLP-1 levels from all test samples were significantly lower than STC-1 cells in Krebs-Ringer buffer (895.36 ± 111.99 pg/ml) ($P < 0.001$).

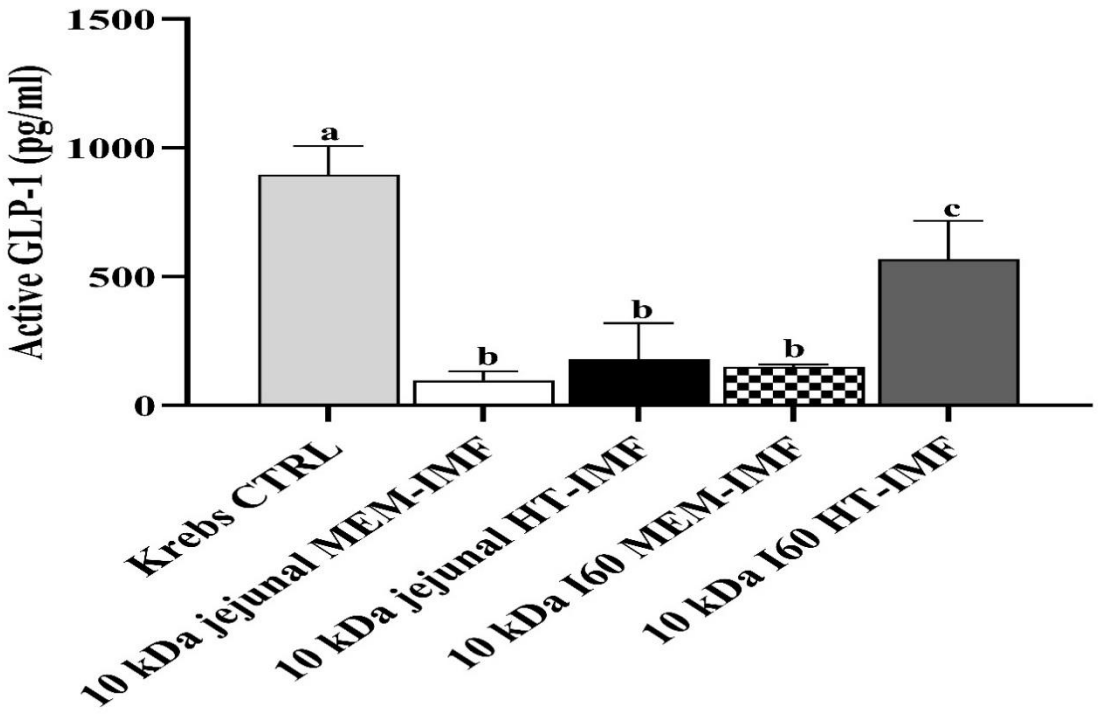


Figure 4.4. Active GLP-1 secretion (pg/ml) from STC cells exposed to 500 μg protein/ml 10 kDa jejunal IMF or 10 kDa I60 IMF. For assays with 10 kDa jejunal IMF at least n = 6 pigs per treatment were included. For assays with 10 kDa I60 IMF, n = 3 samples collected on different days were used. Active GLP-1 levels were measured with technical duplicates on two different days. Values are reported as means ± SD (n = 12). Different letters indicate significant differences between treatment types ($P < 0.05$). Untreated control (Krebs CTRL) was STC-1 cells treated with Krebs-Ringer buffer (11 mM glucose). MEM-IMF, infant milk formula produced by membrane filtration and HT-IMF, high-temperature infant milk formula.

4.3.5. Effect of MEM-IMF peptides on mucin gene expression and protein levels in the mucosal barrier

Bavaro et al. (2021) identified 112 unique peptides in the apical compartments of Caco-2 monolayers treated with I60 MEM-IMF (Bavaro et al., 2021). *In silico* analysis, using a peptide ranker score cut-off of ≥ 0.5 for bioactivity potential, reduced this number to 18. Of these 18 peptides, 4 peptides had a SwissTargetPrediction score ≥ 0.5 for potential μ-opioid receptor modulation, a receptor involved in mucus secretion (Zoghbi et al., 2006). The α_{s1}-CN peptides ^{23}FFVAP^{27} and ^{143}AYFYPE^{148}, α_{s2}-CN peptide ^{100}YQGPI^{105} and β-CN peptide ^{62}FPGPI^{66} were synthesised (Table 4.1). BCM-7 peptide was included as a positive control.

Table 4.1. Peptides identified following *in vitro* gastrointestinal digestion of MEM-IMF

Milk protein	Peptide sequence	Amino acid position	Molecular weight (g/mol)	Peptide ranker score	SwissTargetPrediction score for μ-opioid receptor
β-CN	YPFPGPI (BCM-7)	60 - 66	790.02	0.92	0.89
α_{s1}-CN	FFVAP	23 - 27	579.72	0.88	0.54
α_{s1}-CN	AYFYPE	143 - 148	788.88	0.72	0.53
α_{s2}-CN	YQGPI	100 - 105	576.96	0.56	0.64
β-CN	FPGPI	62 - 66	529.67	0.95	0.50

MEM-IMF, infant milk formula produced by membrane filtration; α_{s1}-CN, α_{s1}-casein; α_{s2}-CN, α_{s2}-casein; β-CN, β-casein.

Treatment of HT29-MTX cells with all peptides at 0.5 mM were not cytotoxic to cells compared to HBSS control (Supplementary Figure 4.3). Some peptides significantly increased cell proliferation at 1 mM (α_{s1}-CN ^{23}FFVAP^{27}), 0.5 mM, 0.25 mM and 0.1 mM (α_{s1}-CN ^{23}FFVAP^{27}, α_{s1}-CN ^{143}AYFYPE^{148} and α_{s2}-CN ^{100}YQGPI^{105}) compared to the HBSS control ($P < 0.05$). At 2.5 mM BCM-7, α_{s1}-CN ^{143}AYFYPE^{148}, α_{s2}-CN ^{100}YQGPI^{105} and β-CN ^{62}FPGPI^{66} significantly reduced cell viability compared to the control ($P < 0.05$). To evaluate the ability of peptides to modulate the mucus barrier, HT29-MTX monolayers were allowed differentiate for 21 days and then treated for 2 h with MEM-IMF peptides. β-CN ^{62}FPGPI^{66} peptide significantly increased TEER compared to HBSS control (387 ± 63 Ω.cm^2 *vs* 288 ± 38 Ω.cm^2) ($P < 0.01$) (Supplementary Figure 4.4). All other peptides had similar TEER values compared to untreated monolayers ($P > 0.05$).

BCM-7 and α_{s1}-CN ^{23}FFVAP^{27} peptides significantly increased *MUC-1* mRNA transcript levels by 5.76-fold and 2.26-fold, respectively, in HT29-MTX monolayers compared to the HBSS control ($P < 0.001$) (Figure 4.5A). Peptides from α_{s1}-CN ^{143}AYFYPE^{148}, α_{s2}-CN ^{100}YQGPI^{105} and β-CN ^{62}FPGPI^{66} had no significant effect on *MUC-1* mRNA levels compared to the control ($P > 0.05$). HT29-MTX monolayer treatment with peptides α_{s1}-CN ^{23}FFVAP^{27} (1.43-fold) ($P < 0.01$) and β-CN ^{62}FPGPI^{66} (1.32-fold) ($P < 0.05$) both significantly increased mRNA transcript levels of *MUC-2* compared to control. *MUC-2* mRNA transcript levels were unchanged in monolayers exposed to BCM-7, α_{s1}-CN ^{143}AYFYPE^{148} and α_{s2}-CN ^{100}YQGPI^{105} peptides ($P > 0.05$). *MUC-5AC* mRNA transcript levels were significantly up-regulated in HT29-MTX monolayers treated with 0.5 mM of each of the peptides; α_{s1}-CN ^{23}FFVAP^{27} (2.24-fold, $P < 0.001$), α_{s1}-CN ^{143}AYFYPE^{148} (1.81-fold, $P < 0.001$), α_{s2}-CN ^{100}YQGPI^{105} (2.18-fold, $P < 0.001$), β-CN ^{62}FPGPI^{66} (1.59-fold, $P < 0.05$) and BCM-7 (1.65-fold, $P < 0.05$) compared to monolayers in HBSS. The mRNA transcript levels of goblet cell transcription factor *KLF4* were significantly higher in HT29-MTX monolayers treated with BCM-7 (2.29-fold, $P <$

0.001), α_{s1}-CN ^{23}FFVAP^{27} (1.49-fold, $P < 0.05$), α_{s1}-CN ^{143}AYFYPE^{148} (1.44-fold, $P < 0.05$), α_{s2}-CN ^{100}YQGPI^{105} (1.78-fold, $P < 0.001$) and β-CN ^{62}FPGPI^{66} (1.43-fold, $P < 0.05$) compared to untreated monolayers (Figure 4.5B). Peptides BCM-7 (1.47-fold, $P < 0.05$), α_{s1}-CN ^{23}FFVAP^{27} (1.71-fold, $P < 0.01$), α_{s1}-CN ^{143}AYFYPE^{148} (1.64-fold, $P < 0.01$) and α_{s2}-CN ^{100}YQGPI^{105} (1.67-fold, $P < 0.01$) all significantly increased mRNA transcripts of *ATOH1* compared to untreated monolayers.

For those peptides eliciting an mRNA response, HT29-MTX MUC-1 and MUC-2 proteins were quantified by western blotting (Figures 4.5C and 4.5D). Significant increases in MUC-1 ($P < 0.01$) and MUC-2 ($P < 0.05$) were detected in in HT29-MTX monolayers treated with α_{s1}-CN ^{23}FFVAP^{27} peptide compared to untreated monolayers. BCM-7 had no significant effect on MUC-1 compared to untreated monolayers ($P > 0.05$). No significant changes were seen in HT29-MTX monolayer MUC-2 protein levels treated with the β-CN derived peptides BCM-7 or ^{62}FPGPI^{66} ($P > 0.05$). Treatment of HT29-MTX monolayers to peptides (BCM-7, α_{s1}-CN ^{23}FFVAP^{27} and ^{143}AYFYPE^{148}, α_{s2}-CN ^{100}YQGPI^{105} and β-CN ^{62}FPGPI^{66}) (81.02 ± 3.04 - 88.19 ± 2.48 ng/ml) had no significant effect on MUC-5AC protein concentrations compared to untreated monolayers (81.81 ± 3.27 ng/ml) ($P > 0.05$) (Figure 4.5E).

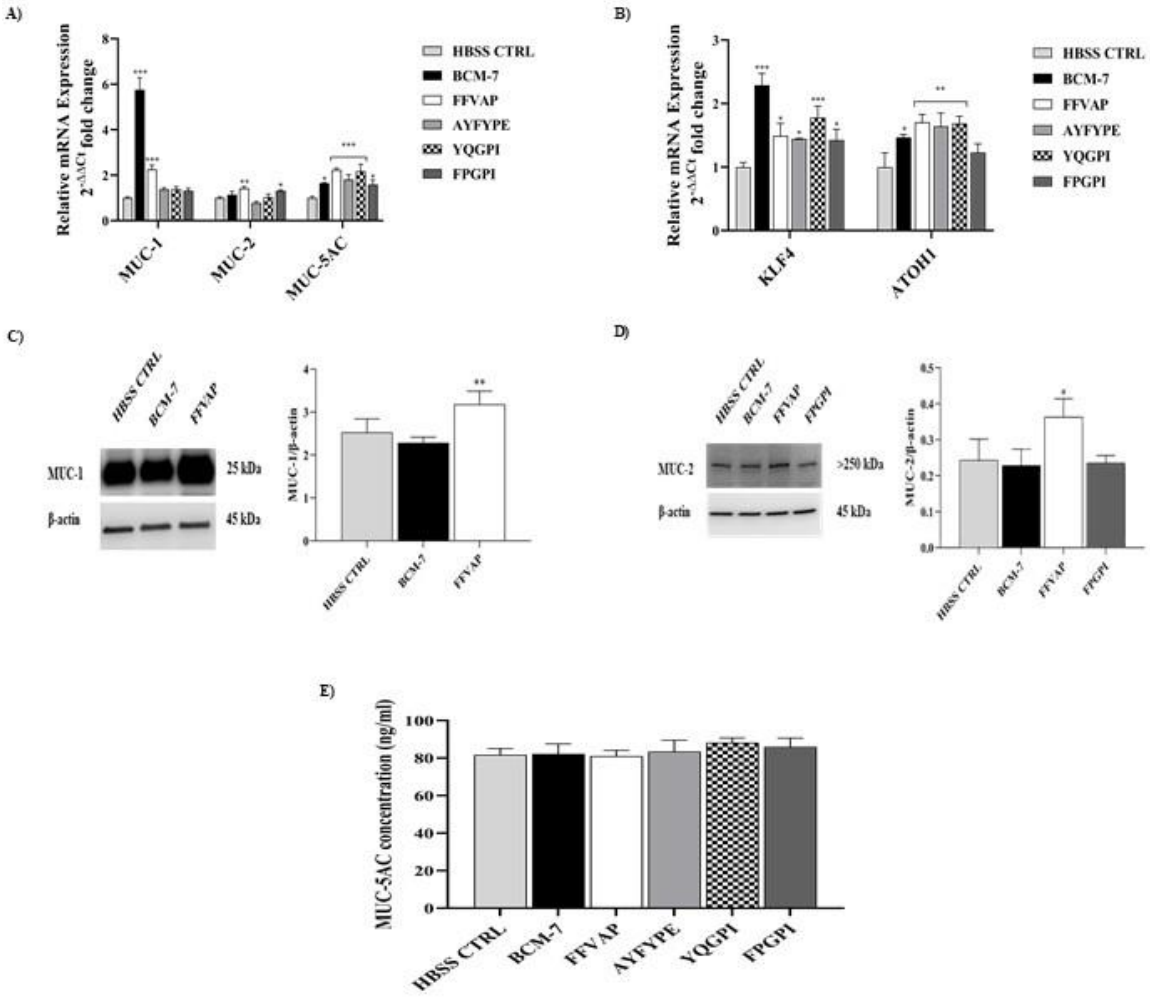


Figure 4.5. Effects of MEM-IMF synthetic peptides on mucus biomarkers in HT29-MTX monolayers. Monolayers were treated with peptides for 2 h at 0.5 mM. The mRNA transcript levels of (**A**) mucin genes; *MUC-1*, *MUC-2* and *MUC-5AC* and (**B**) goblet cell pathway genes; *KLF4* and *ATOH1* and protein levels of (**C**) MUC-1, (**D**) MUC-2 and (**E**) MUC-5AC were measured in HT29-MTX cell lysates. Peptides were from BCM-7, α_{s1}-CN ^{23}FFVAP^{27} and ^{143}AYFYPE^{148}, α_{s2}-CN ^{100}YQGPI^{105} and β-CN ^{62}FPGPI^{66}. Cell treatments for RT-qPCR analysis were performed in triplicate on at least two different days. RT-qPCR analysis was performed with technical duplicates on two different days. Values are reported as means ± SD (n = 12). Protein levels were determined by western blotting or ELISA. Values are reported as means ± SD (n = 6). Statistical differences between peptides and untreated monolayers were analysed by one-way ANOVA and Dunnett's multiple comparisons test. Significant

differences between test samples and untreated monolayers (HBSS CTRL) are represented by asterisks (*); * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. MEM-IMF, infant milk formula produced by membrane filtration and HT-IMF, high-temperature infant milk formula.

4.4. Discussion

Ex vivo and *in vitro*, gastrointestinal digested samples of MEM-IMF reduced the ability of pathogenic bacteria to adhere to goblet cells. This benefit is likely attributed to changes in mucus biomarkers. *Ex vivo* jejunal MEM-IMF increased Caco-2/HT29-MTX monolayer mRNA transcript levels of *MUC-2* and *MUC-5AC*. An *in vitro* gastrointestinal digested sample of MEM-IMF increased MUC-2 protein and mRNA transcripts of *MUC-1* and *MUC-2* in Caco-2/HT29-MTX monolayers. This mucus modulation is likely supported by the bioactive α_{s1}-CN ^{23}FFVAP^{27} peptide in MEM-IMF.

The increase in mucus levels associated with MEM-IMF protected the intestinal barrier from pathogenic bacteria. This link between mucin expression and a reduction in pathogenic attack has been reported by others. MUC-2 knockout (MUC-2$^{-/-}$) mice lack the MUC-2 rich mucus layer (Bergstrom et al., 2010). In wild-type mice (6 - 8 weeks old) infected, by oral gavage, with *S. enterica* (3×10^7 CFU), pathogen burden was lower in the intestinal lumen and cecum compared to MUC-2$^{-/-}$ mice (Zarepour et al., 2013). Seven days post-infection, 50% of MUC-2$^{-/-}$ mice died from infection and the remaining mice lost 15 to 20% of their starting body weight (Zarepour et al., 2013). This link between MUC-2 expression and pathogen numbers is also evident with *Citrobacter rodentium* (Bergstrom et al., 2010; Birchenough et al., 2023). Wild-type mice (6 - 8 weeks old) infected orally with this pathogen (2.5×10^8 CFU), had a 10-fold decrease in *C. rodentium* load in the intestinal lumen 7 days post-infection compared to MUC-2$^{-/-}$ mice ($P < 0.01$) (Bergstrom et al., 2010). In jejunal lumen and mucosa, *C. rodentium* loads and colonisation were significantly lower in wild-type mice (12 - 17 weeks old) versus MUC-2$^{-/-}$ mice, 4 days post-infection ($P < 0.05$) (Birchenough et al., 2023). In our study, an incubation ≤ 4 h is unlikely to cause a goblet cell number increase as goblet cells regenerate every 3 days (Karam, 1999) and HT29-MTX cell doubling time is 24 to 48 h (Lesuffleur et al., 1990). Therefore, MEM-IMF bioactivity on mucins in our acute study must be via modulation

of mucus production pathways. In our chronic study where pigs were fed MEM-IMF diets for 28 days, a significant increase in goblet cell number was observed compared to pigs fed HT-IMF diets (17.39 ± 1.43 goblet cells *vs* 11.7 ± 1.27 goblet cells) ($P < 0.05$) (Dold et al., 2024), suggesting MEM-IMF has immediate and long-term effects on mucus.

Mucin increase can be initiated by the presence of bioactive peptides. Fernández-Tomé et al. (2016) has reported that a 4 h exposure to α_{s1}-CN peptides ^{143}AYFYPEL^{149} (0.1 mM) and ^{144}YFYPEL^{149} (0.5 mM) significantly increased *MUC-5AC* mRNA transcripts in HT29-MTX monolayers by 1.7 ± 0.3-fold and 1.8 ± 0.2-fold, respectively, compared to untreated monolayers ($P < 0.05$) (Fernández-Tomé et al., 2016). HT29-MTX monolayers treated for 4 h with 0.1 mM β-CN ^{108}EMPFPK^{113} and ^{114}YPVEPF^{119} peptides also significantly increased *mucin-4* mRNA transcripts by 142 ± 8% and 152 ± 22%, respectively versus untreated monolayers (Plaisancié et al., 2015). Loukas et al. (1983) has reported that α_{s1}-CN peptides ^{90}RYLGYL^{95}and ^{90}RYLGYLE^{96} have opioid activity *in vivo* (Loukas et al., 1983). Treatment of HT2-MTX monolayers with a derivative, ^{90}RYLGY^{94} (0.1 mM) significantly increased overall mucin secretion by 191% versus untreated monolayers ($P < 0.001$) (Martínez-Maqueda et al., 2012). In our study, MEM-IMF contained 112 unique peptides (Bavaro et al., 2021) which may work individually, or in synergy, to elicit a mucin response. Noteworthy, a 2 h exposure of HT29-MTX monolayers to peptide α_{s1}-CN ^{23}FFVAP^{27} (0.5 mM) from MEM-IMF increased MUC-1 and MUC-2 protein. Breast milk increases production of mucus from goblet cells (Dimitroglou et al., 2022). However, α_{s1}-CN is rarely present in breast milk (Kim & Yi, 2020). A potential mechanism of action may be via the noted upregulation of goblet cell transcription factors KLF4 and ATOH1. In wild-type mice, colonic *MUC-2* mRNA expression and acidic mucins are significantly higher compared to mice with colon-specific deletion of KLF4 (Katz et al., 2002). Ghaleb et al. (2011) has observed that wild-type mice have higher acidic mucins, neutral mucins, MUC-2 and intestinal alkaline phosphatase activity compared

to mice with intestinal-specific knockout of KLF4 (Ghaleb et al., 2011). ATOH1 also modulates mucus production pathways. Images of Alcian blue and periodic acid-Schiff stained ileal tissue showed increases in acidic and neutral mucins in wild-type mice compared to mice with intestine-specific deletion of ATOH1 (Shroyer et al., 2007; Yang et al., 2001). This α_{s1}-CN ^{23}FFVAP^{27} peptide has been previously reported to have angiotensin-converting enzyme inhibitory properties (Maruyama et al., 1985). However, our *in silico* analysis also identified it as a potential opioid bioactive. Indeed, the α_{s1}-CN ^{23}FFVAP^{27} peptide outperformed BCM-7. BCM-7 (2 h, 0.5 mM) significantly increased mRNA transcript levels of *MUC*-1, *MUC-5AC*, *KLF4* and *ATOH-1* but surprisingly did not increase MUC-1 or MUC-2 protein. An 8 h exposure to BCM-7 at 0.1 mM led to a significant increase in MUC-2 (192 ± 4%) protein in colonic rat DHE cells and a significant increase in MUC-5AC (169 ± 3%) protein in HT29-MTX monolayers compared to untreated controls ($P < 0.05$) (Zoghbi et al., 2006). Similar relative abundance of BCM-7 in both MEM-IMF and HT-IMF by LC-MS/MS would suggest it is unlikely to contribute to the mucus differences observed in our study (Bavaro et al., 2021). All peptides used in this study are casein derived peptides. The majority of studied milk bioactive peptides are derived from casein which supported the *in silico* selection of these casein peptides. In addition, whey proteins can bind to surface of casein micelles, reducing protein digestibility and the generation of whey derived peptides.

Jejunal MEM-IMF increased CLDN-1 and OCLN protein in Caco-2/HT29-MTX monolayers compared to jejunal HT-IMF. Previously, CLDN-1 protein was significantly higher in Caco-2 monolayers treated with an *in vitro* gastrointestinal digested MEM-IMF sample compared to HT-IMF ($P < 0.05$) (Bavaro et al., 2021). This suggests MEM-IMF can increase *in vitro* tight junction proteins in acute studies. Tight junction formation is a dynamic process. It is worth highlighting that in this study, tight junction formation was only evaluated at a single time point. However, previously in our pig trial, tight junction formation was examined at different

intestinal locations (jejunum and duodenum), with a significant increase observed in *CLDN-1* mRNA transcripts in the duodenum (Dold et al., 2024). Immunofluorescence revealed OCLN protein was significantly higher in duodenal enteroid monolayers treated with breast milk for 24 h ($P < 0.01$) and 72 h ($P < 0.001$) (20% v/v) compared to monolayers treated with commercial IMF (20% w/v), although these samples were not digested (Noel et al., 2021). Therefore, this preliminary observation with MEM-IMF is also positive in our efforts to produce IMF with similar bioactivities to breast milk.

An *in vitro* gastrointestinal digested MEM-IMF sample reduced GLP-1 secretion from enteroendocrine cells compared to HT-IMF. If this persisted, a reduction in GLP-1 would indicate an increase in appetite (i.e. higher food consumption) suggesting that MEM-IMF may be less satiating. In our dietary intervention study, significant increases in average daily food intake and average daily gain, between day 0 and day 7, were recorded in pigs fed MEM-IMF diets (270 g/day and 200 g/day) versus pigs fed HT-IMF diets (201 g/day and 140 g/day) ($P = 0.01$), albeit no differences were observed at slaughter on day 28 (Chen et al., 2023). Babies who receive MEM-IMF could be hungrier quicker and have higher food intake compared to those fed HT-IMF. Previously, piglets (n = 20) receiving a whey protein isolate (91% native whey protein) had significantly higher postprandial GLP-1 concentrations 150 min after administration compared to piglets receiving a heat-treated (80°C for 10 min) whey protein isolate (8% native whey protein) (Welch-Jernigan et al., 2019). Infants (3 - 20 weeks old) fed a standard IMF had significantly higher weight-gain velocity, a risk factor for obesity, compared to infants fed an extensively hydrolysed IMF (Mennella et al., 2018). A reduction in GLP-1 in babies fed MEM-IMF could be a risk factor for overconsumption and obesity in later life. There is *in vitro* evidence that MEM-IMF is digested faster in the stomach (Chen et al., 2022). From 94 min *in vitro* gastric digestion of MEM-IMF there was less protein aggregates as fat droplets began to separate compared to HT-IMF that formed large, compact protein

aggregates that remained on fat droplet surfaces until the end of gastric digestion (156 min, pH 3.2) (Chen et al., 2022). There was no significant difference in water soluble proteins and peptides < 10 kDa (Chen et al., 2022). Therefore, the fact that MEM-IMF has a faster gastric transit time than HT-IMF together with our GLP-1 results suggests that peptides released during digestion of HT-IMF are more satiating.

Peptides derived from β-lactoglobulin have been classified as allergenic epitopes (Golkar et al., 2019). Previously, 102 β-lactoglobulin peptides were identified in the apical compartments of Caco-2 monolayers treated with I60 MEM-IMF compared to 66 β-lactoglobulin peptides in monolayers treated with I60 HT-IMF (Bavaro et al., 2021). Heat treatment of a whey protein isolate at ≥ 90 °C for 20 min reduced the antigenicity potential of β-lactoglobulin (Bu et al., 2009). The immunoglobulin E binding inhibition, potential allergenicity, of β-lactoglobulin in a milk protein concentrate decreased significantly to 47.84% when heated at 85 °C for 25 min (Xu et al., 2016). Therefore, with higher numbers of β-lactoglobulin peptides present following gastrointestinal digestion of MEM-IMF, additional studies are required in babies to understand the possible allergenicity associated with MEM-IMF.

4.5. Conclusion

In conclusion, *in vitro* work agreed with *in vivo* data providing validation of the Caco-2/HT29-MTX model as a good representation of the human intestinal barrier. Overall, epithelial cells were more responsive to *in vitro* experiments using I60 MEM-IMF and synthesised peptides compared to more complex *ex vivo* jejunal MEM-IMF. However, responses were similar for *S. enterica* adhesion and mRNA transcripts of *MUC*-2, *CLDN-1* and *OCLN*. These similarities reinforce confidence in the ability of *in vitro* cellular models to reflect real-life conditions. Future work should include additional mechanistic studies to understand the ability of MEM-IMF and the α_{s1}-CN ^{23}FFVAP^{27} peptide to modulate mucus pathways. Promotion of intestinal mucus is an important attribute of breast milk in the infant gut. This should also be a capability of next generation IMF.

4.6. References

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Supplementary Table 4.1. Primer sequences for RT-qPCR analysis

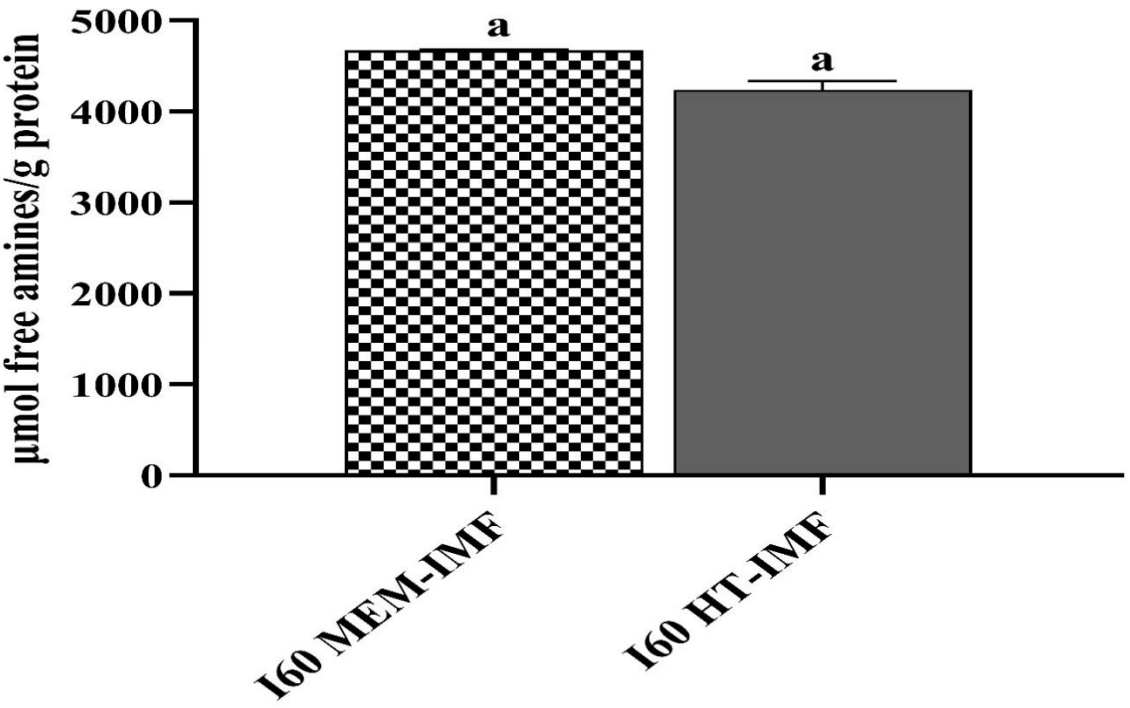
Gene	GenBank accession number	Primer sequences (5'-3')	T_a (°C)	Amplicon size (bp)	Reference
RPLP0	NM_001002.4	F: GCAGCATCTACAACCCTGAA R: GCAGATGGATCAGCCAAGAA	57	235	This paper
CLDN-1	NM_021101.5	F: CGTTGGCATGAAGTGTATGAAG R: CTCTGTGTCACACGTAGTCTTT	57	347	(Kondrashina et al., 2021)
OCLN	NM_002538.4	F: CTGGCCTACAGGAATACAAGAG R: TGATGCTCACAGAGGTTTGG	57	369	(Kondrashina et al., 2021)
ZO-1	NM_003257.5	F: CATCTCCAGTCCCTTACCTTTC R: TCTGCTGGCTTGTTTCTCTAC	57	380	(Kondrashina et al., 2021)
MUC-1	NM_002456.6	F: CCTACCATCCTATGAGCGAGTAC R: GCTGGGTTTGTGTAAGAGAGGC	60	136	This paper
MUC-2	NM_002457.5	F: AAGACGGCACCTACCTCG R: TTGGAGGAATAAACTGGAGAACC	60	238	This paper
MUC-5AC	NM_001304359.2	F: ACAGCGGTGACTTCGACACA R: CCGATGCCTGCTCCCTGTTA	60	183	This paper
KLF4	NM_004235.6	F: CATCTCAAGGCACACCTGCGAA R: TCGGTCGCATTTTTGGCACTGG	60	156	This paper
ATOH1	NM_005172.2	F: CCTTCCAGCAAACAGGTGAATGG R: GAACGACGGGATAACATTGCGC	60	126	This paper

T_a, annealing temperature; bp, base pairs; *RPLP0, ribosomal protein lateral stalk subunit P0; CLDN-1, claudin-1; OCLN, occludin; ZO-1, zona occludens-1; MUC-1, mucin-1; MUC-2, mucin-2; MUC-5AC, mucin-5AC*; *KLF4*, *Krüppel-like factor 4* and *ATOH1*, *atonal BHLH transcription factor 1*.

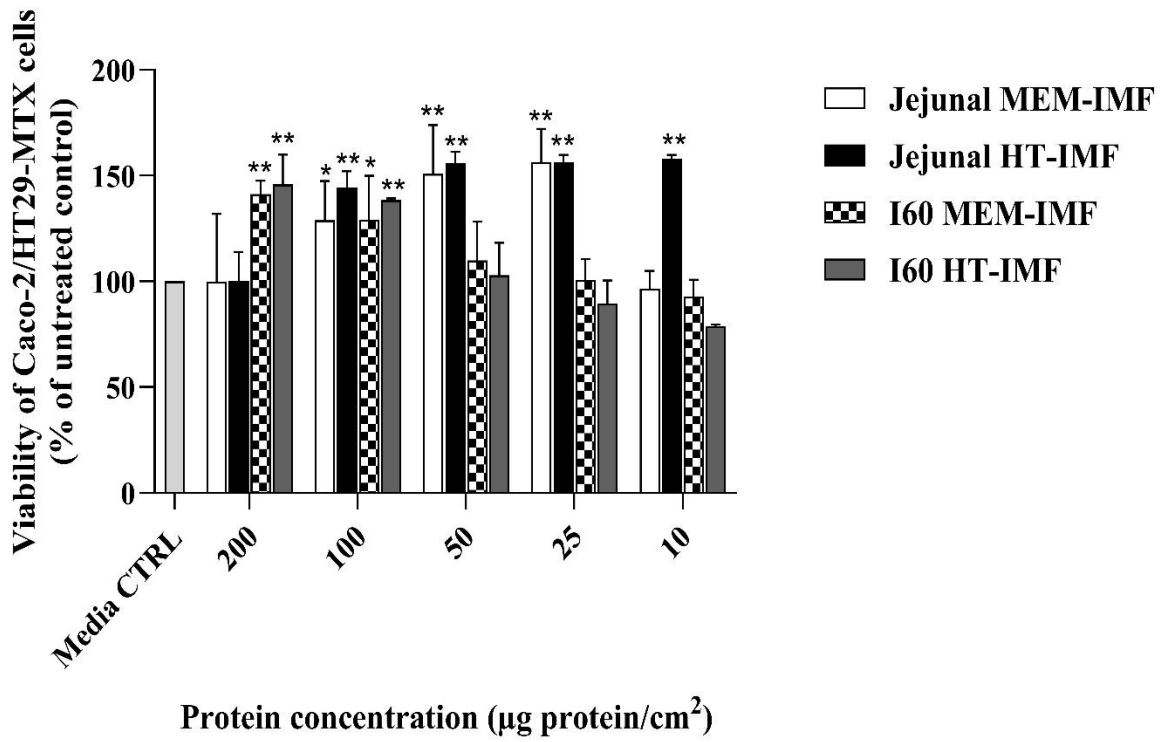
Supplementary Table 4.2. TEER values of 21 day old differentiated Caco-2/HT29-MTX monolayers after 4 h treatment with jejunal IMF and I60 IMF (200 µg protein/cm^2)

Treatment	TEER ($\Omega.cm^2$)
Media CTRL	1142 ± 106[a]
Jejunal MEM-IMF	1071 ± 33[a]
Jejunal HT-IMF	1113 ± 58[a]
I60 MEM-IMF	1135 ± 139[a]
I60 HT-IMF	1187 ± 149[a]

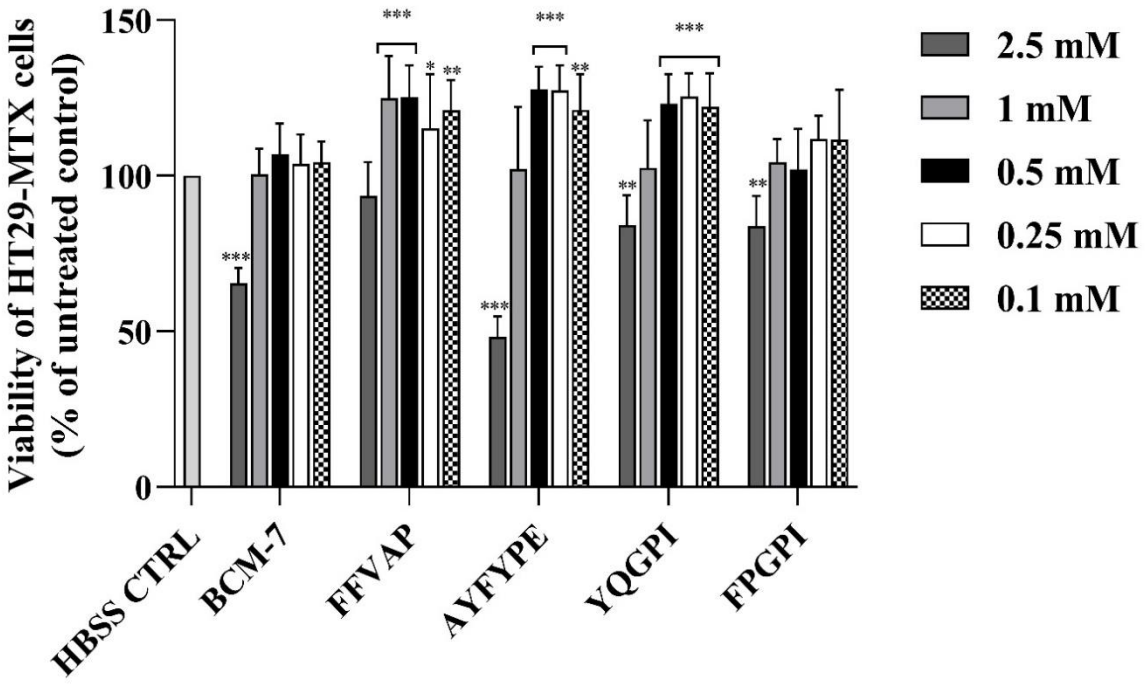
For assays with jejunal IMF at least n = 3 pigs per treatment were included. For assays with I60 IMF, n = 3 samples from different days were used. TEER values were recorded in duplicate on three different days. Values are reported as means ± SD (n = 6). Different letters indicate significant differences between treatment types ($P < 0.05$). The average TEER value on day 21 at 0 h was 1084 ± 19 $\Omega.cm^2$. Media CTRL, complete DMEM media control; MEM-IMF, infant milk formula produced by membrane filtration and HT-IMF, high-temperature infant milk formula.



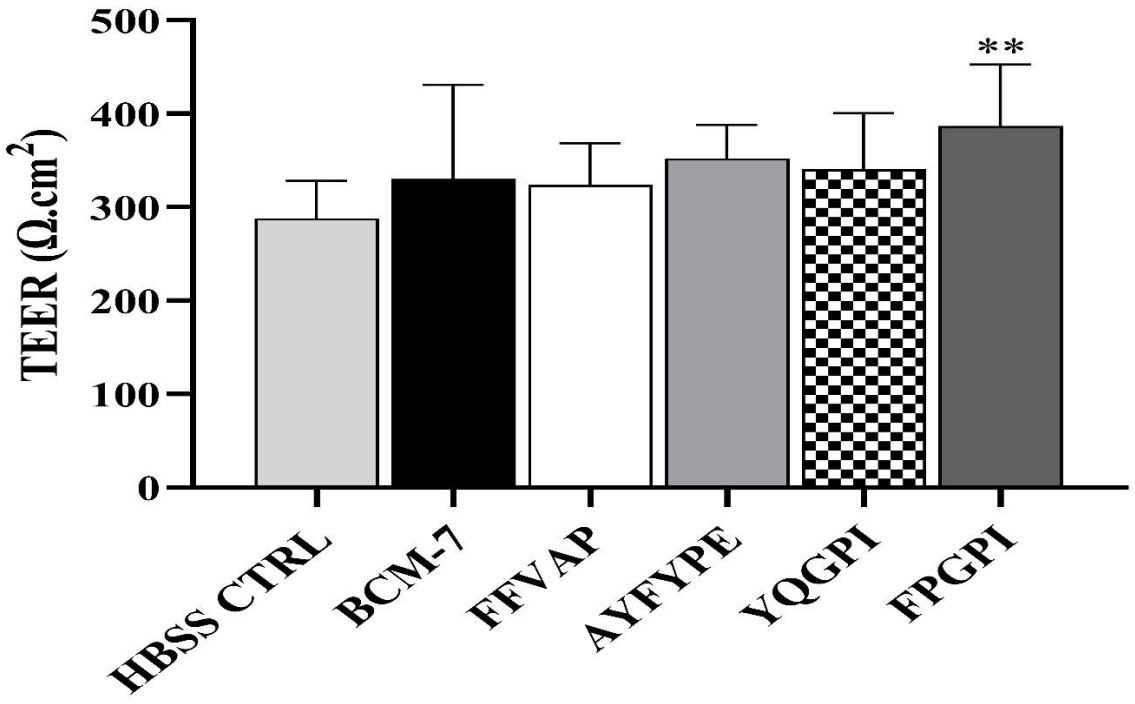
Supplementary Figure 4.1. Concentration of free amine groups (µmol/g protein) in I60 MEM-IMF and I60 HT-IMF samples. The degree of protein hydrolysis was determined using the o-phthaldialdehyde assay. The protein concentration was determined using the Pierce™ BCA Protein Assay Kit. Each measurement was performed in duplicate on two different days. Values are reported as means ± SD (n = 4). Different letters indicate significant differences between treatment types ($P < 0.05$). MEM-IMF, infant milk formula produced by membrane filtration and HT-IMF, high-temperature infant milk formula.



Supplementary Figure 4.2. Cell viability of Caco-2/HT29-MTX co-culture after 4 h incubation with jejunal IMF and I60 IMF (10 - 200 µg protein/cm^2). Media CTRL, complete DMEM media control (100% cell viability. Samples were diluted directly in DMEM media. Cell viability was determined using the MTS assay and expressed as a percentage of untreated cells (complete DMEM media). The SGID control sample had a similar cell viability (95.78 ± 16.13%) compared to media CTRL. For assays with jejunal IMF at least n = 3 pigs per treatment were included. For assays with I60 IMF, n = 3 samples from different days were used. Each measurement was performed in triplicate on two different days. Values are reported as means ± SD (n = 6). Statistical differences between treatments and untreated cells were analysed by one-way ANOVA and Dunnett's multiple comparisons test with asterisks (*) indicating significant differences with untreated cells, * $P < 0.05$ and ** $P < 0.01$. MEM-IMF, infant milk formula produced by membrane filtration and HT-IMF, high-temperature infant milk formula.



Supplementary Figure 4.3. Cell viability of HT29-MTX cells exposed to MEM-IMF synthetic peptides (0.1 - 2.5 mM). Synthetic peptides were prepared and diluted directly in HBSS. Cell viability was determined using the MTS assay and expressed as a percentage of untreated cells (HBSS CTRL, 100% cell viability). Cells incubated with HBSS had a comparable cell viability (> 80%) to a control well with complete DMEM media after 2 h incubation. Each measurement was performed in triplicate on three different days. Values are reported as means ± SD (n = 9). Statistical differences between treatments and untreated cells were analysed by one-way ANOVA and Dunnett's multiple comparisons test with asterisks (*) indicating significant differences with untreated cells; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.



Supplementary Figure 4.4. TEER values of HT29-MTX monolayers following 2 h treatment with MEM-IMF synthetic peptides (0.5 mM). Monolayers were differentiated for 21 days prior to treatment. The average TEER value on day 21 at 0 h for HT29-MTX monolayers was 368 ± 70 Ω.cm^2. MEM-IMF synthetic peptides were diluted in HBSS. TEER values were recorded in duplicate on three different days. Values are reported as means ± SD (n = 6). Statistical differences between treatments and untreated monolayers (HBSS CTRL) were analysed by one-way ANOVA and Dunnett's multiple comparisons test with asterisks (*) indicating significant differences with untreated monolayers; * $P < 0.05$ and ** $P < 0.01$.

Chapter 5

General Discussion

5.1. General Discussion

The primary objective of this thesis was to compare the physiological responses to infant milk formula (IMF) produced using membrane filtration processing (MEM-IMF) to an IMF produced by high-temperature processing (HT-IMF). The aim of Chapter 1 was to comprehensively review the effect on the gut of reducing thermal processing during IMF manufacturing. In Chapter 2, a dietary intervention study was used with young pigs (aged 28 days) fed MEM-IMF or HT-IMF at 35% inclusion for 28 days to understand if MEM-IMF could alter protein digestibility in the upper gastrointestinal tract. In follow on work (Chapter 3), the effect of membrane filtration processing on digestive enzyme activity and gut barrier physiology was examined in the upper small intestine (duodenum and jejunum) of these young pigs. The aim of Chapter 4 was to take a closer look at how MEM-IMF interacts with enterocytes and goblet cells *in vitro*.

Overall, this thesis demonstrated that MEM-IMF differs to HT-IMF as it (a) has faster protein digestion in a young gut, (b) promotes intestinal digestive capacity, (c) significantly increases goblet cell number and mucin biomarkers in the jejunum and (d) significantly reduces pathogen adhesion to an *in vitro* gut barrier. These gut responses for MEM-IMF are similar to gut responses reported with human breast milk. Therefore, we can consider MEM-IMF superior to HT-IMF. As such, MEM-IMF is a promising candidate in efforts to produce an IMF product that more closely resembles breast milk.

The commercial IMF market generates approximately US$55 billion each year (Rollins et al., 2023). In the USA, in 2021, 20.5 ± 1% of newborn infants received infant formula within the first two days of life (Centers for Disease Control and Prevention, 2020). Typically, milk is initially pasteurised at 72 - 75 °C for 15 s followed by higher temperatures to generate an IMF powder. While essential in producing a safe product, free from harmful pathogenic bacteria

(Commission Regulation (EC) No. 2073/2005 on microbiological criteria for foodstuffs (Commission Regulation, 2005)), exposure to high thermal loads will increase denaturation of native milk proteins and promote the Maillard reaction.

Membrane filtration processing produces a safe IMF product, free from harmful pathogenic bacteria. Pilot plant scale production (250 kg) led to higher bacterial loads in MEM-IMF powder compared to HT-IMF powder, albeit meeting the standards set by the Commission Regulation (EC) No. 2073/2005 (Commission Regulation, 2005). To the best of our knowledge, Yu et al. (2021) are the only other group that have published a membrane filtration process (0.8 µm and 0.1 µm pore size membranes) to produce an IMF powder (Yu et al., 2021). Similarly, free from pathogenic bacteria, Yu et al. (2021) did observe higher bacterial loads in their IMF powder produced by membrane filtration versus heat-treated IMF powder (Yu et al., 2021). These IMF powders produced by membrane filtration were produced at pilot plant scale using a discontinuous processing system leaving them vulnerable to post filtration contamination. In both our work and the Yu et al. (2021) study, unpasteurised milk was used in MEM-IMF production (Yu et al., 2021). To reduce bacterial loads in MEM-IMF, a strict, continuous processing system at an industrial scale manufacturing facility with sterile membranes should be configured.

Membrane filtration produces an IMF product with a higher native whey protein content similar to high-temperature processing. The native whey protein content of MEM-IMF (59.9% native whey protein) was significantly higher than HT-IMF (4.5% native whey protein) (Chapter 2). Yu et al. (2021) observed 94 ± 4% native whey protein in their IMF produced by membrane filtration compared to only 42 ± 0% in the extensively, heat-treated IMF (Yu et al., 2021). The processing history and sources of protein ingredients used in IMF manufacturing is critical to understanding the subsequent effect on protein denaturation. Sourcing protein ingredients with limited protein denaturation is essential. The whey protein isolate used in our formulations

already had 8.5% denatured protein (Chen et al., 2021). In addition, in our work, a split-stream process was incorporated. The retentate from the 0.2 µm polyvinylidene difluoride polymeric membrane was mixed with fat, lactose and minerals, heated at 125 °C for 5 s and merged with MEM-IMF permeate. This may explain the differences in native whey protein content in our MEM-IMF compared to Yu et al. (2021).

Thermal processing can promote the formation of harmful Maillard reaction products. Under Commission Regulation (EU) No. 2017/2158, limits have been introduced for acrylamide, a carcinogenic Maillard reaction by-product (Commission Regulation, 2017), with further restrictions on other advanced glycation end products likely to follow. Previously, our group has demonstrated that the membrane filtration process will generate MEM-IMF with similar N(6)-carboxymethyllysine (CML) levels to HT-IMF (3.65 ± 0.74 µg/g MEM-IMF powder *vs* 3.97 ± 0.7 µg/g HT-IMF powder) ($P > 0.05$) (Chen et al., 2021), albeit this was the only advanced glycation end product quantified. The Maillard reaction is likely to occur in our process via the heat treatment of the 0.2 µm membrane retentate with lactose or during spray drying (inlet temperature 185 °C and outlet temperature 80 °C). Additionally, Yu et al. (2021) reported no differences in CML and furosine levels between membrane filtered and heat-treated IMF powders (Yu et al., 2021). A significant reduction in mRNA transcript levels for the *receptor for advanced glycation end products* was observed in the jejunum of MEM-IMF fed pigs compared to pigs fed HT-IMF diets, implying there was less glycation in MEM-IMF versus HT-IMF (Chapter 3). Reducing the susceptibility of IMF to glycation during IMF processing is a goal of next generation IMF to protect infants from harmful and carcinogenic glycation by-products such as acrylamide. Future work with this MEM-IMF should include quantification of early, intermediate and advanced glycation products at each step of membrane filtration processing to understand where the Maillard reaction occurs and to what extent.

Previous work has shown that reducing thermal loads imposed on IMF ingredients and IMF during manufacturing can promote protein digestibility in the gastrointestinal tract and support gut barrier health (Aasmul-Olsen et al., 2024; Halabi et al., 2020; Navis, Muncan, et al., 2020; Navis, Schwebel, et al., 2020; Sun et al., 2022). Chapter 2 revealed that a 35% inclusion diet of MEM-IMF significantly increased water soluble protein and peptide content in the gastrointestinal tract, the degree of protein hydrolysis in the stomach and duodenum and tended to increase the release of free amino acids in the jejunum of young pigs compared to pigs fed HT-IMF diet. Furthermore, in the duodenum, pigs fed MEM-IMF diets had significantly higher trypsin activity compared to those fed HT-IMF diets (Chapter 3). Interestingly, a significant increase in proline concentration was observed in the duodenum lumen of pigs fed MEM-IMF versus HT-IMF diets (Chapter 2). A higher concentration of proline and higher trypsin activity in the duodenal lumen suggests that peptides in MEM-IMF are more accessible to digestive enzymes, increasing proteolysis. However, in the duodenal brush border membrane, a significantly lower aminopeptidase N activity was observed in pigs fed MEM-IMF diets compared to pigs fed HT-IMF diets (Chapter 3). It can be hypothesized that a reduction in protein hydrolysis in HT-IMF forces aminopeptidases to work harder which in turn increases aminopeptidase N activity. Recent work in Wistar Han rats, found no significant effect of membrane filtration or high-temperature processing of IMF on protein digestibility in the stomach or small intestine (Calvez et al., 2024). Membrane filtration processing did increase postprandial (at 1 h) plasma indispensable and total amino acid concentrations (Calvez et al., 2024). Future work should include a dietary intervention study with MEM-IMF where postprandial amino acid concentrations are quantified. Future work should also compare the digestive performance of MEM-IMF with breast milk. As highlighted previously by Charton et al. (2024), the digestive fate of heat-treated IMF and human milk differs greatly during the gastric and early intestinal phase (Charton et al., 2024).

Chapter 3 revealed the responses of the young gut to a MEM-IMF diet. Duodenal brush border membrane lactase activity was significantly higher in pigs fed MEM-IMF diets compared to pigs fed HT-IMF diets along with significant increases in *claudin-1* (*CLDN-1*), *mucin-1* (*MUC-1*) and *mucin-2* (*MUC-2*) mRNA transcript levels. In the jejunum, a significant increase in goblet cell numbers, acidic mucus levels and MUC-2 protein concentrations was observed in MEM-IMF fed pigs compared to pigs fed HT-IMF. Previously, Navis, Muncan et al. (2020) reported an observational increase in goblet cell numbers in the colon of piglets fed an IMF made with a pasteurised whey protein concentrate (WPC) (73 °C/30 s) compared to piglets fed an IMF with extensively heated WPC (73 °C/30 s and 80 °C/6 min) (Navis, Muncan, et al., 2020). The mucus promoting ability associated with MEM-IMF was also observed in our Caco-2/HT29-MTX intestinal barrier model (Chapter 4). Monolayers treated with jejunum lumen collected from pigs fed MEM-IMF diets for 28 days had significantly higher *MUC-2* and *MUC-5AC* mRNA transcripts compared to monolayers exposed to HT-IMF jejunum lumen. An *in vitro* gastrointestinal digesta sample of MEM-IMF also significantly increased MUC-2 protein along with *MUC-1* and *MUC-2* mRNA transcripts in Caco-2/HT29-MTX monolayers. The mucosal barrier creates a defensive shield from attack by pathogenic matter (Yuan et al., 2023). Chapter 4 revealed *ex vivo* jejunal MEM-IMF and *in vitro* digested MEM-IMF significantly reduced the ability of pathogenic bacteria to adhere to HT29-MTX monolayers, compared to HT-IMF test samples ($P < 0.05$). This suggests a direct link exists between MEM-IMF, mucus production in goblet cells and intestinal protection against pathogenic attack. Future studies should examine whether or not consumption of MEM-IMF increases the adherence of gut promoting, probiotic bacteria as well as protecting against other pathogenic bacteria in the young gut.

It is difficult to ascertain what component in MEM-IMF was responsible for increasing mucus *in vitro*, *ex vivo* and *in vivo*. Peptides unique to MEM-IMF may be responsible for this

bioactivity. Previous work in our lab identified by liquid chromatography-mass spectrometry (LC-MS)/MS, 112 unique peptides released during *in vitro* gastrointestinal digestion of MEM-IMF (Bavaro et al., 2021). However, there was no difference in levels of water soluble proteins and peptides between 0.5 kDa and 10 kDa released from MEM-IMF and HT-IMF in the stomach, duodenum, jejunum and ileum of young pigs (Chapter 2). Regardless, the α_{s1}-casein ^{23}FFVAP^{27} peptide, unique to MEM-IMF, significantly increased MUC-1 and MUC-2 protein and mRNA transcript levels of *MUC-1*, *MUC-2* and *MUC-5AC* in HT29-MTX monolayers (Chapter 4). Transcript levels of goblet cell transcription factors, *Krüppel-like factor 4* and *atonal BHLH transcription factor 1* were also significantly up-regulated in monolayers treated with this peptide, suggesting a potential modulation of goblet cell function. Whether or not the α_{s1}-casein ^{23}FFVAP^{27} peptide is present in jejunal MEM-IMF samples could not be ascertained. Future studies should perform additional mechanistic studies to understand the ability of MEM-IMF and the α_{s1}-casein ^{23}FFVAP^{27} peptide to modulate mucus pathways. Peptidomic analysis is also required of gastric, duodenal, jejunal and ileal lumen samples collected from the pigs fed MEM-IMF diets. It is important to note that other studies with extensively heat processed IMF have attributed harmful gut responses to high levels of advanced glycation end products (CML and furosine) (Aasmul-Olsen et al., 2024; Sun et al., 2022). As only CML was quantified as part of our work, we cannot rule out that quantities of other glycation products are lower in MEM-IMF compared to HT-IMF. Lower levels of these compounds may have contributed to the gut responses observed.

In this work, IMF processing had no significant effect on the architecture of the upper small intestine (Chapter 3). It is worth emphasising that the IMF accounted for only 35% of the pig's total diet. In other animal trials, researchers have seen significant effects on small intestinal structure with IMF at 100% inclusion administered via an orogastric feeding tube over a shorter period ($\leq$ 5 days). In our study, IMF was included at 35% to allow us to perform a study over

28 days whilst meeting the recommended dietary requirements for pigs. However, tight junctions were influenced by processing type. *CLDN-1* duodenal mRNA transcript levels were significantly higher in pigs fed MEM-IMF diets compared to those fed HT-IMF diets (Chapter 3). In agreement, *ex-vivo* jejunal MEM-IMF also increased CLDN-1 and occludin (OCLN) protein in Caco-2/HT29-MTX monolayers compared to HT-IMF jejunum lumen (Chapter 4). Future work should include a dietary intervention with MEM-IMF where intestinal permeability is fully investigated (i.e. lactulose mannitol test).

This thesis focused solely on the upper small intestine response to MEM-IMF, future work should also include colonic responses including the gut microbiota. Others have previously examined the influence of IMF and breast milk on the gut microbiota of piglets (Charton et al., 2022). A higher abundance of *Lactobacillus*, *Bifidobacterium* and *Bacteroides* were detected in the faeces of piglets fed human milk compared to a heat-treated IMF (Charton et al., 2022).

5.2. Future Directions

Several question remain unanswered. Membrane filtration processing of IMF could potentially be used to produce a novel food. A novel food is defined as 'any food that was not used for human consumption to a significant degree within the European Union before 15 May 1997' (Commission Regulation, 2015). Before this MEM-IMF powder could be used as a novel food and given to newborn babies, processing improvements are required to reduce bacterial loads and increase sterility of MEM-IMF powders to levels comparable to extensively, heat-treated IMF products. A single pasteurisation step could be included as it has limited impact on protein denaturation. Bacterial loads should also be monitored before and after spray drying. An IMF produced by membrane filtration processing may be classified as an 'ultra-processed food.' Additional research is required to seek alternative, low-temperature processing methods to produce IMF such as high-pressure processing, ultraviolet irradiation, pulsed electric field and ozonation. This thesis recommends that information regarding the processing parameters and temperatures employed during IMF manufacturing, as well as during IMF ingredient production, should be revealed in studies to fully understand the impact of processing on protein digestibility and gut barrier health. Many studies use commercial IMF products, where processing temperatures are unknown, making it difficult to identify causative agents. Studies are warranted in newborn in babies to understand the possible allergenicity associated with MEM-IMF. Furthermore, a comprehensive quantification of early, intermediate and advanced Maillard reaction products by standardised methods are needed for IMF products used in gut response studies. Storage conditions should also be stated. Follow up *in vitro* studies are also required examining gut epithelial cell responses to individual advanced glycation end products and breast milk from human milk banks. A full peptidomic profiling of peptides released during IMF digestion (*in vitro* and *in vivo*) is also required to understand the role of peptides in gut responses. Membrane filtration has no inhibitory effect on indigenous milk enzymes. More

work is required to understand if indigenous milk enzymes are still active in MEM-IMF powders before and after gastrointestinal digestion. This thesis revealed good correlation between *in vitro* and *in vivo* models and indeed between IMF manufacturing batches. The ability of MEM-IMF to increase mucus and tight junction integrity *in vivo* in young pigs (Chapter 3) and *in vitro* in intestinal epithelial cell lines (Chapter 4) is one of the main strengths of this thesis. *In vitro* data that translates to real life conditions will reduce the reliance on preclinical dietary intervention trials. In this work, only one physical form of finished IMF product (i.e. IMF powder) was considered. An additional heat treatment is often used when producing IMF powder (i.e. spray drying). The gastrointestinal protein digestion performance and subsequent physiological benefits from different forms of IMF products may differ. Therefore, it would be interesting to compare the results obtained in this thesis with the corresponding liquid MEM-IMF and HT-IMF products.

To conclude, although we consider MEM-IMF superior to HT-IMF, breast milk is considered the 'gold standard' in infant nutrition. Ultimately, dietary intervention studies with babies must be performed to compare MEM-IMF to breast milk.

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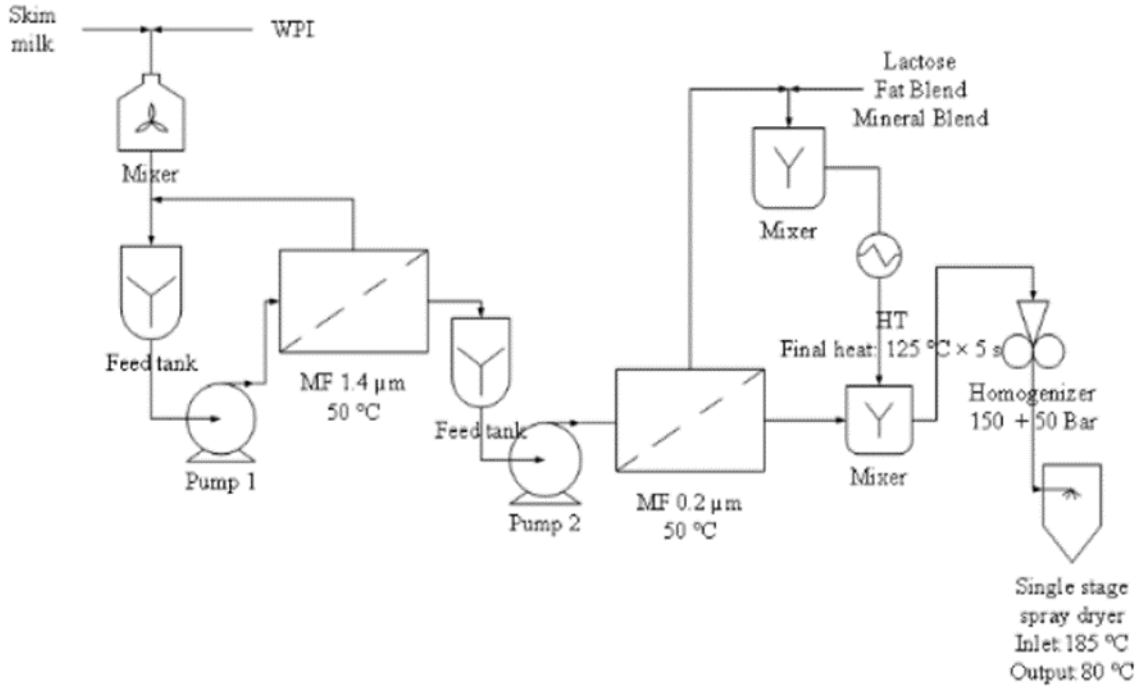
Appendix A

Supplementary material

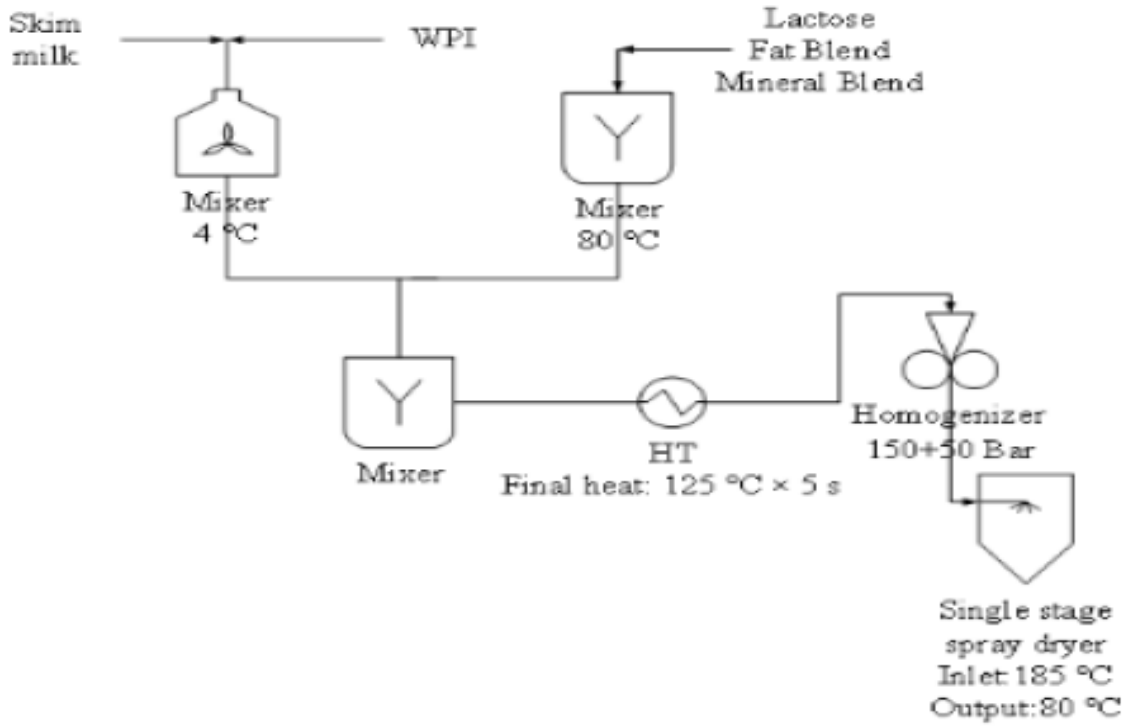
Supplementary Table A1. Macronutrient content of infant milk formula produced by membrane filtration (MEM-IMF) and high-temperature infant milk formula (HT-IMF) powders

Macronutrient	**g/100 g powder**[1]
Protein	10.8
Carbohydrate (lactose)	58.3
Fat	29.1

[1]Both MEM-IMF and HT-IMF powders had identical ingredients.



Supplementary Figure A1. Infant milk formula produced by membrane filtration (MEM-IMF) processing flow chart.



Supplementary Figure A2. High-temperature infant milk formula (HT-IMF) processing flow chart.

Appendix B

Publications from this thesis