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Investigating optimal lipid:protein profiles for infant milk formula

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by

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Declaration

I declare that this thesis, which I submit to Munster Technological University, is my own personal effort. I have not already obtained a degree from Munster Technological University or elsewhere based on this work. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

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Abbreviations

AA	Amino acid
ALA	Alpha linolenic acid
α-lac	Alpha-lactalbumin
αWPC	Alpha-lactalbumin enriched whey powder
AMF	Anhydrous milk fat
AO	Antioxidant
ARA	Arachidonic acid
β-Lg	*β*-Lactoglobulin
BM	Bovine milk
DHA	Docosahexaenoic acid
DWP	Demineralised whey powder
DIAA	Digestible indispensable amino acids
EFSA	European Food Safety Authority
EPA	Eicosapentaenoic acid
FA	Fatty Acid
GI	Gastrointestinal
GIT	Gastrointestinal tract
HM	Human milk
IAA	Indispensable amino acids
IMF	Infant Milk Formula
LA	Linoleic acid
LCPUFA	Long-chain polyunsaturated fatty acids
MFGM	Milk fat globule membrane
OB	Oil blend
PL	Phospholipid
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acid
RCT	Randomised Clinical Trial
ROS	Reactive oxygen species

RNS	Reactive nitrogen species
SFA	Saturated fatty acid
SGID	Simulated gastrointestinal digestion
SM	Sphingomyelin
SMP	Skimmed milk powder
TAG	Triacylglycerols
WHO	World Health Organization
WPC	Whey protein concentrate
WPH	Whey protein hydrolysate
WPI	Whey protein isolate

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Thesis abstract

From the early twentieth century onward, much research has focused on improving infant milk formula (IMF). During the development of IMF, breast milk is commonly considered as the reference and IMF manufacturers are thriving to mimic the composition and/or to match the functionality of breast milk as closely as possible. A variety of bioactive ingredients, found in human breast milk, have been isolated and investigated for their potential functionality and utilised in the production of IMF.

This study evaluated the current knowledge on compositional differences between human breast milk and IMF, and recent advances and developments in IMF production. Following this evaluation, the present research aims to generate novel IMF formulations with different protein and lipid profiles, using novel oils and different whey sources.

It is hypothesised that blending a variety of vegetable oils in suitable proportions will produce a fatty acid profile that more closely mimics that of human milk and that the addition of alpha-lactalbumin (α-lac) enriched whey protein concentrate (αWPC) will improve protein quality and phospholipid (PL) profile in IMF. The bioactive properties of produced formulations are then investigated and compared prior to and following simulated *in vitro* gastro-intestinal digestion (SGID) with an infant model, to allow a more physiologically relevant evaluation of the potential bioactivity of bioavailable compounds. Specific *in vitro* bioactivities which are investigated are anti-infective, anti-adhesion, and antioxidant properties.

IMF prototypes enriched with αWPC resulted in higher α-lac levels and PLs, which were all closer to these reported in human milk, compared to prototypes, containing demineralised whey powder (DWP). This study has shown that there is no bactericidal activity or anti-adhesion effect exhibited by designed IMF prototypes, however, several tested oil blends have demonstrated the ability to protect against cellular oxidative damage induced by hydrogen peroxide (H_2O_2). The results have demonstrated the potential benefits of IMF enrichment with αWPC. This strategy may serve as a viable option to further support the development of optimal IMF for infants.

Chapter 1. Literature review

1.0 Introduction

Infants have a limited diet and therefore it is crucial they receive the right nutrients to promote growth and development in early childhood. Research also suggests that the quality of early nutrition may impact health in adulthood and later life (Pirilä *et al.*, 2011; Ahern *et al.*, 2019). Being a natural first food for babies, breastmilk provides all the energy and satisfies the nutritional requirements of infants until the age of four to six months (Hur *et al.*, 2011). According to the World Health Organization (WHO) breastfeeding is associated with both short-term and long-term health benefits for infants, such as the reduced risk of mortality from infectious diseases and lower morbidity from gastrointestinal (GI) diseases and allergies (Scrimshaw, 2008; Timby *et al.*, 2015). Human milk (HM) protects against a broad range of bacteria and enteric viruses, the most common pathogens causing diarrhoea, which is one of the leading causes of morbidity and mortality in children under five (Isaacs, 2005), positively impacts bone health (Casazza, Hanks and Fields, 2014), supports several physiological functions relating to immune and brain development, lipoprotein metabolism, GI function, and activation of signalling pathways linked to growth (Koletzko, 2017). Furthermore, it is a source of antioxidant agents, including vitamins C and E, phytochemicals, enzymes, and bioactive peptides, which help infants cope with oxidative damage caused by high concentrations of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Aycicek *et al.*, 2006; Tsopmo, 2018).

Breast milk is the best form of nutrition for infants, but infant milk formula (IMF) is an effective milk substitute when breastfeeding is inadequate or not possible (Ahern *et al.*, 2019). Most IMFs are based on cow's milk, but other milk sources can include soya or rice milk. While all milk sources, including animal and human milk, contain a similar percentage of water, the relative amounts of carbohydrates, protein, fat, vitamins, and minerals can vary. Thus, to make IMF more comparable to HM, a variety of ingredients, such as nucleotides, fatty acids, vitamins, minerals, oligosaccharides and probiotics are added (Kent *et al.*, 2015; Martin, Ling and Blackburn, 2016). Manufacturers of IMF strive to produce formulae that have a similar composition and nutritional profile to HM, so that IMF-fed newborns and growing infants are provided with optimum nutrition for growth and development (Kent *et al.*, 2015). However, it is still evident from epidemiological studies that differences remain between breastfed infants and formula-fed infants with regard to both short-term (e.g. susceptibility to infections, cognitive development) and long-term (e.g. development of chronic diseases such as obesity, diabetes, cardiovascular

disease) health outcomes (Ip *et al.*, 2009; Montagne *et al.*, 2009; Lonnerdal, 2016). Additionally, there are still considerable compositional differences between HM and IMF in terms of the fat, protein, and other nutrient content, which can impact digestion and gut biology (Huërou-Luron, 2016). As it is challenging for IMF manufacturers to exactly mimic HM composition, recent studies have focused on developing IMF to contain ingredients that will have comparable health benefits (Koletzko *et al.*, 2011). A variety of bioactive ingredients are being studied and investigated through clinical trials to achieve the goal of similar growth and development patterns in both breast-fed and formula-fed infants. However, there is no consensus if these novel bioactive ingredients added to IMF have the same functional effects as those found in HM (Almeida *et al.*, 2021).

In recent years, the importance of dietary fats and proteins in infant nutrition has gained increasing scientific interest. Rather than just a source of energy, it has become evident that the composition and structure of dietary fats and proteins in the infant diet could have profound influence on infant development, physiology, and health (Carlson, 2009; Delplanque *et al.*, 2015; Lonnerdal, 2016). Modifying the protein and lipids profiles of IMF, using a variety of vegetable oils or bovine milk fats and different proteins, may serve as a potentially effective strategy to improve the composition of IMF and meet the needs of developing infants. To improve the protein quality of IMF recent research is focusing on reducing the overall protein content, matching proportions of the individual whey proteins and caseins found in HM and ensuring provision of all essential amino acids. An ingredient particularly suitable for addition in IMF is an α-lactalbumin enriched whey protein fraction (McSweeney, O'Regan and O'Callaghan, 2013). Combination of various oils, including novel oils, such as β-palmitate, and/or anhydrous milk fat (AMF) allows for matching the type and level of dominant and physiologically functional FAs found in HM.

Furthermore, there is a need to study the digestibility of potentially bioactive components supplemented in IMF to better understand the breakdown mechanisms in the infant GI tract and their bioavailability, which may be affected by thermal processing and storage (Nguyen *et al.*, 2015a; Almeida *et al.*, 2021).

Present review is focused on the IMF current market and IMF design, as well recent advances in IMF production. Up-to-date knowledge of HM composition and infant nutritional requirements, with emphasis on proteins and lipids, is also reviewed.

1.1 Infant requirements for lipids and proteins

The recommended daily requirement of any nutrient is identified as the amount which must be consumed on a regular basis to maintain health in an otherwise healthy individual (EFSA, 2013). Normal growth reflects overall health and nutritional status. Normal growth of infants is the progression of changes in height, weight, and head circumference that are compatible with established World Health Organization (WHO) growth standards, which include length/height-for-age, weight-for-age, weight-for-length, weight-for-height and body mass index-for-age (WHO Child Growth Standards, 2006).

Rapid growth differentiates infancy from all other life stages in terms of nutritional needs. Furthermore, infant nutritional requirements change considerably over the first year of life; with growth accounting for over 50% of protein requirements during the first month of life, reducing to less than 20% by the end of the first year (Duggan *et al.*, 2016). Breast milk naturally meets all the nutritional needs of an infant; therefore, it is used as a gold standard to estimate the nutritional requirements of an infant. These requirements can also be met if a food providing energy (e.g. IMF) contains equivalent types and amounts of nutrients to human breast milk (Roth and Castillo, 2019).

1.1.1 Lipid requirements

Lipids play a critical role in infant nutrition and development (Zou, Pande and Akoh, 2016). Dietary fat is a major source of energy that supports an infants need for energy expenditure, fat oxidation and fat deposition during growth (Krohn, Demmelmair and Koletzko, 2016). It also provides the essential lipid-soluble vitamins (A, D, E, K), as well as omega-6 and omega-3 polyunsaturated fatty acids (PUFA). HM fat contributes 90% of the energy retained by infants during the first 6 months, with numerous lipids known to markedly affect infant growth, development, and health (Koletzko *et al.*, 2011; Delplanque *et al.*, 2015).

During the first month of life energy requirements of healthy, term, normal-weight infants is estimated to be about 120 kcal/kg/d and close to 100 kcal/kg/d during the rest of the first year of life (Krohn, Demmelmair and Koletzko, 2016). Approximately half of an infant's calories should be obtained from fat to meet the demands of growth and development (Zou, Pande and Akoh, 2016). Fat intake in newborns, at 2.5-3.5 g/kg/d, is three to five times higher than that required in adults (Abrahamse *et al.*, 2012)**.** While it is not possible to define a quantitative requirement for total fat (EFSA, 2013), the current recommended total fat intake that is considered adequate for infants from birth to 6 months is 30 g/day and from 6 months to 1 year is 31 g/day (EFSA NDA Panel, 2013). Along

with total fat intake, essential fatty acids (FAs), including linoleic acid (LA) and alpha linolenic acid (ALA), are required as substrates for the synthesis of hormones, eicosanoids, and long chain polyunsaturated fatty acids (LC-PUFAs), especially arachidonic (ARA), eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids. The current recommendation for LA is 4.4 g/day for infants younger than 6 months of age and 4.6 g/day for infants from 7 months to 1 year (Zou, Pande and Akoh, 2016).

1.1.2 Protein Requirements

Protein is an essential component of the diet, supplying the body with nitrogen (N) and amino acids (AAs), as well as other non-protein metabolically active nitrogenous substances (EFSA, 2013). Nitrogen utilisation and therefore protein requirements are critical in maintaining whole body homeostasis, whereas AAs are needed for tissue formation and multiple physiologic functions, such as energy generation, peroxidative protection, neurotransmitter synthesis and lymphocyte proliferation (Roth and Castillo, 2019). Therefore, protein intake of adequate quantity and quality during the first 2 years of life has important effects on growth, neurodevelopment, and long-term health (Michaelsen and Greer, 2014; Roth and Castillo, 2019).

According to the European Food Safety Authority (EFSA, 2013), protein requirement is defined as the minimum intake that will lead to a positive nitrogen balance to allow for growth in normally growing subjects and comprises two components - the maintenance requirement and the growth requirement (EFSA, 2013). However, estimation of adequate protein requirements for infants can be quite challenging, due to body proteins being in a continuous dynamic state of synthesis and catabolism (Roth and Castillo, 2019). Furthermore, factors, such as intake of other nutrients, protein digestibility, AA content of proteins, and protein quality will influence protein requirements (Roth and Castillo, 2019). Currently, the recommended protein intake from birth to 6 months is 9.1 g per day, and from 7 to 12 months is 11 g per day (Zou, Pande and Akoh, 2016).

1.2 Human milk composition

There is consensus that breast milk is the best source of nutrition for infants; it contains all the essential nutrients to support growth and development, as well as providing immunostimulatory components and protection against infection (EFSA, 2014).

HM is a complex food matrix, generally composed of water (~87%), fats (~4%), protein (~1%) and lactose (~7%). Furthermore, it contains vitamins, minerals, digestive enzymes, hormones and immune cells, i.e. macrophages and stem cells (Martin, Ling and

Blackburn, 2016). Classification of HM into three different classes, including colostrum (milk produced during the first (0-5) days after birth), transitional milk (day 6 to 15 postpartum) and mature milk (after day 15 postpartum), reflects the gradual alteration in its composition throughout lactation. For example, colostrum is significantly different from mature milk, containing higher concentrations of whey protein, whilst lower caseins, as well as lower concentrations of both lactose and fat. Colostrum also has greater concentrations of secretory immunoglobulin and growth factors, such as epidermal growth factor, transforming growth factor-beta (TGF-β) and colony-stimulating factor (Andreas, Kampmann and Mehring Le-Doare, 2015). Therefore, HM is a dynamic food source, changing over time to adapt to the growing needs of the infant and in response to various endogenous and environmental factors (Andreas, Kampmann and Mehring Le-Doare, 2015; Martin, Ling and Blackburn, 2016). These factors can include length of lactation, stage of the nursing process, as well as maternal characteristics, such as diet, age, ethnicity. In addition, many studies have reported on the benefits of HM in relation to GI function, reduced risk of infectious diseases, neurological development and impact on the immune system (Demmelmair and Koletzko, 2018). Recently, studies have also suggested that breast milk mitigates infant programming of late onset metabolic diseases, such as obesity and type 2 diabetes (Martin, Ling and Blackburn, 2016).

1.2.1 Lipids

Lipids contribute 40-55% of the total energy content of breast milk in the form of emulsified milk fat globules (Hendricks and Guo, 2014; Andreas, Kampmann and Mehring Le-Doare, 2015), in addition to contributing to taste and aroma (Martin, Ling and Blackburn, 2016). While in general, human milk fat content ranges from 3.5% to 4.5% during lactation, it is the most variable macronutrient component of HM, comprising a complex mixture of different lipids, with quantitative dominance of triglycerides (98% of lipid fraction) (Demmelmair and Koletzko, 2018). Other lipids include diacylglycerides, monoacylglycerides, free fatty acids, phospholipids, cholesterol, and complex lipids. Lipid content is not only dependant on the maternal diet but also changes with stages of lactation, time of day and within feedings (Koletzko *et al.*, 2011), although the reasons for the changing fat content in HM have not been fully elucidated to date. The regulation of milk fat synthesis is not fully understood either, but possibly depends on the availability of substrates and hormones, such as prolactin, growth hormone and insulin (Demmelmair and Koletzko, 2018).

Breast milk contains over 200 fatty acids (Andreas, Kampmann and Mehring Le-Doare, 2015) whose composition largely determines the nutritional and physicochemical properties of HM fat. The FA profile in HM is primarily determined by the milk triglycerides that form the core of the milk fat globule (Demmelmair and Koletzko, 2018). Some fatty acids are present in low concentrations (Table 1.2), while others are more dominant, such as monounsaturated oleic (35%) and saturated palmitic acids (23%) (Martin, Ling and Blackburn, 2016).

Beyond energy, HM also delivers lipids with a specific functionality, such as essential fatty acids (FA), phospholipids, and cholesterol. Healthy development, especially of the nervous and digestive systems, depends fundamentally on these components (Abrahamse *et al.*, 2012)

1.2.2 Proteins

HM contains over 400 different proteins that contribute to its unique qualities (Andreas, Kampmann and Mehring Le-Doare, 2015). The average protein content in breast milk gradually decreases from the second to the seventh month of lactation, after which protein content levels off (Andreas, Kampmann and Mehring Le-Doare, 2015). In HM protein levels range from 1.4-1.6 g/100 mL during early lactation, 0.8–1.0 g/100 mL after 3–4 months of lactation, and 0.7–0.8 g/100 mL after 6 months (Hendricks and Guo, 2014; Lönnerdal et al., 2017).

Caseins, whey, and mucin proteins are three major groups of proteins present in milk. Whey and casein are classified according to their solubility; whey proteins being soluble in the serum fraction of milk and are easier to digest, caseins being present in casein micelles and becoming curds in the stomach. Mucins are present in the milk fat globule membrane (Andreas, Kampmann and Mehring Le-Doare, 2015; Martin, Ling and Blackburn, 2016). The whey:casein ratio in HM fluctuates depending on the stage of lactation. At the beginning of lactation, whey protein concentration is very high, and casein is virtually undetectable, with the whey:casein ratio between 70:30 and 80:20. Casein synthesis in the mammary gland and the volume of milk produced increase as lactation progresses, and the concentration of whey proteins decreases, bringing the ratio down to 50:50 in late stages (Lonnerdal, 2003).

The functions of proteins present in HM are not just limited to their nutritional value, but also includes their multi-functional roles as bioactive compounds, possessing antimicrobial and immunomodulatory properties, enzymatic and probiotic activity, as

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Table 1.1 Concentration and potential health benefits of main proteins in human milk, bovine milk and infant formula.

	HM	BM	IMF	Health Benefits	References
Total protein (g/L)	9-12	18-20	12 -20		
Whey proteins (g/L)	5.3-6.6	~5.0	~7.2		
α-lactalbumin (g/L)	2-3	1.2	1.4-2.3	Source of essential AAs, and peptides with antibacterial, immune-modulating, and antiviral peptides Prebiotic activity; enhancing trace mineral (Fe, Zn) absorption	(Kamau *et al.*, 2009; Chatterton *et al.*, 2013a; Lönnerdal *et al.*, 2017)
β– lactoglobulin (g/L)	None	3-4	3.5	Anti-inflammatory	(Chatterton *et al.*, 2013a)
Immunoglobulins (g/L)	1.2	0.6-1.0	None	GIT protection against enteric antigens Anti-inflammatory and Immune-modulatory	(Chatterton *et al.*, 2013a; Fenelon *et al.*, 2018)
Lactoferrin (g/L)	2-6	0.01-0.1	0.048	Immunomodulatory and antibacterial Iron uptake and modulator of gut permeability	(Manzoni, 2016; Lönnerdal *et al.*, 2017)
Serum albumin (%)	10-15	10-15	no data	Source of AAs	(Fenelon *et al*, 2018)
Osteopontin (mg/L)	~50 -138	~18	~9	Bone remodelling and immune modulatory Cognitive and intestinal development in infants	(Fenelon *et al.*, 2018)
Lactophorin (mg/L)	None	300	no data	Immune stimulating; antibacterial, inhibitor of human rotavirus	(Fenelon *et al.*, 2018)
Enzymes					
Lysozyme (mg/L)	160-460	0.07-0.06	n.d.	Antibacterial activity	(Lönnerdal *et al.*, 2017)
Lactoperoxidase (mg/L)	5.17	13-30	n.d.	Antibacterial	
Superoxide dismutase (U/ml)	11.2	0.06-2.88	n.d.	Free radical scavenger	(Chatterton *et al.*, 2013b)
Alkaline phosphatase(U/ml)	54	250	n.d.	Dephosphorylation of LPS	(Chatterton *et al.*, 2013b)
Casein (g/L)	3.5 -4.4	~30	~4.8		
β-casein	2.6-2.7	9-11	1.62	Enhancing calcium absorption and opioid activity	(Chatterton *et al.*, 2004)
κ -casein	1.2	3-4	0.6	Antibacterial activity	(Lönnerdal *et al.*, 2017)
MFGM Proteins					
MFG-E8 (mg/L)	50-200	28.2-48.4	n.d.	Antibacterial and antiviral	(Lönnerdal *et al.*, 2017)

n.d., not detected; AA, amino acids; HM, human milk; BM, bovine milk; IMF, infant milk formula

well stimulating absorption and utilisation of micronutrients and macronutrients from the milk (Hendricks and Guo, 2014; Andreas, Kampmann and Mehring Le-Doare, 2015; Lönnerdal *et al.*, 2017).

Proteins present in significant concentration in the whey fraction are α-lactalbumin (α-lac), lactoferrin and secretory IgA, all of which promote immunomodulation, followed by serum albumin and lysozyme (Hendricks and Guo, 2014; Andreas, Kampmann and Mehring Le-Doare, 2015). Whey proteins can bind to glycolipids and glycoproteins, which are fundamental in aiding metabolism (Ahern et al., 2019).

α-Lac, one of the major whey proteins in HM, constitutes up to 35% of the total protein content, with highest concentration in colostrum (4.30 mg/ml), which decreases more gradually than concentrations of other proteins and AAs (Lönnerdal et al., 2017). α-Lac and its peptides have shown to have various physiological functions in the developing infant beyond its role as a well-balanced source of essential AAs.

Another dominant whey protein with multiple functions is lactoferrin, which constitutes 20% of true protein in breast milk. Its concentration peaks at 7 mg/ml in colostrum and decrease to 1 or 2 mg/ml in mature milk. Lactoferrin is known to facilitate the uptake of iron into cells, it has both bactericidal and bacteriostatic effects, and is also an effective modulator of inflammatory and immune responses (Lönnerdal et al., 2017).

There are several immunoglobulins found in HM, including sIgA, IgG and IgM. Accounting for 90% of total immunoglobulins, sIgA is the most prominent immunoglobulin throughout lactation, with highest levels in colostrum (5.45 mg/ml at days 0 to 5, 1.50 mg/ml 6 to 15 days after delivery) (Lönnerdal et al., 2017). Transfer of sIgA via breast milk boosts the immunity of the infant through the acquired immunity of the mother (Lönnerdal et al., 2017). sIgA antibodies found in breast milk act against numerous pathogens, such as *E. coli*, *V. cholera*, *H. influenza*, *S. pneumoniaie*, *Clostridium difficile* and *Salmonella*, *C. albicans* rotavirus and influenza.

Casein is present in HM at low concentrations (13% of the total protein) (Andreas, Kampmann and Mehring Le-Doare, 2015). Main types of casein proteins in HM are α-, β-and κ-caseins, with κ-casein stabilizing the former two by forming a colloidal suspension. Casein is a source of AAs and trace elements (calcium, iron, and zinc) as well as bioactive peptides with a variety of physiological functions, such as immunomodulatory, antioxidant, satiating, and GI activity, as well as mucosal development and sleep induction (Almeida *et al.*, 2021). Other notable proteins, their concentrations in HM and their potential biological activities are presented in Table 1.1.

Table 1.2. Typical composition of Fatty Acids in human milk, bovine milk and IMF designed with various oil blends

	Fatty acid composition (% of total fatty acids)				
Fatty Acid	**Human Milk**	**Bovine Milk**	**IMF with veg. fat[a]**	**IMF with milk fat[b]**	**IMF with palm oil-free veg. fat[c]**
Total Saturated	**33.8-46.9**	**53-84**	**43.9**	**48**	**34.8**
Butyric	ND	3.3	ND	2.4	ND
Caproic	0.03-0.79	1.6	ND	1.3	0.2
Caprylic	0.08-0.61	1.3	0.4-2.1	1.7	2.5
Capric	0.72-1.71	3.0	0.1-1.7	2.2	1.8
Lauric	2.31-6.74	3.1	0.2-13.6	6.3	13.4
Myristic	3.98-8.67	14.2	0.9-7.0	7.2	5.2
Pentadecanoic	0.20-0.25	1.03	ND	0.6	ND
Heptadecanoic	0.19-0.41	0.59	ND	0.3	ND
Palmitic	16.6-25	42.9	15.9-31.7	18.9	7.7
Stearic	3.39-6.89	5.7	5.3	6.7	3.2
Arachidic	0.13-0.83	0.14	ND	0.3	0.3
Total Monounsaturated	**35-40**	**13-42**	**38.2**	**30.0**	**43.4**
Myristoleic	0.18-0.26	1.01-1.19	ND	0.8	ND
Palmitoleic	1.62-5.2	1.5	0.-1.1	1.1	0.1
Oleic	26.5-35.6	17.2	37.6	28.1	43.3
Nervonic	0.03-1.21	ND	ND	ND	ND
Total Polyunsaturated	**14-19**	**2-4**	**16.1**	**18.2**	**22.8**
Linoleic	10-24.3	1-2	14	16.7	20.5
Arachidonic	0.7-1.1	0.1	0.3	ND	0.3
Alpha-linolenic	0.1-2.0	0.2-1.3	1.6	1.5	1.8
Gamma-linolenic	0.06-0.83	0.07	ND	ND	ND
Eicosapentaenoic	0.06-0.33	0.07	ND	ND	0.0
Docosahexaenoic	0.2-0.5	ND	0.2	ND	0.2

[a]IMF with vegetable fat - palm, rapeseed, soybean and coconut oils as major fats, [b]IMFs with milk fat - bovine milk fat, corn oil, and other non-specified vegetable fats, [c]IMFs with palm oil-free vegetable fat - high oleic sunflower, coconut, soy oils as major fats. ND, not determined. Data from Hageman *et al.* (2019)

1.3 Infant milk formula

Up to mid-nineteenth century, infants were wet nursed by another woman if the biological mother's milk was not available. The first generation of IMF available to the public in 1865 was designed by German chemist Justus von Liebig and was known as 'Liebig's Perfect Infant Food'; a powder made with malt and wheat flour, and potassium bicarbonate, which had to be diluted with cow's milk (Baker *et al.*, 2021). By 1867 Swiss confectioner Henri Nestlé developed his own powdered breast milk substitute - Nestlé's milk, which was cheaper and more convenient, being diluted with water. This marked the start of an era of IMF commercial production and development. By 1883 a further 27 patented brands of IMF had been launched (Baker *et al.*, 2021). Available powdered IMF used cows' milk, as well as butterfat and corn syrup, as a source of fat and carbohydrates respectively. No vitamins or minerals were added and protein content was far greater than that of current formulas (Duggan *et al.*, 2016). Through continuous innovation infant milk formulations gradually improved from the end of the nineteenth century, such that the composition of IMF is now considerably different to the original products. While overall protein content has been reduced whey proteins are included to improve the whey/casein ratio, and vegetable oils and lactose have replaced butterfat and corn syrup. Furthermore, formulas are now fortified with essential components (e.g. iron, taurine, nucleotides), LC-PUFAs, prebiotics (fructo-oligosaccharides/galacto-oligosaccharides) and lutein (Happe and Gambelli, 2015; Duggan *et al.*, 2016; Lonnerdal, 2016).

From economic perspective IMF markets have steadily expanded reaching ~US$1.5 billion by 1978, US$22.9 billion by 2005, and US$55.6 billion by 2019. In particular, China now has the world's largest and most competitive market, representing 32.5% of global sales by 2019 (Baker *et al.*, 2021). According to Global Market Insights the IMF market is set to grow to over ~US$98 billion by year 2025being driven by a massive growth in manufacturing inputs and increased demand from consistent population growth and the evolving lifestyles of parents (Infant Formula Market Size and Share | Industry Statistics - 2025, 2022).

In Ireland, the IMF industry contributes significantly to the Irish economy. Ireland currently produces approximately 10% of the entire global exports of IMF, and three of the largest infant nutrition companies (Danone, Nestle, Abbott) have manufacturing facilities in Ireland. In 2014, Irish IMF exports were valued at €900 million, and now that Ireland has become the second-largest exporter of IMF to China this figure is set to grow over the next decade (Enterprise Ireland, 2022; EPHA, 2022).

1.3.1 IMF design

IMF production is primarily focused on mimicking human breast milk as closely as possible, with the basic composition of IMF being a combination of five constituents: fat, protein, carbohydrates, vitamins, and minerals. Primary ingredients in IMF include bovine, goat or soy milk solids mixed with whey protein, lactose, vegetable oils and vitamin and mineral premixes (Happe and Gambelli, 2015; Fenelon *et al.*, 2018).

Bovine milk is considered the most adequate substitute for HM and is primarily used to produce IMF. However, it still has considerable differences in terms of protein (Table 1.1), fatty acids (Table 1.2), carbohydrate, mineral, and vitamin content compared to HM (Ahern *et al.*, 2019).

Bovine milk is modified so that the protein level, whey/casein ratio and AA profile closely resembles HM composition (Ahern *et al.*, 2019). Such modifications include fortification with whey protein concentrate (WPC), whey protein isolates (WPI) and/or whey protein hydrolysate (WPH) (Fenelon *et al.*, 2018). However, bovine milk is deficient in essential AAs, such as methionine, cysteine, and tryptophan, therefore, to ensure an adequate supply of these amino acids, the protein content of IMF, made from bovine milk, is still higher (1.3 - 1.5 g/100 g) than that of HM (~0.8 - 1.0 g/100 g) (Fenelon *et al.*, 2018).

Bovine milk fat was used as a staple source of lipids in IMF up until 1970s. However, bovine milk fat contains approximately twice as many fatty acids (400 FAs) as HM, and also has higher levels of saturated fatty acids (SFAs) and lower levels of MUFAs and PUFAs (Table 1.2). Therefore, the use of dairy fats in IMF has gradually been reduced, with manufacturers largely replacing animal fats with vegetable fats in order to provide higher levels of MUFAs and PUFAs (Delplanque *et al.*, 2015). However, the addition of bovine milk fat, as anhydrous milk fat, in combination with vegetable fats is still quite common (Hageman, Danielsen, *et al.*, 2019).

The lipid content in IMF depends on the fat blend used, but, in general, IMF has a much less complex lipid composition than HM. The major differences between HM, bovine milk, and IMF, and even within IMF products, relate to the variety of SFAs, PUFAs, cholesterol, and complex lipids.

To match the type and level of dominant and physiologically functional FAs found in HM, various oil blends are currently incorporated into IMF. Typically, a calculated combination of commercial vegetable oils are chosen as a source of SFAs (C8:0-C18:0), MUFAs (e.g., oleic acid) and PUFAs (Zou *et al.*, 2017). Common vegetable fats that are used in IMF are coconut oil, corn oil, soybean oil, palm oil (olein and kernel), high oleic

sunflower oil, high oleic safflower oil and low erucic acid rapeseed oil (Hageman, Danielsen, *et al.*, 2019). Coconut and palm kernel oils are used as the source of medium chain triacylglycerols, such as lauric and myristic acids, soybean, and rapeseed oils as a source of α-linolenic acid. Palm olein provides mainly palmitic, oleic acid, and some linoleic acid (Happe and Gambelli, 2015). Composition of FAs in the IMF with different vegetable oil blends are presented in the Table 1.2. Addition of bovine milk fat in the form of anhydrous milk fat, that contains triglycerides and other components like cholesterol and fat-soluble vitamins, is also common (Hageman, Danielsen, *et al.*, 2019). Other sources of lipid that can be used include single cell oils, dairy lipids, egg lipids, fish oils and fractionated lipids (Delplanque *et al.*, 2015).

1.3.2 Recent advances IMF production

To refine the content of IMF and simulate the functionality of HM as much as possible, IMF manufacturers continue to innovate their products. Over the last few decades, due to scientific and technological advances, new fields of innovation in neonatal nutrition have emerged. For example, specific bioactive components identified in HM can be isolated and supplemented into IMF thus improving the health of formula fed infants (Ahern *et al.*, 2019). Several studies have reported on the benefits of adding multifunctional bioactive ingredients to IMF, including prebiotics (e.g. human milk oligosaccharides), probiotics (e.g., *Bifidobacterium lactis, Lactobacillus rhamnosus, Bifidobacterium longum*), novel protein fractions (e.g., lactoferrin, osteopontin and α-lac) and specialised lipid fractions (e.g., LC-PUFAS, MFGM and sn-2 palmitate) (Montagne *et al.*, 2009; Ahern *et al.*, 2019; Yu *et al.*, 2019). Table 1.3 provides a summary of bioactive ingredients used to improve the nutritional composition of IMF, with a focus on proteins and lipid profiles.

Studies have reported that adding complex lipids and milk fat globule membranes (MFGM) to vegetable oil-based IMF has the potential to enhance infant development and reduce infections (Gurnida *et al.*, 2012; Tanaka *et al.*, 2013; Timby *et al.*, 2015; Le Huërou-Luron *et al.*, 2016). MFGM is a complex milk fraction composed of lipids (sphingomyelin, cholesterol, phosphatidylcholine, and phosphatidylethanolamine and gangliosides) and proteins (mucin (MUC1), lactadherin, lactoferrin), many of which have recognised health properties (Delplanque *et al.*, 2015). For example, cholesterol stabilizes the structure of cellular membranes, and is incorporated into brain lipids during the first months of life, as well as contributing to the synthesis of bile acids, lipoproteins, vitamin D and hormones (Kinney *et al.*, 1994). Gangliosides constitute up to 10% of brain lipids,

Table 1.3. Examples of bioactive proteins and lipids added for improvement of IMF nutritional composition

Bioactive ingredient	Characteristics	References
α-lac	Addition to IMF results in plasma tryptophan concentrations similar to those in breastfed infants Addition of α-lac-enriched WPC to IMF results in formulations with cholesterol, gangliosides and PLs levels similar to HM Antibacterial, immune-modulating, and antiviral properties as well as prebiotic activity and an ability to enhance trace mineral (Fe, Zn) absorption	(Ren, Stuart and Acharya, 1993; Heine *et al.*, 1996; Davis *et al.*, 2008; Sandström *et al.*, 2008; Kamau *et al.*, 2009); Moloney *et al.* 2020)
Lactoferrin	Non-heme iron binding protein Antimicrobial and immunomodulating effects, influence on iron absorption Bifidogenic activity and intestinal development Increased memory and learning abilities due to lactoferrin enriched formula	(Demmelmair *et al.*, 2017; Ahern *et al.*, 2019)
Osteopontin	One of small integrin-binding ligand, N-linked glycoproteins mediating cell-matrix interactions and cell signalling Identified as a linking protein and crucial factor in extracellular bone biomineralization Bone remodelling, immune modulatory functions, and cell proliferation	(Demmelmair *et al.*, 2017)
LC-PUFAs	Fatty acids of carbon chain longer than 20 with multiple double bonds, mainly DHA (22:6 n-3) and ARA (20:4 n-6) Important for optimal brain development, including visual and cognitive functions Anti-inflammatory properties and perform immune functions in allergy, asthma, and pulmonary function	(Wei, Jin and Wang, 2019)
MFGM	Triple membrane structure covering on milk fat globule, which consists of proteins and lipids Provide considerable amounts of free and esterified cholesterol, gangliosides, sphingomyelin, protein etc. Have potential health benefits that include development of cognitive functions Antimicrobial and antiviral properties	(Hernell *et al.*, 2016; Koletzko, 2017; Demmelmair and Koletzko, 2018)
AMF	Potential positive effect on digestive physiology and microbiota development SCFAs and MCFAs in milk fat, that are not present in vegetable fat, are more efficiently absorbed Contains components lacking in vegetable fats (trace amounts of ether lipids, hydrocarbons, fat-soluble vitamins, flavour compounds)	(Delplanque *et al.*, 2013; Bourlieu and Michalski, 2015; Hageman, Danielsen, *et al.*, 2019)
Sn-2 palmitate	Chemically or enzymatically synthesised, specifically structured TAG with palmitic acid (C16:0) predominantly enriched in sn-2 position, Better absorption of FAs (myristic, palmitic, stearic acids) Reduces calcium faecal loss and positively influences fat absorption and metabolism	(Bourlieu, Bouzerzour, *et al.*, 2015; Wei, Jin and Wang, 2019)

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with high concentrations in the cerebral cortex (Koletzko, 2017), while sphingomyelin plays an important role in the structure of the brain cell membrane (Tanaka *et al.*, 2013).

In the late 20th century, it was discovered that HM fat is rich in TAGs with palmitic acid at the sn-2 position (> 70%) on the glycerol backbone, compared to the lower levels in bovine milk fat (40-45%) or vegetable oils (<10%) (Happe and Gambelli, 2015; Wei, Jin and Wang, 2019). While palm oil or bovine milk fat-based IMF can be designed to have a comparable FA composition, the positioning of the FA in the TAGs differs. To address this sn-2 palmitate, or β-palmitate, is a chemically or enzymatically modified TAG employed in the production of IMF to mimic the positional distribution of FAs in the TAGs found in HM.

Supplementation of IMF with anhydrous milk fat (AMF) is also becoming more prevalent (Moloney, O'Connor and O'Regan, 2020). Incorporation of AMF modifies the structure of the fat emulsion and its composition in terms of polar and apolar lipid fractions. The high proportion of TAG in AMF with palmitic acid positioned at the sn-2 position of the glycerol backbone allows for design of IMF that better mimics the structure and composition of HM which could also improve physiological properties (Delplanque *et al.*, 2015). However, bovine milk fat contains low levels of linoleic acid, therefore, to reach the required amount of linoleic acid addition of vegetable fat is needed (Hageman, Danielsen, *et al.*, 2019).

Strategies to improve the protein content of IMF include supplementing with purified BM proteins to match their levels in HM and/or improve the functionality of the IMF. In particular, supplementation with proteins such as lactoferrin, osteopontin and α– lac. These are multifunctional proteins, abundant in HM, and commercially available ingredients that have been deemed safe for inclusion in IMF(Ahern *et al.*, 2019). α– Lac, one of the major human milk proteins, is available as an enriched whey fraction, its supplementation to IMF has shown to improve protein quality, simultaneously reducing total quantity of proteins (Sandström *et al.*, 2008). Moreover, it has shown to exhibit multiple bioactive properties with health benefits for the infants (Lönnerdal, 2011).

Despite the efforts to improve nutritional composition of IMF, it is acknowledged that IMF cannot provide all the numerous immune protective and bioactive factors present in HM. In addition, lipid structures in IMF are still different from those in HM, which can lead to differences in the kinetics of digestion and assimilation of nutrients (Bourlieu *et al.*, 2017). Therefore, further alterations in the composition of IMF need to be considered.

Extensive amount of evidence indicates that it is reasonable to study the effects that

supplementation of ingredients reviewed in this section to IMF have on infants. Research on these nutritive and non-nutritive components demonstrates their numerous beneficial effects on growth, GI health, and neurodevelopment in early infancy. However, instead of separate enrichment with many different fractions, appropriate use of whey-based ingredients and combination of various oil blends may serve as a useful strategy to increase the concentration of key nutrients in IMF. For example, modified whey protein powder (WPCs) with concentrated MFGM- derived components can be produced during processing, as can WPC enriched in α- lactalbumin. For instance, WPC for use in IMF can be enriched in α- lactalbumin to increase its content of IMF to be closer to that of HM (McSweeney, O'Regan and O'Callaghan, 2013). Importantly, studies comparing IMF enriched with different complex lipid sources (vegetable oils, dairy lipids) indicate they have different biological effects (Du *et al.*, 2012; Delplanque *et al.*, 2013; Timby *et al.*, 2014). Details will be elaborated on further in Section 5.

1.3.3 Bioactive components in IMF

As has been previously mentioned there are many different bioactive components found in HM addition of which provides new opportunities to improve health and nutrition of a formula fed infant (Section 1.3.2). In this section, animal and clinical studies on lipids and proteins as potential bioactive components in IMF are discussed.

1.3.3.1 Lipids as bioactive components in IMF

Despite knowledge on the types of phospholipids (PLs) in HM, there is a lack of studies investigating their functions or requirements in infants. It has been proposed that PLs may protect DNA, proteins, and lipids from oxidative damage, and positively effect on memory, learning capacity and attention, and function of the nervous system (Cilla *et al.*, 2016). However, the EFSA Panel on Dietetic Products, Nutrition and Allergies consider that these compounds lack sufficient characterization to warrant claims (EFSA NDA Panel, 2009). Nevertheless, there is now increasing interest in milk PLs, their bioactivity and their potential effect on nutritional status and health benefits for infants. Numerous animal studies have demonstrated that supplementing the diet with bovine lipid components influences brain development and cognitive function (Hageman, Danielsen, *et al.*, 2019), but few clinical trials have investigated cognitive development in infants fed with IMF with modified lipid composition (Gurnida *et al.*, 2012; Tanaka *et al.*, 2013).

PLs present in human breast milk and bovine milk are primarily associated with the MFGM (Demmelmair and Koletzko, 2018). Several animal studies and clinical trials have

investigated how the addition of bovine MFGM and its individual components to infant diets affect health and development, and cognitive function (Table 1.4). Oshida *et al.*, (2003) suggested that dietary sphingomyelin (SM), which is a major sphingolipid in human and bovine milks and an important structural component of neurons and lipid bilayers, may contribute to myelination of the developing rat central nervous system in the experimental setting of low activity. Few studies suggest a positive association between individual MFGM PLs, including SM and gangliosides, and cognitive development of infants. For instance, Tanaka *et al* (2013) have observed that administration of SM-fortified milk, administered for the first 8 weeks of age, has improved neurobehavioral development of infants at age 12- and 18-months, assessed by Bayley Scales of Infant Development (Second Edition), which evaluates mental and psychomotor parameters using the cognitive, motor, and verbal subscale as an indicator of neural development (Ortega-Anaya and Jiménez-Flores, 2019), Fagan test scores, and sustained attention test scores compared to that of the control group. Gurnida et al. (2012) have demonstrated positive effect of ganglioside supplemented IMF and cognitive functions, such as hand and eye coordination, performance IQ and general IQ as assessed by Griffith Scales. Findings of Timby *et al* (2014) suggest that MFGM supplementation of IMF has a positive effect on cognitive function tested with the Bayley Scales of Infant and Toddler Development. Importantly, addition of the MFGM fraction to IMF has been proven to be safe and well tolerated by infants from birth to 4 months (Billeaud, 2015).

Supplementation of IMF with MFGM may also protect infants from infection. Timby *et al* (2015) have demonstrated that supplementation of formula with bovine MFGM reduced the risk of acute otitis media (middle ear infection) and the use of antipyretics in formula-fed infants. Authors also have suggested that MFGM has immunomodulatory effects on humoral response against pneumococcus vaccine. In Peruvian study (Zavaleta *et al.*, 2011) supplementation with MFGM enriched protein have significantly reduced the incidence of bloody diarrhea in infants aged 6-12 months. Le Huërou-Luron *et al.* (2016) in their animal study have demonstrated that addition of MFGM fragments positively modifies the faecal microbiota composition and accelerates the immune system maturation. Bhinder *et al.* (2017) have also shown that the gut microbiota of rats fed with IMF supplemented with MFGM resembled microbiome levels found to be in mother's milk fed pups. Furthermore, the authors found that addition of MFGM to formula

Table 1.4. Studies reported on safety and health benefits of milk gat globule membrane (MFGM*) and its individual lipids

Clinical Study	Country	Results	Reference
RCT; n = 119, SF enriched with a lipid-rich MFGM fraction (Fonterra Co-operative Group Limited), or with a protein-rich MFGM fraction Dosage -NS	France	Formula tolerance rates were high (94%) in all groups. Adverse event and morbidity rates were similar across groups except for a higher rate of eczema in the MFGM-P group (13.9% vs control [3.5%], MFGM-L [1.4%]) Proved to be safe	(Billeaud, 2015)
Rat model, control formula, mother's milk and 1.2 g/L or 6 g/L MFGM* enriched formula.	Canada	MFGM-enriched formula promotes development of the intestinal epithelium and microbiome and protects against *C. difficile* toxin induced inflammation. Dose dependant.	(Bhinder *et al.*, 2017)
Prospective, double-blind, RCT; n=160 infants <2 months of age; MFGM*-supplemented, low-energy, low-protein experimental formula (EF) up to 12months	Sweden	MFGM-supplemented IMF narrows the gap in cognitive development between breastfed and formula-fed infants.	(Timby *et al.*, 2014)
Double-blind RCT, n=160 EF supplemented with bMFGM* or SF from <2 months until 6 months of age.	Sweden	Reduced risk of acute otitis media, decreased antipyretics use, immunomodulatory effects on humoral response against pneumococcus vaccine**.**	(Timby *et al.*, 2015)
Double-blind RCT, n=24 VLBW sphingomyelin-fortified milk (SM 20% of all phospholipids in milk)	Japan	Positive association with the neurobehavioral development of LBW infants.	(Tanaka *et al.*, 2013)
Double-blind RCT; n=91. EF supplemented with complex milk lipid with ganglioside content of 11 to 12 μg/ml until 24 weeks of age.	Indonesia	Increased ganglioside serum levels, Beneficial effects on cognitive development in healthy infants aged 0–6 months.	(Gurnida *et al.*, 2012)
Double-blind RCT; n=550 (6 to 11 months old) Supplementation with 40 g/day MFGM protein fraction*	Peru	Reduced episodes of bloody diarrhoea No differences between groups in anaemia, serum ferritin, zinc, or folate.	(Zavaleta *et al.*, 2007)

RCT -Randomised Clinical Trial, VLBW -very low birth weight, SF – standard formula, EF – experimental formula, bMFGM -bovine milk fat globule membrane, NS -not stated

promotes development of the intestinal epithelium and protects against inflammation induced by *C. difficile* toxin.

Overall, it is plausible, based on existing evidence from model studies and clinical trials, that addition of bovine lipid fractions or any component of MFGM to IMF potentially would provide additional bioactivity to IMF and possibly narrow the nutritional gap between conventional IMF and HM. However, the scientific base of knowledge is still limited. The number of studies on lipid fraction and MFGM or its components assessed in infants and children is small, the interventions are heterogeneous, including varying concentration of provided ingredients, which are given for varying length of times, also age and stage of growth varies greatly. Therefore, more randomised clinical studies of high quality are needed. Moreover, future studies should aim to elucidate the digestive fate of individual lipid components to better understand their metabolic fate in different regions of the GI tract.

1.3.3.2 Significance of alpha-lactalbumin as bioactive component in IMF

Owing to the different composition of proteins in bovine and HM, protein levels in IMF need to be higher than HM to cover the AA's needs of infants (Fleddermann *et al.*, 2014; Brück, 2017). Thus, formula-fed infants have higher total protein intake than breast fed infants. High protein intake has been associated with metabolic stress in infants that may lead to a higher risk of childhood overweight and obesity (Koletzko *et al.*, 2009). Increasing evidence from RCTs demonstrated that higher protein content of whey-predominant IMF is associated with higher weight, weight-for-length, and BMI later in childhood (up to 7 years follow-up) compared to that of breast milk and lower protein IMF (Koletzko *et al.*, 2009; Alexander *et al.*, 2016; Gutierrez-Castrellon, 2018). Results of these studies suggested that lower protein intake during infancy, might constitute a potentially important approach to reducing the risk of childhood overweight and obesity. However, reducing the total protein concentration of IMF may reduce the plasma concentration of the formula's limiting AA tryptophan and may affect concentrations of blood urea nitrogen and serum insulin (Kelleher *et al.*, 2003). Several studies have demonstrated that it is possible to reduce total protein concentration in IMF without compromising its nutritional quality through supplementation with bovine α-lac. Numerous clinical trials comparing formulae enriched with α-lac to control formulae (no α-lac) and HM show that α-lac supplementation produces IMF with improved protein quality (Lien, Davis and Euler, 2004; Davis *et al.*, 2008; Sandström *et al.*, 2008; Dupont *et al.*, 2010; Trabulsi *et al.*, 2011; Fleddermann *et al.*, 2014; Oropeza-Ceja *et al.*, 2018).

Lowering total protein (14 g/l) and increasing α-lac (2.2 g/l) content can result in plasma concentrations of EAAs similar to those in breastfed infants (Lien, 2003). These changes can also improve GI tolerance relative to control formula (Davis et al. 2008), increase plasma concentrations of nearly all AAs relative to breast-fed infants (Davis et al. 2008; Sandström et al. 2008). Therefore, further reduction of protein concentration in formula may be feasible through the addition of α-lac. Trabulsi *et al* (2011) also reported that experimental low protein (12.8 g/L) α- lac enriched IMF supported age-appropriate growth similar to that of breast milk-fed infants in terms of weight gain, weight-for-age Z-score (WAZ) and weight-for-length Z-score (WLZ) assessed at 120 days, while weight gain of formulae- fed infants was lower than that of standard formula fed infants. In contrast, Fleddermann *et al* (2014) reported that weight gain was lower in control formula (CF)-fed infants (15 g/L protein) compared to intervention with α- lac enriched IMF (EF, 13 g/L protein) supplemented with LC-PUFAs measured at the same time point. However, in the latter study other components were modified in the IMF, therefore making the comparison between the two more complex. Of interest, authors hypothesised that α- lac enabled fat absorption of DHA, an essential fatty acid in terms of cognitive function (Fleddermann *et al.*, 2014, 2018).

In addition to adequate growth support, supplementation of IMF with α-lac, alone or with other bioactive components, may confer GI benefits. Brück *et al* (2006) investigated the prebiotic effect of α-lac and casein glycomacropeptide (GMP)-supplemented IMF in healthy term infants. While results were inconclusive, with a large fluctuation of bacterial counts within groups, it was speculated that formulas enriched with α-lac and GMP may confer an increase of the indigenous microflora only when levels are below 10% of the faecal flora. More recently, DuPont *et al* (2010) showed that an α-lac-enriched and probiotic-supplemented IMF formula proved to be adequate for infants with colic in terms of growth and reduction of GI side effects.

Increasing the proportion of α-lac in IMF is particularly beneficial because α-lac has comparatively high tryptophan content. This amino acid is a precursor of vital components such as serotonin and cysteine. Serotonin regulates the sleep-wake rhythm, whereas cysteine is a component of glutathione, a vital intracellular compound of the neonatal antioxidant system (Brück, 2017). Sandström et al (2008) reported that feeding infants with IMF enriched with α-lac protein fraction, which contained ~20% more tryptophan than did breast milk and standard formula, resulted in plasma tryptophan concentrations that were not significantly different from those of breastfed infants. Similar results were

seen in the study where formula was enriched with α-lac resulting in higher tryptophan level and consequentially higher plasma tryptophan levels in infants fed EF than CF group (Heine *et al.*, 1996).

In another study experimental α-lac-enriched and symbiotic-supplemented IMF provided better GI tolerance and had a protective effect against the occurrence of mild atopic dermatitis in infants at 6 months of age compared to the standard formula, however this effect may not be attributed to α-lac alone, but rather its combination with prebiotics and probiotics used in this study (Rozé *et al.*, 2012).

Health-related quality of life (HRQOL) was assessed in prospective observational cohort study by Hays *et al* (2016) in Chinese infants who received IMF enriched with α-lac, increased sn-2 palmitate and oligofructose. Overall conclusion was that HRQOL was regarded as high between breastfed, formula-fed, and mixed-fed infants. Small differences in HRQOL concept scores were observed in categories such as temperament and mood in formula fed infants. In conclusion, evidence to date suggests that α-lac enrichment in IMF is safe as well as confers health benefits and improves lipid profile of IMF it is reasonable to suggest that such enrichment would modify bioactivity of IMF. It is noteworthy that some of the biological effects of the different bioactive components could be due to synergies between these compounds and may not manifest after addition of isolated components to IMF. Therefore, future studies should focus on the synergistic or antagonistic effect of novel combinations, for example α-lac with other known fractions, within food matrices, e.g., lipids, such as phospholipids and sphingomyelin.

1.4 *In vitro* Digestion models

Understanding how IMF is digested and how bioactive ingredients are released from this food matrix is central to elucidating the metabolic pathways that are stimulated to promote growth and development in formula-fed infants. Digestion can affect the half-life of biologically active milk ingredients, change their biological activity, and release active fragments with new biological properties (Chatterton *et al.*, 2004). To support the design of optimal IMF it is important to consider its fate during digestion in the GI tract (GIT) and its potential subsequent effect on infant health, therefore, there is a need to study the digestibility of IMF to better understand the degradation mechanism of its components as well as the bio-accessibility of the digested nutrients in the GIT (Nguyen *et al.*, 2015a).

Clinical investigation of digestive processes frequently requires procedures such as the use of nasogastric and nasoduodenal tubes, or the drawing of blood samples (Abrahamse *et al.*, 2012). Due to the invasive nature of these procedures and the ethical implications associated, the application of *in vitro* digestion models has become an attractive alternative. *In vitro* approaches allow investigations on basic mechanisms of infant digestion and to observe the digestibility, structural changes, and the release of nutrients under simulated GI digestion (Abrahamse *et al.*, 2012; Nguyen *et al.*, 2015a).

The GI tract is a complicated semi-continuous set of bioreactors that is intertwined with influences from dynamic events associated with the haematological, hormonal, and nervous system. This complex physiology makes recreation of the entire GI function in an *in vitro* model limited. Furthermore, composition and subsequent digestive secretion, digestion and absorption, and the interaction between the host, the food and micro-bacteria in the digestive system cannot be mimicked (Nguyen *et al.*, 2015a; Shani-Levi *et al.*, 2017). However, many aspects of luminal digestion can be employed *in vitro* models using reliable and detailed information on physiologically relevant conditions gathered from humans. (Shani-Levi *et al.*, 2017).

In vitro models can be static single phase, semi-dynamic or complex and dynamic, multicompartmental systems (Abrahamse *et al.*, 2012). Several dynamic digestion models have been developed in recent years (e.g., Human Gastric Simulator, Dynamic Gastric Model, TIM advanced gastric model (TNO's advanced gastric compartment). These models include regulation of the pH change, gastric emptying, peristaltic movements, dynamic flows of food as well as concentration and secretion of digestive enzymes in the different compartments, which allows to address complex aspects of digestion (Ménard *et al.*, 2014; Nguyen *et al.*, 2015a).

Static models are designed so that the final digestive products remain in reaction vessels during the digestion process, and other physical movements like shear, mixing, falling of gastric pH, and absorption process are not employed (Nguyen *et al.*, 2015a). Static *in vitro* digestion methods are oversimplified and do not consider the dynamic aspects of the digestive process. However, they are easy to use, cheap, and do not require specific equipment. Therefore, these models have been used to compare the digestion of related foods under the same conditions to study the digestion of pure compounds or to unravel the interactions between constituents at the molecular level (Bohn *et al.*, 2018).

In vitro models have been increasingly applied to understand digestibility, structural changes, and kinetics of digestion under closely simulated physiological conditions in the

human GI tract (Hur *et al.*, 2011). However, proposed variety of protocols differed in the experimental conditions (pH and duration of the different steps, amount of digestive enzymes and bile etc), which made it impossible to compare results between studies (Bohn *et al.*, 2018). In order to harmonise *in vitro* digestion experiments, a network of international scientists was recently involved in EU funded COST project FA1005 INFOGEST (http://www.cost- infogest.eu/) (Brodkorb *et al.*, 2019). As a result, consensus was reached and developed protocol for static adult *in vitro* model was published by Minekus *et al* (2014), that has since been widely used internationally. However, functionality of human gastro intestinal tract (GIT) develops in the first year of life with newborns (<28 days of life) and infants up to six months possessing an immature digestive system compared to older infants (>6 months) or the fully mature GI of an adult (Shani-Levi *et al.*, 2017). Digestive capabilities of infants differ to that of adults by many factors, i.e. limited stomach capacity with higher pH gradient (3.2-6.5), immaturity of gastric and intestinal enzymes, such as pepsin, α-amylase, pancreatic triglyceride lipase, as well as limited gallbladder secretions and lower bile salts concentration (Bourlieu *et al.*, 2014; Nguyen *et al.*, 2015a). Therefore, various efforts are ongoing to develop and harmonise *in vitro* models that would simulate infant digestion. Ménard *et al* (2014) developed a simple *in vitro* dynamic GI digestion system, for studying IMF digestion. This model was validated by comparing the kinetics of proteolysis obtained *in vitro* with *in vivo* data collected from piglets, with good correlation demonstrated between the two (Ménard *et al.*, 2014). However, there is no physiologically-relevant harmonised model of *in vitro* static digestion at the infant stage (Shani-Levi *et al.*, 2017). Same authors proposed an *in vitro* static digestion model for the full-term newborn with parameters relying on *in vivo* data published in the literature and compared this infant model with the adult international consensus model regarding the digestive kinetics of commercial IMF. This model represents a first step toward the establishment of a consensus static infant digestion model (Ménard *et al.*, 2018).

1.4.1 In vitro digestion studies *of human milk, bovine milk and IMF*

As *in vitro* models are now considered a good predictor of *in vivo* behaviour they have been employed to investigate behaviour of HM or IMF during static *in vitro* digestion. These models are widely used as an alternative to *in vivo* digestion to obtain data in structural changes, rheology, digestibility, and bioavailability of infant foods (Nguyen *et al.*, 2015a). Numerous studies to date have compared the digestibility of IMF and HM to determine how bioactive components are released following digestion. However, limited

studies focus on the health-promoting properties of the IMF or HM digesta. This section reviews recent and relevant research on the formation and fate of bioactive components released after the digestion from milk feeds intended for infants (Table 1.5).

Digestibility of bovine milk and IMF differs to that of HM. Chatterton *et al.* (2004) compared the protein breakdown of HM, WPC and IF during *in vitro* gastric digestion using gastric juice of neonate. Authors have found that several proteins in HM and bovine WPC were resistant to gastric proteolysis, which suggested that intact proteins may be biologically active in the infant intestine, or could be digested further in the intestine, potentially releasing biologically active peptides. In contrast proteins in IMF were found to be more susceptible to gastric proteolysis, which may possibly be due the heat-treatment involved in IMF production. This results in different AA profiles generated post digestion. Protein digestion and the bioavailability of indispensable amino acids (IAAs) from IMFs have also been investigated. Similarities and differences in the endogenous and postdigestion peptide profiles of HM and IMFs have been described by Hernández-Ledesma *et al*. (2007). IMFs had different protein composition and markedly different peptide profiles compared to HM. Authors identified only one peptide (HLPLPL sequence) which had known potent ACE-inhibitory properties, in both HM and IMF digesta. This study reported that the digestion of IMF can produce bioactive peptides with equivalent function to that generated in HM. However, this peptide was found only in IMF produced with hydrolysed whey protein as a source, suggesting that the release of bioactive peptides depends on the composition of IMF. The same unique peptide profiles were observed and confirmed by another study (Su *et al.*, 2017)**.** One study revealed that the true ileal protein digestibility and digestible indispensable amino acids (DIAAs) of bovine milk-based IMF were similar to those of HM, indicating that protein quality of IMF was comparable to that of HM. While slower initial protein digestion was observed, whether this has physiological consequences in infants following complete digestion of IMF was not investigated (Maathuis *et al.*, 2017).

Bioaccessibility of sialic acid and gangliosides, bioactive components related to brain development, was assessed by Lacomba *et al.* (2011), comparing their contents in HM and bovine milk based IMF and follow-on liquid formula (FF). Bioaccessibility refers to the fraction of the total amount of a substance potentially available for absorption. The study reported that the bioaccessibility of sialic acid was greatest in HM, while the bioaccessibiltiy of gangliosides was greatest in FF. This may be explained by the presence of emulsifiers in the liquid formula that may have affected *in vitro* GI digestion. Authors

suggested that the enrichment of IMF with raw sialic acid and gangliosides may be warranted to match their content in HM. It has been shown that HM has a greater lipid bioaccessibility during the gastric digestion than IMF (Bourlieu *et al.*, 2017). Furthermore, it has been demonstrated that the lipid droplet size distribution varies distinctly between IMF and HM: IMF fat droplets are smaller than those in HM (Fondaco *et al.*, 2015). This difference between fat droplet size may influence the initial rate of lipolysis and the release of FFAs. Overall rate of lipolysis and the amount of bio-accessible FFAs was found to be higher in HM, which may be attributed to endogenous maternal bile salt-stimulated lipase (BSSL) and the unique interfacial properties of the MFGM. Similar results were obtained by Santillo et al. (2018), who have investigated FFA profile in IMF and milk from different species, grouping FAs according to the length of the carbon chain. Authors found that percentage distribution of FAs post digestion did not reflect the patterns found in undigested milks. Total FFAs released post digestion were highest in HM, that included short-, medium- and long- chain FFAs, as well as mono- and poly-unsaturated FFA were the lowest in bovine milk and intermediate in IMF.

This body of data suggests that the efficiency with which IMFs are digested, based on blends of different vegetable oils, differ from that of human breast milk (Lueamsaisuk *et al.*, 2014). However, it is acknowledged that the bioactive components released from IMF depend on the source of lipids used in its design. For instance, in the study conducted by Hageman *et al.* (2019) the FAs released from IMF containing either a vegetable fat blend with palm oil (IF1) or a mixture of AMF and vegetable fat (IF2) was compared to HM. Results demonstrated that release of FFA was much slower from HM during the gastric phase compared to both IFs, which is in line with findings in previous studies (Fondaco *et al.*, 2015; Santillo *et al.*, 2018). The FFA profile after intestinal phase was different, with higher levels of unsaturated FAs (C18:1 and C18:2) and lower levels of saturated FAs released from HM, compared to both IFs. Total amount of released FFAs did not differ between IFs, however more SCFA and MCFA, and less LCSFA, especially palmitic acid, were released from IF2, suggesting that the combination of AMF and vegetable oil blend in the IMF contribute to FFA profile similar to HM. Similar results were found by Liu *et al.* (2021) who also demonstrated differences in FFAs profile in IMF designed with different sources of fats. Composition of IMF supplemented with the bovine MFGM and/or phospholipid-enriched dairy source was shown to be more similar to that of HM after gastric digestion.

Overall, the structure of IMF impacts lipolysis and proteolysis during *in vitro* gastric digestion. Difference in protein quality and lipid emulsions may modify the kinetics of digestion of IMF in newborns and influence protein and lipid metabolism (Nguyen *et al.*, 2015b, 2018; Wada and Lönnerdal, 2015b; Cheong *et al.*, 2018).

Together the above data may explain some of the functional differences between HM and IMF, at the same time allowing identification of some functional aspects between bovine and HMs which contribute to the effectiveness of modern IMF. These studies are also useful in the exploitation of different source materials and novel ingredients for design and optimisation of IMF. This information may be useful in the ongoing development of IMF to be more like HM in composition and function.

Table 1.5. Data collected on *in vitro* digestibility of human milk (HM), bovine milk (BM) and infant milk formula (IMF).

Analyte	Digestion	Investigation	Main findings	Reference
HM, WPC (Lacprodan-80) and IMF (SMA, Wyeth)	*In vitro* and *in vivo* proteolysis with human gastric juice	Effects of *SGID* on novel milk protein ingredients and their stability	• Proteins IgA, α_{S1}-CN, MUC-1, OPN, LF, β-CN in HM and BM are resistant to proteolytic enzymes, but more susceptible in IF • *In vivo* digestion results indicated a close correlation between *in vivo* and *in vitro* investigations • HM and BM α-lac appears to be digested in the same manner	Chatterton *et al.*, 2004
8 IFs of various protein sources and HM	*In vitro* digestion with pepsin and pancreatin	Peptides' release and their (ACE)-inhibitory activity	• Markedly different peptide patterns were obtained from HM and the IF • Peptide patterns in IF's digesta dependant on the protein source in IF • One peptide (HLPLPL) was the same in the digesta of HM and IF (with hydrolysed proteins)	Hernández-Ledesma *et al.*, 2007
A powder IF and a liquid follow-on formula (FF) and HM	SGID (gastric and intestinal)	Effects of SGID on sialic acid and gangliosides	• Greater decrease in sialic acid and ganglioside contents in gastric phase • Decrease in total and individual contents of gangliosides after intestinal stage • Bioaccessibility of sialic acid in HM -87%, IF 77% and FF 16% • Bioaccessibility of gangliosides in FF 51%, HM 29% and IF 5%.	Lacomba *et al.*, 2011
Different Similac (Abbott Nutrition, OH, USA) IFs (n=4) and HM	TIM-1 (TNO, Zeist, The Netherlands)	Physico-chemical properties and correlation with *in vitro* FFA's bioaccessibility	• Lipid droplet size distribution varies distinctly between IF and HM • The rate of lipolysis was higher in HM • Significantly lower FFA bioaccessibilities for IF's (except Advance formula) than for HM	Fondaco *et al.*, 2015
Standard IF (CN + WP-based); e hydrolyzed CN-based IF, and partially hydrolyzed WP-based IF	SGID (gastric and intestinal phase)	Peptidomic analysis of released bioactive peptides by LC-MS	• *In vitro* digestion of the standard and hydrolyzed IFs released a multitude of bioactive peptides, • Standard IF released a larger variety of bioactive peptides than the hydrolyzed IFs	Wada and Lönnerdal, 2015a
IFs with hydrolysed and non-hydrolysed proteins	SGID in the presence of proteases only	Comparison of the digestibility and microstructural changes	• Hydrolysed proteins completely digested in the small intestine • Non-hydrolysed proteins (caseins, α-lac, β–lactoglobulin, only partially digested) • Significantly higher digestibility of hydrolysed proteins than non-hydrolysed	Nguyen *et al.*, 2016
HM, goat and BM-IFs (GIF and CIF)	dynamic GID (tiny-TIM)	Kinetics of true ileal protein digestion and (DIAAS)	• Ileal protein digestibility and DIAAs of IF were similar to those of HM • Protein quality of IF comparable to HM	Maathuis *et al.*, 2017

Table 1.5. continued

HM and Canterbury Pure IF (0–6 months, Synlait, New Zealand)	SGID	Peptidomic profiling by LC-MS/MS	• Identified both similarities and differences in the endogenous and post-digestion peptide profiles of HM and IF	Su *et al.*, 2017
A powdered standard IF (Similac Advance; Abbott Nutrition, Abbott Park, IL) and HM	*In vivo* digestion using a suckling rat pup model	Peptidomic analysis to identify release of peptides	• α-lac and β-casein in HM, and β-lactoglobulin and β-casein in IF were the main sources of peptides • β-CN is the greatest source of releasing peptides for both HM and IF • Larger numbers of β-CN-derived peptides were identified for IF than HM	Wada *et al.*, 2017
Model IF adapted to the energy and protein requirements of piglets	*In vivo* piglet model	The effect of protein composition on the digestion behavior	• Different concentrations of milk proteins can be critical to the digestion properties of the food matrix • Different concentrations of milk proteins may affect the nutritional properties of the components	Tari *et al.*, 2018
Mature HM, Stage 1 IFs (*2), ready-to-drink liquid IF (*2)	SGID	Lipid profiling, particle size and rate of lipolysis	• Higher GI lipolysis rate of mature HM fat • IF with lowest phospholipids content demonstrated lowest GI lipolysis rate.	Cheong *et al.*, 2018
HM, Liquid IF and different animal milks including bovine	SGID	FFAs analysis	• The total FFA was highest in HM, lowest in IF and intermediate in others • SCFFA were highest in HM and lowest in IF • MCFFA and LCFFA highest in bovine and the lowest in IF • LCFA were highest in IF and intermediate in HM •	Santillo *et al.*, 2018
IF1 containing AMF and an IF2 with vegetable fats; HM	SGID (gastric and duodenal phase)	Release of FAs and concentration of the residual TAG	• Addition of AMF to IF1 changed triglyceride structure but did not influence the release of total FA's • More SCFA and MCFA, and less LCSFA, especially palmitic acid, were released from an IF1 than IF2	Hageman, Keijer, *et al.*, 2019
IFs containing different fat sources and HM	SGID	Lipid profiles and release of FAs	• HM had a higher proportion of MAG and DAG linked to LCFA • Higher release of total FFAs from HM • IMF supplemented with the bovine MFGM and/or phospholipid-enriched dairy source was more similar to that of HM	Liu *et al.*, 2021

HM human milk, WPC whey protein concentrate, HPLC-MS high-performance liquid chromatography mass spectrometry, GI gastrointestinal, ACE - angiotensin converting enzyme, AO -antioxidant, FFA – free fatty acids, SCFA -Short chain fatty acids, MCFA - Medium chain fatty acids, LCFA -Long chain fatty acids, TAG, triacylglycerols; DAG, diacylglycerols; MAG, monoacylglycerols, SGID simulated gastrointestinal digestion

Conclusion:

Despite the great advances in the optimisation of IMF composition, it cannot provide all the numerous immune protective and bioactive factors that are present in HM. Differences between HM and IMF exist in terms fat, protein, and other nutrient content, which may affect nutrient digestion, gut biology and subsequent health promoting properties. Furthermore, differences exist between breastfed infants and formula-fed infants with regards to health outcomes such as illnesses, cognitive development, and metabolic disorders in later life. Efforts continue to 'humanise' IMF, making its nutritional composition as close to HM as possible. However, the purpose of IMF design has advanced over recent years from just mimicking its nutrient composition, which is qualitatively incomparable, to approximation of the nutritional characteristics that HM offers, as well as physiological functions associated with HM. To achieve this goal, IMF containing novel ingredients with potent biological functions are being developed with new techniques. There are many bioactive substances that are abundant in HM but found at lower concentrations in bovine milk, some of which are added to IMF. These ingredients are bioactive compounds, including MFGM, LC-PUFAs, complex lipids such as phospholipids, multifunctional proteins (lactoferrin, α-lac), prebiotics and probiotics. However, evidence that addition of these ingredients to IMF provides long term effect and health benefits for infants, is either limited or not always convincing. A major challenge in the development of IMFs is the evaluation of their nutritional effects on infants. *In-vitro* digestion models that stimulate gastrointestinal conditions might be valuable as a quick route to understand the digestion of lipids and proteins used in the design of the IMF. Preliminary studies comparing digestibility of HM, bovine milk and IMF of various sources have shown the differences in digesta profile in regard of FA, peptide, and AA profiles, which may indicate different biological effects. Thus, it is important to identify how these different profiles can potentially impact infant health. Incorporating combinations of bioactive ingredients into IMF leads to a greater diversity of peptides and FAs post digestion. However, studies investigating potential health effect of digested IMF are limited. Future studies should aim to elucidate how bioactive ingredients are released, and their bioaccessibility and bioavailability in GI tract should be evaluated, to allow optimal IMF design. Modifying the lipids and protein profiles of IMF using combination of different oils and whey protein sources, for example α-lac enriched WPC, may allow to produce IMF with the same functionality of the HM, such as anti-infectious activity and ability to protect against oxidative damage.

Chapter 2. Development and compositional analysis of infant formula prototypes with different lipid and protein profiles

Note: *Details relating to the design and composition of the IMF products described in this thesis are subject to a confidential agreement between MTU and Nestlé Ltd (Ireland) and cannot be disclosed /discussed or published without the consent of both collaborating partners.*

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Abstract

The nutritional composition of human milk serves as a model for infant milk formula (IMF) manufacturers. To attain the protein and lipid requirements needed for sufficient growth and development of an infant their quantity and quality are very important when designing IMF. To improve nutritional composition of IMF one of the potentially effective strategies could be modification of IMF protein and lipid profile, using a combination of various oil blends and/or different whey protein sources. In the present study combinations of different oil blends and protein sources were used to produce IMF prototypes with varying lipid and protein profiles. Ten IMF prototypes were designed with 5 different oil blends (OB) and 5 were supplemented with 90 % demineralised whey powder (DWP, 12.6% protein) another 5 with alpha-lactalbumin (α-lac) enriched whey powder concentrate (αWPC, 80% protein).

Fatty acid (FA) profile of IMF prototypes varied depending on the OB used. Phospholipid (PL) levels were determined to be higher in αWPC and this translated to the products produced with this ingredient. Total PL levels were significantly (p<0.05) higher in Group B (39.11 – 53.61 mg/100 ml) compared to Group A (17.68 – 22.55 mg/100 ml), with some Group B prototypes comparable to HM. β-lg content was found to be significantly (p<0.05) lower in Group B prototypes. Group B prototypes, enriched with αWPC had significantly higher α-lac levels compared to Group A prototypes, containing DWP. Prototypes containing αWPC, as expected, had levels of α-lac and PLs higher than those compared to prototypes designed with DWP. In the case of αWPC enriched IMF α-lac (0.24 –0.33 g/100ml) and PLs, such as sphingomyelin (9.82 -13.86 mg/100 ml), phosphatidylcholine (11.38-14.62mg/100ml), phosphatidylethanolamine 10.68-15.33 mg/100 ml), phosphatidylinositol (2.71-3.6 mg/100 ml), and phosphatidylserine (4.53 – 6.29 mg/100ml) were all closer to these reported in human milk compared to the prototypes produced without αWPC.

Key words: IMF, prototypes, proteins, lipids, DWP, αWPC, phospholipids

2.1 Introduction

Due to advancements in scientific research and technologies, significant progress has been made in recent years with regards to making IMF resemble human breast milk as much as possible, to provide formula-fed infants with similar macro-and micro-nutrients to support their growth and development (Fil *et al.*, 2021). However, the nutritional profile of IMF is still different to human milk (HM) in terms of fat and protein content, as well as other nutrients (Le Huërou-Luron *et al.*, 2016; Lonnerdal, 2016). The basic composition of IMF is a combination of five main constituents: fat, protein, carbohydrates, vitamins, and minerals. While the lactose content, the main carbohydrate in human milk, remains fairly constant in mature milk (21 days postpartum) (Montagne *et al.*, 2009), protein and fat profiles can change over time and are influenced by the diet (Lonnerdal, 2016; Demmelmair and Koletzko, 2018) and this provides a challenge to IMF manufacturers in terms of replicating HM.

To adapt to the needs of growing infants, the composition of IMF is modified according to the age of an infant. IMF products are categorised as follows: Stage 1 formula targets infants aged 0-6 months; Stage 2, or follow-on formula, is intended for infants between 6 and 12 months; Stages 3-5, or growing-up formula, targets toddlers and young children from 1 to 6 years of age (Blanchard, Zhu and Schuck, 2013; Happe and Gambelli, 2015). Bovine milk fractions and their derivatives are commonly used to produce IMF as they are considered the most adequate substitute for human milk (Ahern *et al.*, 2019). Ingredients that contribute to the protein content and profile of IMF include bovine skimmed milk powder (SMP), whole milk powder (WMP), and/or milk protein concentrate (MPC), which typically provide the casein component of IMF. In addition, demineralised whey powder (DWP), whey protein concentrate (WPC) and whey protein isolate (WPI), are added to provide the whey protein fraction (Fenelon *et al.*, 2018; Walshe, O'Regan and O'Mahony, 2021). The impact of each ingredient on the final composition must be considered. The quality of fats is determined by the composition of fatty acids (FAs), the degree of saturation, the position of the FAs on the glycerol backbone and the content of trans FAs. Protein quality is expressed by its nitrogen and indispensable amino acids content. To attain the protein and lipids requirements needed for sufficient growth and development of an infant, their quantity and quality are very important when designing IMF.

Bovine milk is higher in protein and mineral content than HM and differs in its casein:whey ratio (Fenelon et al., 2018). Therefore, to reduce protein and mineral content in IMF, when using SMP, lactose and/or DWP can be added, which supports a casein:whey ratio that matches human milk (40:60) (Fenelon *et al.*, 2018). In addition, bovine milk has a different amino acid (AA) profile than HM and is deficient in essential AAs such as methionine, cysteine, and tryptophan, therefore, the protein content in most bovine milk-based IMF is higher (13–15 g/L) than HM (9.5 -11 g/L), to ensure sufficient quantities of all essential AAs and to meet the requirements of infants (Davis *et al.*, 2008; Fenelon *et al.*, 2018). The higher protein content of IMF is hypothesised to contribute to growth differences observed between IMF- and HM-fed infants (Lien, Davis and Euler, 2004). Recently, IMF manufacturers have explored the use of α-lactalbumin (α-lac) enriched WPC to improve the essential AA profile of formulations, while reducing overall protein content. α-lac is the predominant whey protein found in HM and is a rich source of essential AA's (Trabulsi *et al*., 2011). Recent advances in dairy technology allows for the generation of WPC fractions with a higher concentration of α-lac (69%) than standard bovine whey, and a 59% reduction of β-lactoglobulin (β-lg) (Davis *et al.*, 2008; Trabulsi *et al.*, 2011). Furthermore, α-lac enrichment of IMF has potential health benefits for infants, which include antimicrobial, antiviral and immunostimulatory (Fenelon *et al.*, 2018).

The main strategies used to improve the lipid profile of IMF are based on matching fatty acid profiles to those found in HM and improving their positional distribution in milk fat triglycerides (e.g., high sn-2 palmitic acid) to better mimic HM lipids. To achieve this, a wide range of vegetable oils and fat from animal sources (such as goats and bovine milk fat) are used in IMF design (Happe and Gambelli, 2015). However, vegetable oils and animal fat have FA profiles that differ to that of HM, therefore, a combination of oils is usually required to achieve target FA specifications. The vegetable oils that are most commonly used in IMF manufacture include palm olein, sunflower, palm kernel, rapeseed, soybean and coconut (Happe and Gambelli, 2015). However, supplementation of IMF with specific lipid components, such as long-chain polyunsaturated fatty acids (PUFA), sn-2 palmitate, medium-chain triacylglycerols, and milk fat globule membrane (MFGM), has recently emerged as a novel approach to improve IMF lipid profiles (Wei, Jin and Wang, 2019). Several other lipid fractions are also being investigated in terms of their biological significance and potential health benefits (Wei, Jin and Wang, 2019). For example polar lipids in IMF have gained interest due to their presence in HM and their

associated bioactive properties, in particular their potential role in brain and cognitive development (Moloney, O'Connor and O'Regan, 2020; Fil *et al.*, 2021). The main polar lipids include glycolipids (e.g. gangliosides), sphingolipids (e.g. sphingomyelin (SM)) and phospholipids (PL's) (e.g. phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS)) (Fil *et al.*, 2021). Studies reporting on the importance of these lipids in the infant diet refer to their broad functionality, including their contribution to myelination within the central nervous system (Oshida *et al.*, 2003) and their positive effects on cognitive (Gurnida *et al.*, 2012) and neurobehavioral (Tanaka *et al.*, 2013) development in infants.

IMF are typically produced using bovine milk ingredients which have lower levels of PL's (~20 mg/100 ml) compared to those found in HM (15-47 mg/100ml) (Garcia *et al.*, 2012). Enrichment methods, such as solvent extraction, supercritical fluid extraction, ultrafiltration or electrodialysis with bipolar membranes, may be used to recover PLs in dairy by-products (Price *et al.*, 2020; Faucher *et al.*, 2021). Supplementation with milk fat globule membrane (MFGM), is one strategy used by manufacturers to improve the PL profile of IMF. Adding MFGM increases the levels of PL which has potential health benefits, including positive immune and cognitive outcomes (Fontecha *et al.*, 2020). Another strategy recently proposed by Moloney *et al.* (2020) is the addition of α-lac-enriched WPC, which alters the nutritional composition of IMF such that the cholesterol, gangliosides and PLs levels are similar to human milk. Recent developments in milk protein fractionation and isolation technologies allow for α-lac-enriched WPC production. For that different methods can be used, including membrane filtration, selective precipitation and ion-exchange chromatography, which are proprietary to each manufacturer (Moloney *et al.*, 2018; Barone *et al.*, 2020). The addition of α-lac-enriched WPC to IMF provides a significant opportunity to improve both protein quality (Davis *et al.*, 2008) and PLs profile (Moloney, O'Connor and O'Regan, 2020), and potentially leads to better health outcomes for formula-fed infants.

In the current study a range of IMF prototypes were designed to contain different oil blends and whey protein sources. Compositional analysis of the prototypes will confirm differences between PL profiles and FFA content. In addition, prototypes containing WPC enriched with α-lac will be analysed to determine if this strategy can improve the lipid and protein profiles of IMF, without the addition of ingredients such as MFGM. The IMF prototypes generated will be used in further studies to compare their bioactive properties and determine if there are optimal lipid and protein profiles for promoting infant health.

2.2 Materials and methods

Skimmed milk powder (SMP, 36% protein), 90 % demineralised whey powder (DWP, 12.6% protein), α-lac enriched WPC (αWPC, 80% protein), lactose powder and the selection of various oil blends were provided by Nestlé, Ltd (Ireland). All materials and reagents were sourced from Merck (Sigma-Aldrich, Ireland), unless otherwise stated.

2.2.1 Design of IMF prototypes

Ten infant formula (IF) prototypes representing stage 1 formulation (suitable for infants from birth to 6 months of age) were designed to contain the same total fat (27.9 $g/100g_{powder}$) and total protein (10.4 $g/100g_{powder}$) but different lipids and protein profiles. All prototypes were produced at the Bio-functional Food Engineering (BFE) pilot plant facility, Moorepark Technology Limited, Co. Cork. Formulations were designed with the following ingredients: skimmed milk powder (SMP, 36% protein), 90 % demineralised whey powder (DWP, 12.6% protein), α-lac enriched WPC (αWPC, 80% protein) and lactose powder.

Five oil blends (OB) were prepared using combinations of oils as follows: OB1 contained soybean, high oleic sunflower, and coconut oils in the ratio of 20:20:60, respectively; OB2 contained the same oils as OB1 but in different ratio (33:21:25) and included palm oil (21%); OB3 contained a mix of vegetable oils with addition of anhydrous milk fat (AMF, 45%), OB4 contained OB1 oils (13:25:15) with addition of a novel fat, designated sn-2 fat l (46 %); OB5 contained similar OB1 oils in a similar ration to OB4 (13:25:15) but sn-2 fat l was replaced with another novel fat, designated sn-2 fat 2 (46%). These novel two oils (sn-2 fat 1 and sn-2 fat 2) differed in terms of their FA configuration.

Using these oil blends five Group A prototypes (1A-5A) and five Group B prototypes (1B-5B) were designed such that they had different whey protein sources: DWP was added to Group A and αWPC was added to Group B products. Therefore, (1) prototypes within the same group contained the same whey protein source but different oil blends (OB1-OB5); (2) each group A prototype has a corresponding Group B prototype which had the same oil blend but was different in terms of whey protein source.

2.2.2 Production of IMF prototypes

Twenty-liter batches of each IMF prototype were produced in the pilot plant facility as follows: one third of the oil blend, heated to ~45 °C, was added to distilled hot water (~76 °C) and mixed by agitation for 5 minutes. Lactose, SMP and the appropriate whey protein source (DWP or αWPC) were then added separately followed by the remaining two-thirds of the oil blend, and the mix was then agitated for an additional 10 minutes. The mixture

was then thermally processed at 85 °C x 22 sec and homogenised using an in-line two-stage valve homogeniser, using first- and second-stage pressures of 2500 and 500 psi respectively. The mixture was spray dried using a pilot-scale Anhydro 750 Micraspray dryer with inlet and outlet temperatures of 185 °C and 85 °C for Group A prototypes (1A-5A), and 175 °C and 95 °C for Group B prototypes (1B-5B).

2.2.3 Compositional analysis

Compositional analysis of the raw ingredients and the ten prototypes was carried out at the Nestlé Ltd facilities, (NDC Askeaton, Ireland) in accordance with validated protocols. Total fat content of the IMF prototypes and the oil blends were determined by the base hydrolysis method (Methods 989.05, 932.05, 986.25, 945.48B, AOAC International 2005), phospholipid analysis was conducted using LI - 08.115 method (MISC_4025) and total ash was determined according to the method 923.03 (AOAC, 2005). The α-lac and β-lg content of IMF prototypes was determined by HPLC, and Free Fatty Acids (FFA) by HI00.0251.512-Fatty acids in milk products by GC-FID. Individual powder ingredients were analysed for carbohydrate (USDA, 1973), fat (Methods 989.05, 932.05, 986.25, 945.48B, AOAC), free fat (GEA Niro Method No. A10, 2005), protein (Dumas Method, N x 6.25), cholesterol (Method, 994.10, AOAC), free amino acids (using Zorbax Eclipse-AAA columns and the Agilent 1100 HPLC) and moisture (Methods 925.09 and 926.08, AOAC).

2.2.3.1 Protein determination by Kjeldahl method

Total protein was determined using the Kjeldahl method of nitrogen analysis as described by Guo et al., (2013), with some modifications. Briefly, 0.5 g of each IMF prototype, 5 ml of H_2O, 20 ml 98 % sulfuric acid, 2 Kjeldahl catalyst tabs (S 5.005g, VWR Chemicals, Ireland) and 5 ml of 33 % H_2O_2 were placed into a Kjeldahl digestion tube. Tubes were then placed in the digestion unit (DK 20 heating digester, VELP Scientifica), which was set at 420 °C for 1 h. Tube with no IMF powder sample was used as the blank control. Following digestion, mixtures were then allowed to cool and distilled for 4 minutes in the distillation unit (UDK 139, VELP Scientifica) with parameters set at as 50 ml of H_2O and 70 ml of 35 % NaOH. Flask containing 4 % of H_3BO_3 solution with bromocresol green and methyl red indicators was used to absorb the distilled product. The distillate was then titrated with 0.1M HCl and Nitrogen (%) was calculated as follows:

$$\% N = \frac{(Vsample - Vblank) * N * M(N)}{m(g) * 10}$$

Where V sample (ml) = titrant consumption of sample, V blank (ml) = titrant consumption of blank, N (mol/L) = concentration of the standard acid, M(N) (g/mol) = atomic mass of nitrogen = 14.00674, m (g) = weight of the sample. Protein (%) was then calculated with a nitrogen-to-protein conversion factor of 6.25 (EFSA NDA Panel, 2014).

2.2.3.2 Energy determination by heat of combustion

Energy values of all IMF prototypes were measured using an isoperibol oxygen bomb calorimeter (Parr Model 6200). Energy values were measured in approximately 1 g of powder, and each prototype was analysed at least in triplicate, to achieve an intraassay variation of less than 2%.

2.3 Results and Discussion

2.3.1 Raw materials analysis

Phospholipids (PLs) are vital components in the brain myelination process as well as regulation of inflammation and in signalling pathways, involved in the infants' brain and cognitive development (Cilla *et al.*, 2016). PLs are residual lipids in whey products and usually not detected (Faucher *et al.*, 2021) or only found in trace amounts, 0.27g/100g_{powder} (Svanborg *et al.*, 2015). In the current study total PL levels were determined to be higher in αWPC (4.97 g/100$g_{powder)}$ compared to DWP (0.22 g/100g_{powder}) (Table 2.1), confirming that α-Lac enriched WPC retains higher levels of PLs. Svanborg *et al* (2015) have also reported that PLs were detected in SMP in the range of 0.16 – 0.25 g/100g_{powder}, which is similar to the levels found in this study (0.20 g/100g_{powder}). The higher content of PLs is likely due to the preferential retention of PLs during the manufacturing process of αWPC (method cannot be disclosed for proprietary reasons), which involves retaining components including lipids and PLs (Moloney *et al.*, 2018). The incorporation of αWPC into IMF has been shown to be a viable strategy to increase the content of these key nutrients in IMF without the requirement for fortification using separate enriched fractions, such as MFGM (Moloney *et al.*, 2020).

It has been reported that levels of α-lactalbumin (α-lac) in αWPC can vary depending on the method of manufacture and have been reported as 28.4 %, 24.4 % and 73.4 % (w/w) for membrane filtration, membrane filtration coupled with selective precipitation and ion-exchange chromatography-based separation methods respectively (Barone *et al.*, 2020). In the current study levels of α-lac was found to be higher in αWPC at 30.5 g/100g_{powder} (30.5 % w/w) compared to DWP at 1.97 g/100g_{powder} (1.97% w/w). There are no studies to date reporting specifically on the α-lac content in DWP, however at 15.63 % w/w of total protein (12.6 g/100 g_{powder}) it is within the range of what has been reported of α-lac

% of total protein in WPC (15-25 % (w/w)) (Sousa *et al.*, 2012; Anand, Khanal and Marella, 2013).

Table 2.1 Compositional analysis of individual powdered raw materials

Component	αWPC[a]	SMP[b]	DWP[c]
Energy	1750 kJ/100g	1530 kJ/100g	1640 kJ/100g
Fat (g/100g)	9.2	1.1	1.6
Carbohydrates (g/100g)	4.1	54.6	80.4
Protein (g/100g)			
Total protein	78.7	33.1	12.6
α-lactalbumin	30.5	1.51	1.97
β-lactoglobulin	14.61	4.69	5.79
Phospholipids (g/100g)	4.97	0.20	0.22
Phosphatidylcholine (PC)	1.21	0.05	0.07
Phosphatidylethanolamine (PE)	1.50	0.05	0.04
Phosphatidylinositol (PI)	0.34	0.01	0.02
Phosphatidylserine (PS)	0.49	0.02	0.03
Sphingomyelin (SM)	1.43	0.07	0.07

[a] αWPC: α-lac enriched whey protein concentrate, [b] SMP: skimmed milk powder, [c] DWP: demineralised whey powder

2.3.2 Analysis of IMF prototypes

Total fat was determined for the ten prototypes and ranged between 29.2–31.5 $g/100_{powder}$ and total protein ranged from 10.12-10.39 $g/100_{powder}$ (Table 2.2).

Table 2.2 Compositional analysis of IMF prototypes[a]

Component	Group A					Group B				
	1A	**2A**	**3A**	**4A**	**5A**	**1B**	**2B**	**3B**	**4B**	**5B**
Oil blend[b]	**OB1**	**OB2**	**OB3**	**OB4**	**OB5**	**OB1**	**OB2**	**OB3**	**OB4**	**OB5**
Fat (g/100g)	29.8	30.7	30.2	30.2	30.3	29.7	29.8	29.2	31.5	29.8
Protein (g/100g)	10.39	10.39	10.39	10.37	10.38	10.20	10.21	10.12	10.17	10.16
Phospholipids (g/100g)	0.13	0.16	0.13	0.12	0.13	0.37	0.35	0.27	0.34	0.36
Energy (kcal/100g)	555.27	546.30	537.68	555.10	538.70	564.89	551.12	552.76	555.18	536.07

[a] Group A prototypes (1A-5A), and Group B prototypes (1B-5B) were designed such that they had different whey protein sources: demineralised whey powder (DWP, 12.6% protein) was added to Group A prototypes, α-lac enriched WPC (αWPC, 80% protein) was added to Group B prototypes.

[b] Prototypes contained one of 5 oil blends (OB1-OB5): OB1 contained soybean, high oleic sunflower, and coconut oils in the ratio of 20:20:60, respectively; OB2 contained the same oils in different ratio (33:21:25) in addition to palm oil (21%); OB3 contained vegmix with anhydrous milk fat (45%) OB4 contained OB1 oils (13:25:15) with addition of novel sn-2 fat 1 (46 %); OB5 contained OB1 oils (13:25:15) with addition of sn-2 fat 2 oil (46%).

2.3.2.1 α-lactalbumin and β-lactoglobulin

With respect to the protein quality of IMF two main proteins are of interest, namely α-lac and *β*-lg. α-Lac, a protein found in the milk of all mammals, is a major protein in HM,

and is particularly rich in essential AAs tryptophan, lysine, and cysteine. *β*-Lg is absent from HM and is rich in the essential AAs valine and threonine. Consequently, human milk, bovine milk and bovine milk-based IMF differ in AA composition substantially. Furthermore, β-g is an allergenic protein, associated with 66 % of milk allergies (Micinskia *et al.*, 2013), thus its reduction in IMF is preferred to lower the allergenic potential of IMF , which can be achieved by increasing levels of α-lac (Lien, 2003).

Previous studies have reported that the concentration of α-lac in human milk is highest in colostrum (0-5 days) at 0.43 g/100ml median value, and decreases gradually over time to ~0.26 g/100ml by days 90-360 (Lönnerdal *et al.*, 2017). The levels of α-lac were significantly (p<0.05) higher in Group B prototypes (0.24 –0.33 g/100ml) compared to Group A prototypes (0.15 – 0.21 g/100ml) (Table 2.3), with α-lac levels in Group B prototypes close to those reported in mature human milk. Data from this study confirms that incorporating αWPC into IMF improves α-lac levels and protein quality. This is in line with previous studies where α-lac content in IMF enriched with α-lac was reported to be 0.22 g/100ml (Lien, Davis and Euler, 2004; Davis *et al.*, 2008; Wernimont *et al.*, 2015), 0.23g/100ml (Trabulsi *et al.*, 2011), 0.25 g/100ml (Brück, Redgrave, *et al.*, 2006) and 0.29 g/100ml (Dupont *et al.*, 2010). Whereas in standard formula, α-lac levels were reported to be 0.11 g/100ml (Brück, Redgrave, *et al.*, 2006), 0.12 g/100ml (Lien, Davis and Euler, 2004) and 0.13 g/100ml (Davis *et al.*, 2008), which are close to the levels found in this study for Group A prototypes. Therefore, α-lac levels in IMF prototypes produced with αWPC have increased, compared to the ones produced with DWP, and are closer to α-lac levels found in human milk.

Table 2.3 α-lactalbumin and β-lactoglobulin levels in IMF prototypes[a]

Protein	Prototypes									
	1A	**2A**	**3A**	**4A**	**5A**	**1B**	**2B**	**3B**	**4B**	**5B**
α-lactalbumin										
g/100g	1.15	1.05	1.21	1.06	1.48	2.31	2.19	1.68	1.86	2.24
g/100ml	0.17	0.15	0.18	0.15	0.21	0.33	0.32	0.24	0.27	0.32
β-lactoglobulin										
g/100g	3.93	3.69	4.19	3.83	4.21	1.80	1.65	2.99	2.45	1.75
g/100ml	0.57	0.54	0.61	0.56	0.61	0.26	0.24	0.43	0.36	0.25

[a] Protein levels per 100 mL are based on the reconstitution instructions for the IMF at 14.55 g/100 mL for Group A prototypes and 14.33 g/100 mL Group B.

It has been previously demonstrated that α-lac-enriched formula can have reduced levels of β-lg (Heine *et al.*, 1996; Lien, Davis and Euler, 2004; Davis *et al.*, 2008). In this study β-lg content was found to be significantly (p<0.05) lower in Group B prototypes compared

to Group A prototypes (Table 2.3). Furthermore, it was observed that in Group A prototypes, β-lg levels (0.54 -0.61 g/100ml) were higher than α-lac levels (0.15-0.21 g/100ml). Conversely, for most Group B prototypes α-lac levels were higher (0.24-0.33 g/100ml) than β-lg levels (0.24-0.43 g/100ml), with the exception of prototype 3B (Table 2.3). It may be that the addition of AMF to the oil blend contributed to the higher β-lg levels observed. Of interest, increasing α-lac and decreasing β-lg potentially supports reducing levels of threonine and increasing levels of cysteine, tryptophan, tyrosine, and phenylalanine, thus bringing AA levels in IMF closer to those found in HM.

2.3.2.2 Phospholipid content

Total PL levels ranged at 17.68 – 22.55 mg/100 ml for Group A prototypes and at 39.11 – 53.61 mg/100 ml for Group B. An average total PL levels of Group B prototypes at 339.6 ±40.07 mg/100 g powder (48.66 ± 5.74 mg/100ml) was significantly (p<0.05) higher than of Group A prototypes at 132.18 ± 13.29 mg/100g powder (19.166 ± 1.93 mg/100ml), even though the total fat was kept at the same target for all the IMFs, at 27.9 g/100 g (Table 2.4).

PL levels in human milk varies depending on lactation stage, maternal diet and geographical location (Wei, Jin and Wang, 2019). Claumarchirant *et al.* (2016) reported that total PL amounts in HM were highest in transitional milk at 48.62 ± 5.82 mg/100 ml, and then decreased over time from 39.93 ± 2.49 (1 month), 34.71 ± 2.19 (3 months), and 28.66 ± 3.35 mg/100 mL (6 months). PL content of Group A prototypes was below the level generally found in HM. PLs in Group B prototypes 3B and 4B (39.11 and 48.02 mg/100ml, respectively) were closer to the levels found in transitional HM and at 1 month, respectively. PL levels in 1B, 2B and 5B (Table 2.5) were higher than HM, but did not exceed the maximum level of 2 g/L that is regulated by European Food Safety Authority (EFSA, 2014).

Claumarchirant et al. (2016) reported that PL levels can vary greatly in IMFs depending on the manufacturing processes and ingredients used. In their study authors reported PL content of 13 different IMF in the range of 25.11 – 58.07 mg/100 ml.

In terms of PLs, the addition of αWPC to the IMF prototypes increased total PL content (Table 2.4). PL levels measured in Group B prototypes were comparable to previous studies that used a similar strategy. For example, Moloney *et al*., (2020) reported PL

Table 2.4. Composition of phospholipids in IMF prototypes

Prototypes	PC		PE		PI		PS		SM		Total PLs	
	mg/100g powder	mg/100ml	mg/100g powder	mg/100ml	mg/100g powder	mg/100ml	mg/100g powder	mg/100ml	mg/100g powder	mg/100ml	mg/100g powder	mg/100ml
1A	33.80	4.90	19.40	2.81	11.60	1.68	12.90	1.87	49.90	7.24	127.6	18.50
2A	45.10	6.54	25.50	3.70	12.00	1.74	11.00	1.60	61.90	8.98	155.5	22.55
3A	39.60	5.74	27.50	3.99	11.90	1.73	11.10	1.61	38.20	5.54	128.3	18.60
4A	32.20	4.67	25.40	3.68	11.80	1.71	11.80	1.71	40.70	5.90	121.9	17.68
5A	38.40	5.57	23.10	3.35	12.90	1.87	11.50	1.67	41.70	6.05	127.6	18.50
1B	102.00	14.62	107.00	15.33	25.20	3.61	43.20	6.19	96.70	13.86	374.1	53.61
2B	100.00	14.33	98.90	14.17	24.40	3.50	43.90	6.29	84.10	12.05	351.3	50.34
3B	79.40	11.38	74.50	10.68	18.90	2.71	31.60	4.53	68.50	9.82	272.9	39.11
4B	88.20	12.64	91.00	13.04	21.30	3.05	36.60	5.24	98.00	14.04	335.1	48.02
5B	97.90	14.03	104.00	14.90	25.50	3.65	42.70	6.12	94.50	13.54	364.6	52.25

IMF prototypes of standard line (1S-5S) produced with 90 % demineralised whey powder and of premium line (1P-5P) with α-lac enriched WPC. Abbreviations: SM, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PL, phospholipid. Phospholipids levels per 100 mL are based on the reconstitution instructions for the IMF at 14.55g/100 mL for standard line and 14.33g/100 mL for premium line.

levels, in IMF designed with αWPC, of 46 mg/100ml and by Claumarchirant *et al.*, (2016) reported levels of 54.79, 56.18 and 58.07 mg/100ml.. While the levels reported in the latter study are slightly higher, this may be due to the addition of MFGM-enriched powder, which is also known to be a good source of PL (ref) Also, both Moloney et al (2020) and Claumarchirant *et al.* (2016), reported that soya lecithin was included in IMF studied, which is often added to improve the emulsion stability of the IMF and as a source of LCPUFA (EFSA NDA Panel, 2014; Fenelon *et al.*, 2018). Therefore, it may be another likely source of PLs in the IMF in the aforementioned studies compared to IMF prototypes produced in the current study, where no lecithin was used.

Compared to Group A prototypes, levels of individual PLs in the Group B prototypes (Table 2.4) are closer to those reported in human milk up to 6 months postpartum, which range from 7.96 - 16.49 mg/100ml (SM) , 8.29 -15.90 mg/100ml (PE) , 3.12 – 6.32 mg/100ml (PI), 3.39 – 6.74 mg/100ml (PS) (Claumarchirant *et al.*, 2016). PLs are important nutrients in the infant diet as they can protect DNA, proteins, and lipids from oxidative damage, have a positive effect on memory and learning capacity, and support the nervous system (Cilla *et al.*, 2016). Thus, supplementing IMF with αWPC represents a viable method to increase PL content, which has potential health benefits for formula-fed infants.

2.3.2.4 Fatty Acid composition

FA composition in IMF is determined by the fats and oils used in their production. Total FA in the study prototypes ranged from 4.70 - 5.03 g/100ckal. As expected, the FA profiles of the prototypes varied depending on the oil blends used (Table 2.5). Butyric acid (C4:0) Butyric acid (C4:0) was highest in the prototypes designed with OB3 (3A and 3B) containing AMF, which included 3A (1.95 %FA) and 3B (1.96 %FA). This fatty acid is present in bovine milk fat, but not in vegetable fat and only found in trace amounts in HM (Hageman, Keijer, *et al.*, 2019). Anti-carcinogenic effects and anti-inflammatory and immunosuppression properties of butyric acid are well known (German and Dillard, 2006) and may have potential have beneficial effects on health (Korhonen, 2011), however further confirmation and human clinical studies are required. Prototypes 3A and 3B also had highest content of SAFA (44.44 and 44.51 %FA) and TFA (1.55 and 1.52 %FA) and were lowest in MUFA's (34.74 and 34.68 %FA) (Table 2.5). TFAs are not synthesised by the human body, moreover, evidence indicates their adverse effects on blood lipids and lipoproteins and higher intakes of TFA correlate with increased risk of coronary heart disease. (EFSA NDA Panel, 2013). Levels of TFA in the prototypes were within the

accepted range defined by EFSA (no more than 3 %FA). It has been previously reported that IMF designed with AMF compared with an IMF designed with only vegetable fat contains higher levels of butyrate, SAFA and MCFAs, and lower levels of MUFAs (Hageman, Danielsen, *et al.*, 2019).

Of interest, levels of α-linolenic acid (ALA, C18:3 n-3) in all prototypes were at the levels above what is recommended by EFSA as minimum FA level (0.05 g/100 kcal) in IMF but within the recommended range. Prototypes with addition of AMF (3A and 3B) had highest levels of this fatty acid, at the maximum recommended by EFSA (0.1 g/100 kcal). These protypes also had measurable levels of other important PUFAs, such as ARA (0.003 g/100kcal) and EPA (0.003 g/100kcal), which were either not detected or detected at lower levels in other prototypes. However, levels of linoleic acid (LA, C18:2 n-6) are lowest in these prototypes (0.73 and 0.69 g/100kcal or 14.76 and 14.77 %FA). Linoleic acid (LA, C18:2 n-6) was highest in prototypes designed with OB5 (5A and 5B at 0.94 g/100kcal; 18.74 %FA) which contain a novel sn-2 fat 2, which makes it also highest in total PUFA. LA and ALA are essential FAs, from which LC-PUFA are synthesised by endogenous enzymes. These FA are found in HM at 7.53-24.29 %FA and 0.35-4.06 %FA respectively (EFSA NDA Panel, 2014; Hageman, Danielsen, et al., 2019). EFSA regulations classify the supplementation of IMF with LC-PUFA's as optional but, when added, there are associated with specific regulatory requirements. Prototypes designed with OB5 also had the highest levels of palmitic acid (5A and 5B at 1.18 and 1.16 g/100kcal or 23.48 and 23.07 %FA). Palmitic acid is a predominant SUFA in HM found in the range of 15.43-29.00 %FA, which can vary according to the stage of lactation and geographical distribution (refs). In the current study levels of palmitic acid were within this range for all prototypes, except for prototypes 1A and 1B designed with OB1, which had lower levels at 11.30 and 11.11 %FA. When a mixture of only vegetable oils is used in IMF production a source of palm oil should be added to attain a similar level of palmitic acid as found in HM (Hageman, Danielsen, *et al.*, 2019). Previous studies have reported that IMF designed with sunflower oil, coconut oil, and/or soy oil have lower levels of palmitic acid levels compared to IMF designed with AMF or palm oil (Hageman, Danielsen, *et al.*, 2019). Prototypes designed with OB1 (1A and 1B), contained the highest levels of MUFA (51.89 and 50.40 %FA respectively), of which oleic acid was the highest (51.46 and 49.99 %FA) compared to other prototypes, which is also higher than that found in HM 28-43 %FA (Zou *et al.*, 2017).

Table 2.5. Fatty Acid (FA) composition of IMF prototypes designed with different oil blends

Fatty Acid	1A		2A		3A		4A		5A		1B		2B		3B		4B		5B	
	g/100kcal	% Total FA	g/100kcal	% Total FA	g/100kcal	% Total FA	g/100kcal	% Total FA	g/100kcal	% Total FA	g/100kcal	% Total FA	g/100kcal	% Total FA	g/100kcal	% Total FA	g/100kcal	% Total FA	g/100kcal	% Total FA
Short-chain FAs (SCFA)																				
C4:0 Butyric acid	0.002	0.04	0.002	0.05	0.10	1.95	0.002	0.04	0.002	0.05	0.003	0.05	0.003	0.06	0.09	1.97	0.003	0.05	0.003	0.06
Medium-chain FAs (MCFA)																				
C6:0 Caproic acid	0.01	0.17	0.01	0.19	0.06	1.21	0.01	0.16	0.01	0.16	0.01	0.18	0.01	0.19	0.06	1.21	0.01	0.16	0.01	0.17
C8:0 Caprylic acid	0.08	1.64	0.09	1.81	0.07	1.36	0.07	1.41	0.07	1.47	0.08	1.71	0.09	1.75	0.06	1.35	0.07	1.41	0.08	1.50
C10:0 Capric acid	0.06	1.26	0.07	1.39	0.10	2.07	0.05	1.09	0.06	1.13	0.07	1.32	0.07	1.35	0.10	2.05	0.05	1.09	0.06	1.17
C12:0 Lauric acid	0.46	9.23	0.51	10.29	0.28	5.56	0.39	7.95	0.42	8.36	0.48	9.64	0.48	9.88	0.26	5.51	0.40	7.93	0.43	8.53
Long-chain FAs (LCFA)																				
C14:0 Myristic acid	0.19	3.89	0.22	4.43	0.35	7.06	0.18	3.54	0.19	3.69	0.21	4.21	0.22	4.48	0.33	7.09	0.18	3.55	0.19	3.81
C14:1 N-5 cis myristoleic acid	0.001	0.01	0.001	0.01	0.03	0.59	<0.001	0.01	0.001	0.01	0.001	0.01	0.001	0.01	0.03	0.58	0.001	0.01	0.001	0.01
C15:0 Pentadecanoic acid	0.002	0.04	0.002	0.05	0.03	0.60	0.002	0.05	0.002	0.05	0.002	0.05	0.002	0.05	0.03	0.59	0.003	0.05	0.003	0.05
C16:0 Palmitic acid	0.57	11.30	0.95	19.31	0.90	18.06	1.13	22.86	1.18	23.48	0.55	11.11	0.96	19.57	0.85	18.18	1.15	22.96	1.16	23.07
C16:1 N-7 cis palmitoleic acid	0.01	0.11	0.01	0.12	0.04	0.89	0.004	0.08	0.004	0.08	0.01	0.11	0.01	0.12	0.04	0.88	0.004	0.08	0.004	0.09
C17:0 Margaric acid	0.002	0.05	0.003	0.07	0.01	0.28	0.004	0.07	0.004	0.08	0.003	0.06	0.003	0.07	0.01	0.27	0.004	0.07	0.004	0.07
C18:0 Stearic acid	0.15	2.99	0.18	3.55	0.28	5.67	0.20	4.10	0.22	4.34	0.17	3.43	0.18	3.60	0.27	5.68	0.21	4.15	0.21	4.25
C18:1 N-9 cis oleic acid (+n-7 cis)	2.58	51.46	1.97	39.84	1.64	32.90	1.95	39.37	1.78	35.39	2.48	49.99	1.97	40.15	1.55	32.88	1.95	39.09	1.79	35.52
C18:2 N-6 cis linoleic acid	0.75	14.94	0.79	16.08	0.73	14.76	0.81	16.27	0.94	18.74	0.75	15.21	0.78	15.87	0.69	14.77	0.82	16.33	0.94	18.77
C18:3 N-3 cis alpha-linolenic acid	0.06	1.16	0.06	1.31	0.10	1.96	0.07	1.32	0.06	1.17	0.06	1.19	0.06	1.27	0.09	1.95	0.07	1.31	0.06	1.14
C20:0 Arachidic acid	0.01	0.23	0.01	0.27	0.01	0.26	0.01	0.29	0.01	0.30	0.01	0.25	0.01	0.26	0.01	0.26	0.01	0.29	0.01	0.29
C20:4 N-6 cis arachidonic acid aa (ARA)	<0.002	<0.002	<0.002	<0.002	0.003	0.05	<0.002	<0.002	<0.002	<0.002	0.001	0.01	0.001	0.01	0.003	0.06	0.001	0.01	0.001	0.01
C20:5 N-3 cis eicosapentanoic acid (EPA)	<0.002	<0.002	<0.002	<0.002	0.002	0.03	<0.002	<0.002	<0.002	<0.002	<0.002	<0.002	<0.002	<0.002	0.001	0.03	<0.002	<0.002	<0.002	<0.002
C22:0 Behenic acid	0.03	0.52	0.02	0.31	0.01	0.25	0.01	0.24	0.02	0.35	0.02	0.50	0.02	0.33	0.01	0.24	0.01	0.24	0.02	0.34
C22:6 N-3 cis docosahexaenoic acid (DHA)	<0.002	<0.002	<0.002	<0.002	<0.002	<0.002	<0.002	<0.002	<0.002	<0.002	<0.002	<0.002	<0.002	<0.002	<0.002	<0.002	<0.002	<0.002	<0.002	<0.002
C24:0 Lignoceric acid	0.01	0.20	0.01	0.14	0.01	0.11	0.01	0.11	0.01	0.13	0.01	0.16	0.01	0.17	0.01	0.11	0.01	0.11	0.01	0.13
Total LCFA	4.38	87.41	4.24	85.97	4.25	85.34	4.41	89.06	4.44	88.51	4.30	86.82	4.24	86.46	4.01	85.36	4.45	89.03	4.44	88.23
TOTAL FA	5.01		4.93		4.98		4.96		5.02		4.95		4.90		4.70		4.99		5.03	
Total SCFA and MCFA	0.62	12.34	0.68	13.74	0.60	12.14	0.53	10.64	0.56	11.17	0.64	12.90	0.65	13.22	0.57	12.09	0.53	10.64	0.57	11.42
Fatty acids, Mono unsaturated (MUFA)	2.60	51.89	1.98	40.22	1.73	34.74	1.97	39.72	1.79	35.72	2.50	50.40	1.99	40.55	1.63	34.68	1.97	39.45	1.80	35.85
Fatty acids, Poly unsaturated (PUFA)	0.81	16.11	0.86	17.40	0.84	16.90	0.87	17.60	1.00	19.93	0.81	16.44	0.84	17.18	0.80	16.92	0.88	17.68	1.00	19.96
Fatty acids, Saturated (SAFA)	1.58	31.55	2.06	41.86	2.21	44.44	2.08	41.90	2.19	43.58	1.62	32.67	2.05	41.75	2.09	44.51	2.10	42.07	2.19	43.45
Fatty acids, Total trans (TFA)	0.01	0.20	0.06	1.25	0.08	1.57	0.02	0.48	0.02	0.46	0.01	0.24	0.01	0.23	0.07	1.52	0.02	0.50	0.02	0.44

Conclusion

Ten IMF prototypes were developed that contained different oil blends and protein sources (αWPC or DWP). As expected, FA profiles varied, depending on the oil blends used in the formulations. Of interest, important PUFAs, such as ARA and EPA, were detected in prototypes with anhydrous milk fat (AMF). The strategy used in this study confirms that supplementing IMF with αWPC serves as an effective method to increase levels of phospholipids and α-lactalbumin, with levels comparable to those found in HM. Prototypes with higher levels of α-lactalbumin had lower levels of the allergenic β-lactoglobulin protein. Furthermore, this approach provides enrichment of IMF with high quality protein. This strategy demonstrates that modifying IMF ingredients, particularly the whey protein, can help develop products that are more comparable to human milk. Produced prototypes will be further analysed to explore their potential biological activities *in vitro* and investigate if different oil blends and protein sources are associated with different bioactive properties.

Chapter 3. Investigating the antimicrobial and anti-adhesive properties of IMF prototypes with different lipid and protein profiles

Abstract

The infant's gut is vulnerable to pathogens. Adherence of pathogenic bacteria to intestinal epithelial cells can lead to invasive neonatal sepsis, which is associated with a high risk of pneumonia, septicemia, and meningitis. Formula-fed infants are more susceptible to infections compared to breast-fed infants. The addition of food substances with anti-infective properties activity to infant milk formula (IMF) may serve as an effective strategy to reduce the incidence and severity of invasive bacterial infections. In this study, ten IMF prototypes were designed with 5 different oil blends (OB) and 5 were supplemented with alpha-lactalbumin enriched whey powder (α-WPC). IMF prototypes were digested using a simulated gastrointestinal *in vitro* digestion (SGID) infant model. The antimicrobial potential of undigested and digested IMF prototypes was evaluated by well diffusion assay and anti-adhesion properties were assessed *in vitro* using an intestinal human colorectal adenocarcinoma cell culture model (HT-29, clone 34). Screening of the undigested and the SGID digests against *Escherichia coli*, *Listeria monocytogenes* and Group B Streptococcus strains indicated no antimicrobial activity against the strains tested. No significant ($p<0.05$) anti-adhesion effect was observed for any IMF prototypes in HT-29 cell line clone 34. Overall, there was no significant difference between the combination of tested oil blends and whey protein sources based on their antimicrobial activity.

Key words: pathogenic bacteria, IMF, SGID, anti-infective, anti-adhesion, HT-29 cell line

3.1 Introduction

Neonatal sepsis is associated with a high risk of pneumonia, septicemia, and meningitis (Simonsen *et al.*, 2014). Neonatal pathogens, such as *Escherichia coli* (*E. coli*), Group B streptococcus **(**GBS) and *Listeria monocytogenes* are involved in early-onset neonatal sepsis, where GBS is the most common etiologic agent, while *E. coli* is the most common cause of mortality (Simonsen *et al.*, 2014). Formula-fed infants are reported to have a higher incidence of infections compared to breast-fed infants (Ip *et al.*, 2009; Grote *et al.*, 2016; Lönnerdal *et al.*, 2017). It may be that compositional differences between human milk (HM) and infant milk formula (IMF) contribute to this observed effect (Lönnerdal *et al.*, 2017). The antimicrobial properties of HM are reported to be primarily linked to immunoglobulins and other protective factors such as lipids, lactoferrin, lactoperoxidase, lysozyme, α-lac, and oligosaccharides (Isaacs, 2001; Schlievert *et al.*, 2019), as well as other antimicrobial agents released following gastrointestinal digestion, such as bioactive peptides, amino acids (AAs) and fatty acids (FAs) (Dolan, Boesman-Finkelstein and Finkelstein, 1986; Newburg, 2005; Bielecka, Cichosz and Czeczot, 2022). These antimicrobial molecules contribute to protective mechanisms by reducing the bacterial load in the gastrointestinal (GI) tract, via immune modulation, as well as alterations to the gastrointestinal microbiome (Trend *et al.*, 2015).

Due to compositional differences, some of the endogenous antimicrobial agents are lacking in IMF, thus modifying the lipid and protein content of IMF to make it more comparable to HM, is a strategy to potentially reduce the incidence and severity of invasive bacterial infections (Isaacs, Litov and Thormar, 1995; Trend *et al.*, 2015; Wei, Jin and Wang, 2019).

Several studies have confirmed the antimicrobial activity of individual bovine milk components; milk fat globule membrane (MFGM) (Schroten *et al.*, 1992; Guri *et al.*, 2012; Ross *et al.*, 2016; Douëllou *et al.*, 2018), oligosaccharides (Altamimi, Abdelhay and Rastall, 2016; Ryan *et al.*, 2018), α-lac and β-lactoglobulin (β-lg) and their peptides (Pellegrini *et al.*, 1999; Pihlanto-Leppälä *et al.*, 1999; Biziulevičius *et al.*, 2006; Brück, Kelleher, *et al.*, 2006; Théolier *et al.*, 2013), casein and serum albumin (Biziulevičius *et al.*, 2006), lipids and fatty acids (Isaacs, Litov and Thormar, 1995; Isaacs, 2001; Sprong, Hulstein and Van Der Meer, 2001). However, limited number of studies have investigated the antimicrobial properties of IMF, pre- and post-digestion.

Lipid-dependent antimicrobial activity of human and bovine milk and IMF is associated with specific long-chain unsaturated fatty acids (e.g. oleic acid), medium-chain saturated

fatty acids (e.g. lauric acid) and their monoglycerides, which are released in the GI tract as a result of lipolytic activity (Isaacs, Litov and Thormar, 1995; Isaacs, 2001).Studies demonstrate that the antimicrobial activity of bovine milk lipids can species-specific and dependent on lipid type, structure and FA composition. For example, *Escherichia coli* (*E. coli*) was shown to be inactivated by mixtures of medium-chain FAs but resistant to long-chain unsaturated FAs (Isaacs, 2005) Sprong et al. (2001) reported that sphingolipids and triglycerides, containing carbon-10 ($C_{10.0}$) and carbon-12 ($C_{12.0}$) saturated fatty acids, have strong bactericidal activity against food-borne pathogens, including *E. coli*, *Salmonella* and *Shigella* species. In the same study saturated C-18 fatty acids ($C_{18.0}$) demonstrated antimicrobial activity against *Listeria monocytogenes* and *Campylobacter jejuni* (Sprong, Hulstein and Van Der Meer, 2001). Isaacs et al (1995) reported that monoglycerides with a chain length of 8 and 10 carbons inactivated Group B streptococcus (GBS). Importantly, these studies highlight that the susceptibility of bacteria to the antimicrobial effects of lipids and FAs can be species and strain specific.

Milk proteins have also shown the potential to inhibit the growth of bacteria. The most investigated milk proteins include lactoferrin, lysozyme and their proteolytic fragments (Bielecka, Cichosz and Czeczot, 2022). There are limited studies reporting on the antimicrobial potential of peptides encrypted within α-lactalbumin (α-lac) (Brandelli, Daroit and Corrêa, 2015). Of interest, Pellegrini et al. (1999) demonstrated that enzymatic digestion of bovine milk α-lac with trypsin and chymotrypsin (but not with pepsin) have yielded bactericidal peptides that were mostly active against Gram-positive bacteria (e.g. *B. subtilis, S. aureus*), and weakly active against some Gram-negative bacteria (e.g. *P. aeruginosa*), with no activity observed against *E.coli* (ATCC 25922) (Pellegrini *et al.*, 1999). α-Lac peptides were negatively charged, and the authors concluded that that their weaker activity against Gram-negative bacteria may be due to the electrostatic repulsion between these peptides and negatively charged lipopolysaccharide on the outer membrane of bacteria. However, Pihlanto-Leppälä et al. (1999) demonstrated the antimicrobial effect of digested α-lac against genetically modified *E. coli* (JM103). In another study, Biziulevičius et al. (2006) also reported the antimicrobial potential of α-lac hydrolysates against 19 different bacterial strains, including 4 strains of *E.coli* (K12, M17, MRE-600, and O2).

The anti-infective properties of milk components, such as proteins, may be due to their ability to prevent pathogen adhering to host epithelial cells. Brück et al. (2006) demonstrated the anti-adhesion properties of the human and bovine milk α-lactalbumin

protein. Reduced microbial adhesion of GI infections causing pathogens such as *Salmonella typhimurium*, *E. coli*, and *Shigella flexner* to intestinal cells was observed in the presence of undigested and digested α-lac. Of interest, the degree of pathogen adhesion to intestinal cells varied depending on whether trypsin, pepsin, or chymotrypsin were used for digestion as well as the concentration of the treatment (Brück, Kelleher, *et al.*, 2006).

Milk is a complex food matrix, which following digestion releases an abundance of antimicrobial peptides (Bielecka et al., 2022). It is possible that several bioactive compounds, released following digestion, have antimicrobial properties that can potentially work in synergy with one another, such as fatty acids (linoleic acid, lauric acid, and oleic acid) with antimicrobial peptides (Isaacs, 2005) or lysozyme (Bielecka, Cichosz and Czeczot, 2022). Therefore, a mixture of compounds with an antimicrobial potential, e.g., lipids and antimicrobial peptides, in IMF may effectively inactivate pathogens. It is of interest, to investigate bioactive properties of IMF post digestion, as the digestion kinetics and bioactive properties of individual nutrient content may be affected by compositional, physical, and structural properties of a food matrix. The structure of a food matrix is influenced by the composition of proteins and lipids and the processing method used in food production. Hence, kinetics of protein and lipid degradation during digestion and subsequent release and absorption of AAs and FAs is influenced by the structure of food matrix and protein and lipid composition (Guo *et al.*, 2017; Phosanam *et al.*, 2021).

This study aims to investigate the antimicrobial and antiadhesion properties of IMF formulations designed with different oil blends and whey sources and compared pre- and post-digestion to identify if addition of α-lac-enriched whey powder and different oil blends affected the antimicrobial properties of the formulations.

3.2 Materials and methods

All materials and reagents were sourced from Merck (Sigma-Aldrich, Ireland), unless otherwise stated.

3.2.1 Batch sterility check

Ten IMF prototypes, containing different lipids and protein, were produced, as described in Chapter 2 (Section 2.2.2). Briefly, five prototypes (Group A: 1A-5A) were prepared with 90 % demineralised whey powder (DWP, 12.6% protein) but contained different oil blends (OB). Five prototypes (Group B: 1B-5B) contained the same oil blends as Group

A but were prepared with a different protein source, α-lac enriched WPC (αWPC, 80% protein) (Table 2.2).

A sterility check of all IMF prototypes (n=10) was performed according to the FDA's Bacteriological Analytical Manual (BAM) laboratory procedure for microbiological analyses of foods and cosmetics, which conforms to AOAC Official Methods of Analysis, sec. 966.23. Briefly, 45 ml of phosphate-buffered saline (PBS) was added to 5g of IMF powder and mixed until homogenous. Decimal dilutions of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} were prepared using sterile PBS by transferring 1 ml of the previous dilution to 9 ml of diluent PBS. An aliquot of 1 ml of each dilution was then pipetted into separate Petri dishes and 15 ml of plate count agar (PCA) was added and mixed thoroughly. When solidified, Petri dishes were inverted and incubated for 48 ± 2 h at 35°C. Following incubation colony forming units (CFU) were counted and the aerobic plate count (APC) was calculated for plates between 25- 250 CFU as described by Maturin and Peeler (2001):

$$N = \frac{\Sigma c}{[(1 * n1) + (0.1 * n2)] * (d)}$$

where N = Number of colonies per g of product, Σc= Sum of all colonies on all plates counted, n_1= Number of plates in the first dilution counted, n_2= Number of plates in the second dilution counted, d = Dilution from which the first counts were obtained. Distilled water was used as a negative control and all experiments were carried out in triplicate.

3.2.2 Simulated gastrointestinal digestion of IMF prototypes

To investigate and compare the potential antimicrobial and antiadhesion properties of the IMF prototypes post digestion static gastrointestinal *in vitro* digestion (SGID) model simulating the digestive conditions of a full-term infant, was used to digest all prototypes. This model included two consecutive phases: a gastric phase and an intestinal phase, which was established based on the model previously described by Menard et al (2018) with modifications. Considering that infant formula is liquid, the oral phase was omitted, as suggested for the adult international consensus (Minekus *et al.*, 2014). With each independent gastrointestinal digestion trial, an undigested control was included.

Gastric phase

Meal to gastric secretions ratio was set to be 63:37 v/v (Ménard *et al.*, 2018). Simulated gastric fluid (SGF) was composed of 94mM NaCl and 13mM KCl and adjusted to pH 5.3. Rabbit gastric extract was used as a source of gastric lipase and was added at 19 U/ml gastric content. Pepsin was added to provide activity of 268 U/ml gastric content. The

gastric phase was carried out for 60 minutes at 37°C in a shaking incubator set at 50 rpm. The reaction was completed in the dark and following gastric digestion the pH was increased to 7 by the addition of 1M NaOH to deactivate gastric enzyme activity.

Intestinal phase

The ratio of gastric phase contents and intestinal fluids was 62:38 v/v respectively (Ménard *et al.*, 2018). Simulated intestinal fluid (SIF) (164 mM NaCl, 10 mM KCl, 85 mM Na_2HCO_3) was adjusted to pH 7. $CaCl_2$ (3mM) and porcine bile extract (3.1mM) were added separately prior to the beginning of the intestinal phase. Pancreatin was added to ensure the required trypsin activity and intestinal lipase activity of 16 U/ml and 90 U/ml intestinal content, respectively. The intestinal phase was carried out for 60 minutes at 37°C in a shaking incubator set at 50 rpm. Samples were then immediately heated to 85 °C for 3 minutes. Following this, digests were centrifuged at 12000 rpm for 30 minutes, supernatants were collected, filtered (0.4µm), and stored at -20 °C until further analysed.

3.2.3 Anti-microbial investigations

Well diffusion assay was carried out according to a method described by Mothershaw et al. (2004). Fresh overnight bacterial cultures of *Escherichia coli* ATCC 25922, *Listeria monocytogenes EGD-e, and Group B Streptococcus* (MTU clinical strain, MTU-105) were diluted in Ringer's buffer to prepare suspensions at concentrations of 10^8 CFU/mL (McFarland standard 0.5). These cultures were inoculated onto BHI agar plates using a sterile swab to provide a lawn of growth. Wells of approx. 5 mm in diameter were cut out using a sterile cork borer and 90 µL of filter sterilised (0.2 µm) IMF prototypes were added to the wells. Digestates were added undiluted, and undigested IMF were reconstituted at 2.5% (w/v). Higher than 2.5% (w/v) concentrations of undigested IMF were initially tested, however were difficult to filter- sterilise. Penicillin G (1 mg/ml and 0.1 mg/ml) was used as a positive control, with the negative control consisting of sterile H_2O. Plates were then incubated at 37 °C for 24 h and then analysed for zones of inhibition. Interpretation of growth inhibition was based on measurement (mm) of zones of clearing.

3.2.4 Antiadhesion investigations

The HT-29 cell line (human colorectal adenocarcinoma, clone 34) was obtained from Nestlé (Ireland) Limited. Cells were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 1% (v/v) non-essential amino acids and 1% penicillin-streptomycin. Cells were maintained at 37 ◦C in a humidified atmosphere with 5% carbon dioxide (CO_2).

Subtoxic concentrations of IMF prototypes were determined by assessing their effects on the viability of the HT-29 cells using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. For this, 100 µl of cells ($1x10^5$ cells/ml) were seeded into 96 well plates and incubated for 24 hours at 37 °C. Following this, cells were washed with phosphate-buffered saline (PBS) and exposed to various concentrations (0.5-5 % v/v) of digested prototypes for 24 hours at 37 °C. After incubation cells were washed twice with PBS, then DMEM media (100 µl) and MTT solution (10 µl, 5 mg/ml) were added, and further incubation was allowed for 4 hours at 37 °C. Subsequently, 100 µl of dimethyl sulfoxide (DMSO) per well was added and absorbance was measured at 570 nm. Blank wells contained culture media, MTT reagent, and solvents only (no cells), and were used to correct for any background colour. Control wells included media with untreated cells, and % cell viability was determined as follows: (absorbance of tested samples/absorbance of untreated control cells) x 100.

3.2.4.1 Anti-adhesion assay

Anti-adhesion properties were assessed according to described methods with some modifications (Rhoades *et al.*, 2006; Hotchkiss *et al.*, 2015). The potential antiadhesion properties of all ten prototypes were investigated and compared using a Gram-negative bacteria (*E. coli*, ATCC 25922) and a Gram-positive bacteria (Group B *Streptococcus*: MTU-105, supplied by Hayes *et al.* (2017)). Briefly, bacterial cultures were grown in BHI medium for 24 h at 37 ^oC, and then cell pellets were harvested by centrifugation (8000rpm for 8 min), washed twice with Dulbecco's PBS buffer, and re-suspended in DMEM culture media (supplemented with 2% FBS) to a final concentration of $1 x10^8$ cells/ml.

HT-29 cells were seeded into 12-well tissue culture plates at a density of $1 x 10^5$ cells/ml/well and grown to 90-95% confluency. Monolayers were then washed twice with PBS buffer. Digested prototypes (0.5 ml of 2% v/v prepared with DMEM media) were added (in duplicate wells) and then bacterial suspensions (0.5 ml) were added to every well, and plates were incubated for 1 h at 37 ^oC, 5% CO2. After the incubation period, supernatants were removed, and cells were gently washed with PBS buffer (x3) to remove non-attached bacteria. Following incubation, cell monolayers were treated with 70 µl of 0.25% trypsin–EDTA solution, the plate was rocked to ensure even coverage, and then incubated at 37 ^oC for 5 min. PBS (1 ml) added to each well and mixed until the cell monolayer was completely dislodged, and clumps were broken up (determined visually). Bacterial counts were determined by plating onto PCA agar, after incubation under

aerobic conditions for 24 h at 37 ^{o}C. Results were expressed as the percentage of bacteria adhered relative to the number of bacteria added ((CFU bacteria adhered/CFU bacteria added) *100%).

3.3 Results and discussion

3.3.1 Batch sterility check

IMF is not a commercially sterile product even if produced under controlled conditions, therefore some level of microorganisms may still be present in the powder (Buchanan and Oni, 2012). A sterility check of the IMF prototypes was performed to make sure that prototypes produced in the pilot plant were in accordance with standards set out by Codex Alimentarius Commission (Codex Alimentarius Commission, 2008). Four prototypes from Group A (1A, 2A, 4A and 5A) were found to be contaminated with bacterial levels outside accepted criteria of ≤ 500 CFU per gram of powder (Table 3.1). While these prototypes are not intended for use by infants, to prevent interference with subsequent assay prototypes were filter-sterilised (at 0.45 µm) when appropriate.

3.3.2 Anti-microbial investigations

The antimicrobial properties of the IMF prototypes (pre and post digestion) were investigated using a well diffusion assay, and against *E. coli,* Group B Streptococcus and *Listeria monocytogenes* strains. Results indicated that none of the IMF prototypes, neither pre- nor post- digestion prototypes had a bactericidal effect against the strains tested, as none of the prototypes caused a zone of growth inhibition. All strains were susceptible to the positive control Penicillin G, as evidenced by clear zones of inhibition (Figures 3.1, 3.2 and 3.3).

Previous studies have demonstrated the presence of antimicrobial peptides released upon hydrolysis of α-lac and β-lg bovine milk proteins and IMF (Pellegrini *et al.*, 1999; Pihlanto-Leppälä *et al.*, 1999; Biziulevičius *et al.*, 2006; Brück, Redgrave, *et al.*, 2006). However, to exhibit antimicrobial effect bioactive components must be present at certain concentrations. For instance, in the study conducted by Pihlanto-Leppälä et al. (1999) undigested bovine milk proteins (α-lac and β-lg) did not exhibit antimicrobial activity at concentrations of 100 mg/ml, however bacteriostatic effects for α-lac and β-lg hydrolysates (digested with combination of trypsin and pepsin) were observed at 25 mg/ml when tested against an *E. coli* strain (JM103). Interestingly, α-lac hydrolysed with enzyme alcalase promoted bacterial activity in the former study, suggesting that the

Table 3.1. Sterility check of IMF prototypes.

Prototypes[a]	Oil Blends[b]	MAB (CFU*10^3/g)[c]
1A	OB1	20.1±0.76
2A	OB2	1.5±0.09
3A	OB3	0
4A	OB4	0.78±0.05
5A	OB5	1.4±0.1
1B	OB1	0
2B	OB2	0
3B	OB3	0
4B	OB4	0
5B	OB5	0

[a] Group A prototypes (1A-5A), and Group B prototypes (1B-5B) were designed such that they had different whey protein sources: demineralised whey powder (DWP, 12.6% protein) was added to Group A prototypes, α-lac enriched WPC (αWPC, 80% protein) was added to Group B prototypes.
[b] Prototypes contained one of 5 oil blends (OB1-OB5): OB1 contained soybean, high oleic sunflower, and coconut oils in the ratio of 20:20:60, respectively; OB2 contained the same oils in different ratio (33:21:25) in addition to palm oil (21%); OB3 contained vegmix with anhydrous milk fat (45%) OB4 contained OB1 oils (13:25:15) with addition of novel sn-2 fat 1 (46 %); OB5 contained OB1 oils (13:25:15) with addition of sn-2 fat 2 oil (46%).
[c] MAB, Mesophilic Aerobic Bacteria; CFU, Colony Forming Units. Results expressed as mean ±SD

antimicrobial activity of hydrolysates is dependent on the enzyme involved in the hydrolysis as well as their concentration levels in the tested samples.

Pellegrini et. al (1999) have demonstrated that antimicrobial properties of bovine milk α-lac, digested with trypsin and chymotrypsin, were dose dependent as well as species and strain specific: at concentrations 1 x 10^{-6} and 5.5 x 10^{-8} mol per assays did not show antimicrobial activity against *E. coli* ATCC 25922 but *Bacillus subtilis* BGA was the most susceptible to α-lac hydrolysates among all other bacteria tested. In contrast to its polypeptide fragments, undigested α-lac has shown no antimicrobial effect on any of the tested bacterial strains in the former study. Théolier et al. (2013) reported that hydrolysed whey protein isolate (WPI) had no microbial inhibitory effect against an *E. coli* strain, however at 75 mg/mL the hydrolysate inhibited a *Listeria ivanovii* isolate. Of interest, lower concentrations of the hydrolysate were ineffective against the same *Listeria ivanovii* strain.

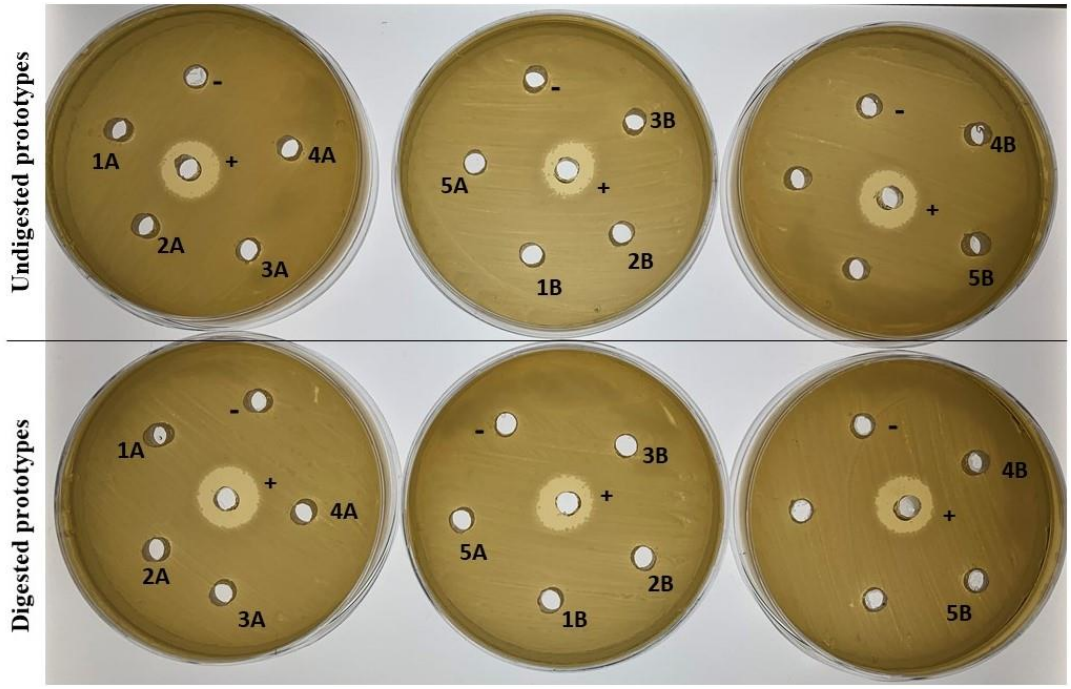

Figure 3.1. Antimicrobial investigations of IMF prototypes against *E. coli* (#ATCC 25922). Figure shows representative samples from Group A prototypes (1A-5A) and Group B prototypes (1B-5B). Controls are indicated by '-': negative control (H_2O) and '+': positive control (Penicillin G, 1mg/ml).

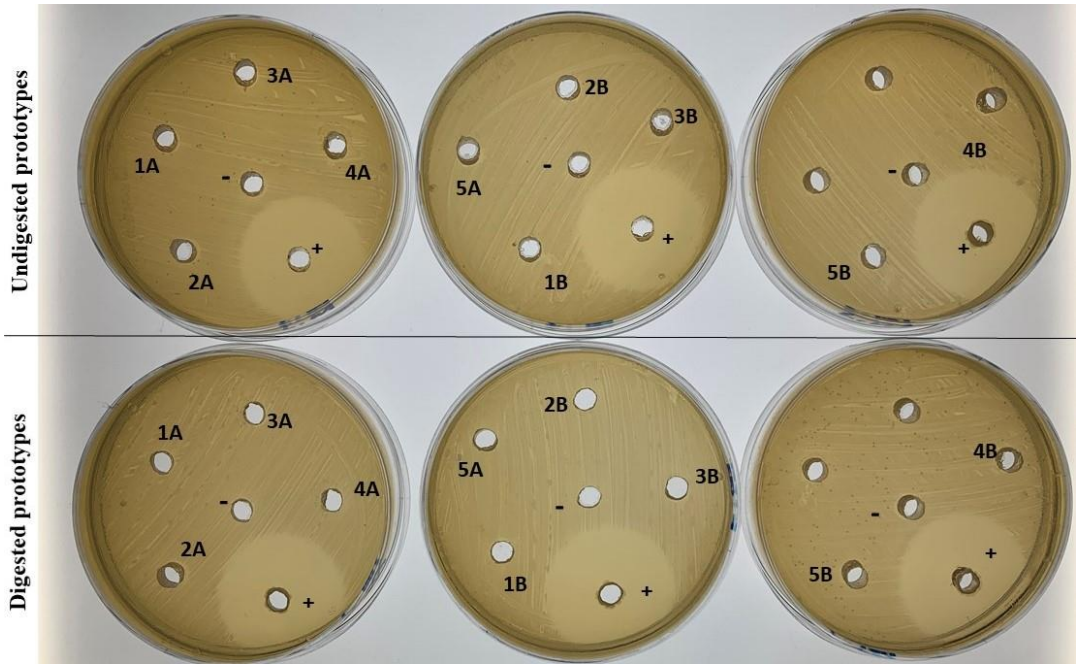

Figure 3.2. Antimicrobial investigations of IMF prototypes against *Listeria monocytogenes* (EGD-e). Group A prototypes (1A-5A) and Group B prototypes (1B-5B). Controls are indicated by '-': negative control (H_2O) and '+', positive control (Penicillin G 0.01mg/ml). Group A prototypes (1A-5A) and Group B prototypes (1B-5B)

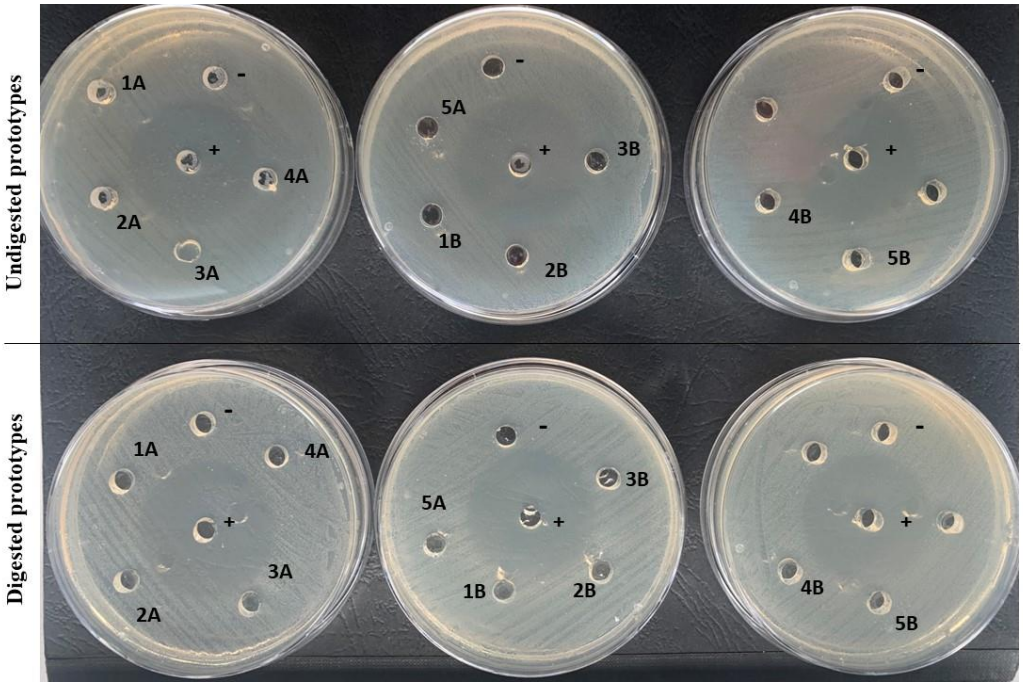

Figure 3.3. Antimicrobial investigations of IMF prototypes against Group B Streptococcus (MTU-105). Group A prototypes (1A-5A) and Group B prototypes (1B-5B). Controls are indicated by '-': negative control (H_2O) and '+': positive control (Penicillin G 0.01mg/ml).

Bactericidal properties of milk lipids have been reported and is dependent on the lipid type, its FAs' chain length, concentration and, also, are bacterial strain and species specific. Sprong et al (2001) demonstrated the antimicrobial activity of sphingosine, an 18-carbon amino alcohol with an unsaturated hydrocarbon chain, which forms a primary part of sphingolipids. The study reported that concentrations of 25 µmol/litre were effective against *C. jejuni* and *L. monocytogenes* strains, whereas higher concentrations of 100 µmol/litre were needed to inhibit *E. coli* and *S. enteritidis* strains. Therefore, it could be that the lack of antimicrobial activity observed in the present study may be due to fact that the bioactive components in the samples were not present in sufficient quantities to exhibit antimicrobial effects on the strains tested.

3.3.3 Cell viability

To determine non-toxic concentrations for use in the antiadhesion assay the viability of the intestinal cells was determined with digested IMF prototypes in HT-29 cells. In general HT-29 cell viability, as measured by the MTT assay, was above 90% in the presence of most IMF prototypes at tested concentrations (0-2 % v/v) (Table 3.2). Group B prototype, 1B, a significant ($p<0.05$) decrease in cell viability was observed at 1% v/v (89.26±5.84 %) and 2% v/v (82±7.37 %). As cell viability was >80% for all prototypes at 2%, this was selected as test concentration for anti-adhesion investigations.

3.3.4 Anti-adhesion properties of IMF prototypes

The potential antiadhesion properties of the prototypes were investigated using a human adenocarcinoma intestinal cell line (HT-29, clone 34) and one Gram negative bacteria (*E. coli*, #ATCC 25922) and one Gram positive bacteria (Group B *Streptococcus*: MTU 105, supplied by Hayes et al. 2017).

Data indicated that the IMF digestates, at the chosen concentration tested (2% v/v), did not exhibit antiadhesion properties against *E. coli*. Indeed, for some prototypes adherence increased, although not significantly (Figure 3.4). No antiadhesion effect of IMF digestates was observed against GBS (Figure 3.5) either.

Brück et al. (2006) demonstrated the dose-dependent anti-adhesion properties of purified human and bovine α-lactalbumin (both digested and undigested) against Gram-negative bacteria such as *E. coli*, *Salmonella typhimurium*, and *Shigella flexneri* strains in intestinal CaCo-2 cells.

Table 3.2 Investigating the effect of IMF digestates (0 -2 % v/v) on HT-29 cell viability

Sample	**Cell viability (% of control)**			
(%v/v)	0	0.5	1.0	2.0
1A	100 ± 0	100 ± 28.74	100 ± 3.88	99.32 ± 13.23
1B	100 ± 0	105.03 ± 17.92	89.26 ± 5.84	82 ± 7.37*
2A	100 ± 0	114.56 ± 12.63	101.91 ± 7.28	96.94 ± 1.3
2B	100 ± 0	98.87 ± 10.47	99.38 ± 11.39	89.48 ± 26.72
3A	100 ± 0	106.68 ± 5.07	95.14 ± 18.48	99.43 ± 23.31
3B	100 ± 0	115.75 ± 18.73	108.88 ± 14.67	90.68 ± 7.47
4A	100 ± 0	120.6 ± 17.73	109.34 ± 10.66	95.23 ± 9.57
4B	100 ± 0	101.28 ± 6.38	97.51 ± 1.63	86.5 ± 4.86*
5A	100 ± 0	99.83 ± 23.22	93.2 ± 12.62	93.71 ± 19.1
5B	100 ± 0	110.54 ± 7.22	106.66 ± 10.29	105.46 ± 6.19

Values represent the mean±SD of three independent experiments, expressed as a percentage relative to untreated HT-29 cells. Statistical analysis by Student's TTEST (MS Excel). *Denotes statistically significant difference between sample and untreated cells control; $p<0.05$.

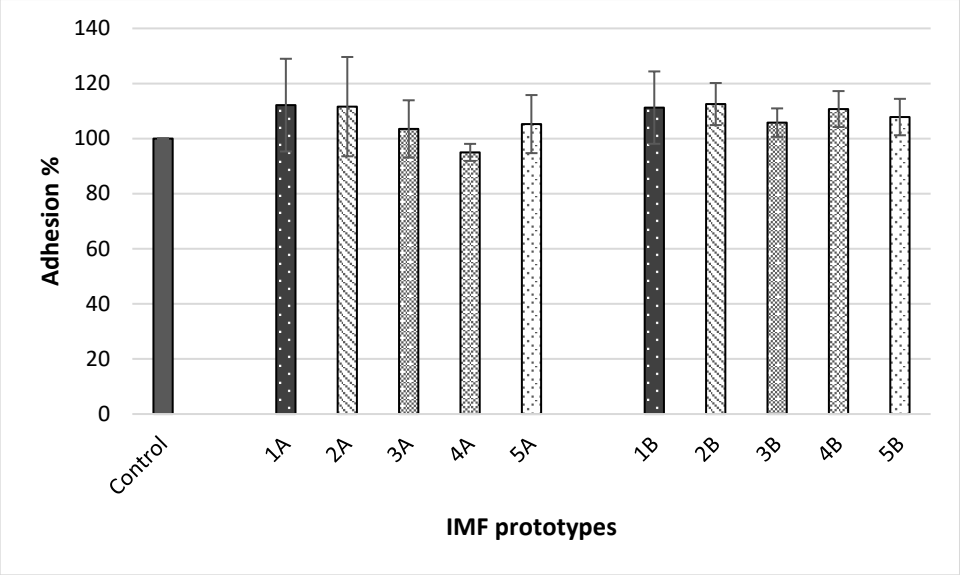

Figure 3.4. Effect of IMF prototypes, post-digestion, on the adhesion of *E. coli* to HT-29 clone 34 cells. Control was *E. coli* in the media with no sample. Data are mean ± standard deviation of assays carried out on three separate occasions in triplicate. No significant difference was observed.

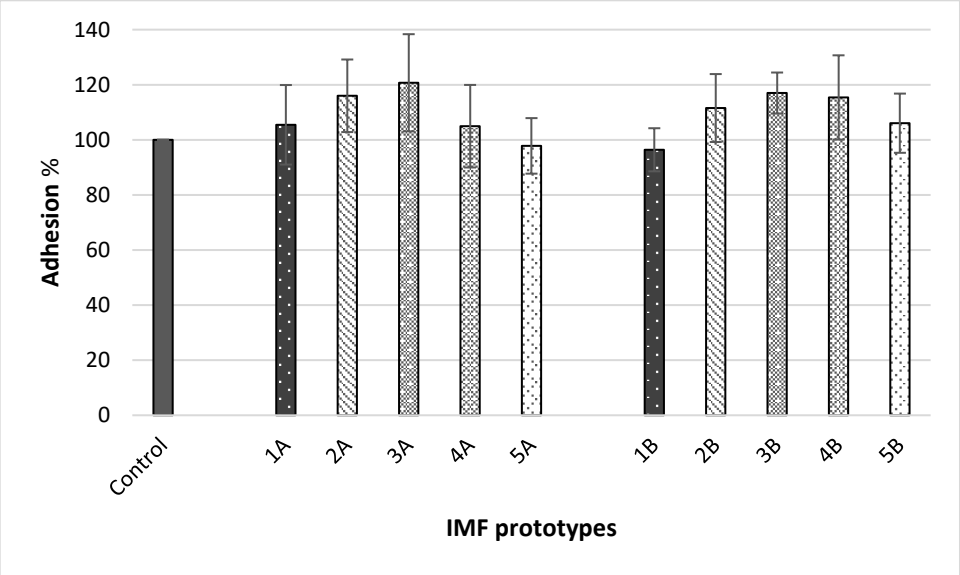

Figure 3.5. Effect of IMF prototypes on the adhesion of GBS to HT-29 clone 34 cells. Control was GBS in the media with no samples. Data are means ± standard deviation of assays carried out on three separate occasions in triplicate. No significant difference was observed

Of interest the degree of bacterial inhibition was species and strain specific. Undigested protein at all test concentrations (0.25, 0.05, and 0.01 mg/ml) were effective against *Shigella flexneri* adhesion to the cells, effective against *Salmonella typhimurium* only at higher concentrations and had no effect against *E. coli*. Of interest, enzymatic treatment influenced the bioactive properties of the digestates, as evidenced by differences in anti-

adhesion properties. Pepsin- and pancreatin-digested proteins all had an inhibitory effect against both *E. coli and Salmonella typhimurium* but only at higher concentrations against *Shigella flexneri.* Nonetheless, the higher concentrations of hydrolysates dictated a higher degree of bacterial inhibition (Brück, Kelleher, *et al.*, 2006). Diversity of peptides released from whey proteins using different enzymes has been previously documented (Sibel Akalin, 2014; Brandelli, Daroit and Corrêa, 2015), but their mechanism of action is still not fully elucidated and may depend on numerous factors, such as amino acid sequence, secondary structure, net charge, isoelectric point, and amphipathicity (Brandelli, Daroit and Corrêa, 2015). For example, digestion of bovine α-lac with trypsin generated antimicrobial peptides with the sequence EQLTK and GYGGVSLPEWVCTTF/ALCSEK, digestion with chymotrypsin - CKDDQNPH/ISCDKF, whereas no peptides with antimicrobial activity were obtained with pepsin digestion (Pellegrini *et al.*, 1999). These data suggest that bovine α-lac-derived peptides are associated with anti-adhesion properties. In the current study, while IMF prototypes contain α-lac, with higher levels present in the Group B prototypes, anti-adhesion properties were not evident, suggesting either a lack of bioactive peptides in the digestates, or ineffective concentrations of peptides with antiadhesion properties.

Milk-derived sphingolipids are associated with anti-adhesion properties (Douëllou, Montel and Thevenot Sergentet, 2017; Yoon *et al.*, 2018). Bacteria bind to glycosphingolipids on host cells to initiate adherence and cell invasion (Douëllou, Montel and Thevenot Sergentet, 2017). Milk sphingolipids can mimic these binding sites, and prevent bacteria from invading host cells (Douëllou, Montel and Thevenot Sergentet, 2017). However, to date there are no studies investigating the antiadhesion properties of individual milk phospholipids or IMF with different phospholipid composition. Noteworthy, bacterial anti-adhesion to intestinal cells is mainly attributed to milk immunoglobulins (Naaber *et al.*, 1996; Casswall *et al.*, 2002; Brooks *et al.*, 2006; Ulfman *et al.*, 2018), oligosaccharides (Simon *et al.*, 1997; Maldonado-Gomez *et al.*, 2015), and MFGM components, namely its protein mucin 1 (Schroten *et al.*, 1992), sphingolipids and gangliosides (Sánchez-Juanes *et al.*, 2009; Douëllou, Montel and Thevenot Sergentet, 2017). It may be possible that higher concentrations (v/v) of the digestates, potentially containing higher concentrations of bioactive peptides would have promoted anti-adhesion effects, however, higher than 2% v/v of the digests would have been toxic to the HT-29 cells (data not shown). Different cell line could be employed for further analysis for this purpose, e.g. Caco-2 cells or Caco-2/HT29-MTX coculture that are routinely used

for anti-adhesion studies (Naaber *et al.*, 1996; Brück, Kelleher, *et al.*, 2006; Verhoeckx *et al.*, 2015). Furthermore, while isolated individual milk bioactive components pre- and post-digestion may have a potential for inhibition of bacterial growth or adhesion, it is important to note that within the food matrix, such as IMF, they interact differently with other nutrients, e.g., competing for enzymes or receptor binding sites in the intestine, which may have biological significance (Institute of Medicine, 2004). In addition, processing of IMF modifies the structure of milk fats and proteins, thereby affecting digestion patterns, bioaccessibility and bioavailability of resulting fatty acids and peptides (Bourlieu, Ménard, *et al.*, 2015; Bavaro *et al.*, 2021), therefore, affecting potential bioactivities including anti-adhesion properties..

Conclusion

This study has shown that there is no bactericidal activity exhibited by IMF prototypes in their intact form nor following digestion as assessed by well diffusion assays against tested bacterial strains. Data from this study suggested that the IMF prototypes did not display bactericidal effects against the Gram-negative and Gram-positive strains tested. Following SGID, none of the IMF prototypes had shown anti-adhesion effect in HT-29 cells at tested concentrations. The lack of antimicrobial and anti-adhesion effects observed in this study against *Escherichia coli* ATCC 25922, *Listeria monocytogenes (EGD-e), and Group B Streptococcus* (MTU-105) may be due to low concentrations of antimicrobial compounds or may be due to strain-specific effects. Clinical isolates often show different susceptibility to antimicrobial agents, therefore, to identify if the antimicrobial potential of the IMF prototypes was species and/or strain dependant it may be warranted to test other strains. GI digestion potentially releases antimicrobial bioactive peptides and fatty acids.

Overall, while there was no antimicrobial or anti-adhesion effect observed, it appears that this study is first of its kind to examine named bioactivities of IMF prototypes designed with different novel oils and protein sources following SGID digestion. It may be concluded that modification of lipids and protein profiles in IMF had no effect on their antimicrobial potential under the conditions used in this study.

Chapter 4. Investigating the antioxidant properties of IMF with different lipid and protein profiles

A manuscript based on this chapter has been prepared and will be submitted to the *International Journal of Food Sciences and Nutrition*

Abstract

Neonates need an adequate supply of dietary antioxidants to prevent damage evoked by oxidative stress. Evaluating the impact of different protein and lipid profiles on the antioxidant (AO) properties of infant milk formula (IMF) will help to identify optimal blends for its rational design. In this study, ten IMF prototypes were designed with 5 different oil blends (OB) and 5 were supplemented with alpha-lactalbumin enriched whey powder (α-WPC). IMF prototypes were digested using a simulated gastrointestinal *in vitro* digestion (SGID) infant model. AO capacity was assessed pre- and post- digestion using chemical-based assays (FRAP, DPPH, TPC), and cellular AO assays (SOD, GSH) were also used for post-digestion prototypes. When compared, pre- and post- digestion AO activity of IMF prototypes varied across different assays. TPC significantly ($p<0.05$) increased post-digestion for prototypes supplemented with α-WPC and did not change for the ones without. Following SGID, FRAP and DPPH significantly ($p<0.05$) decreased for all prototypes and group A prototypes, respectively.

Following SGID, a combination of α-lac with anhydrous milk fat, OB1, and OB4 significantly ($p<0.05$) improved FRAP, TPC, and SOD activity respectively. Significant ($p<0.05$) intracellular protection against oxidative stress was observed in IMF containing novel oils (OB4 and OB5), which protected against H_2O_2-induced GSH and SOD depletion in U937 cells. Results of this study suggest that different combinations of α-lac and various oil blends have an impact on the AO activity of IMF which is retained following SGID.

Key words: IMF, SGID, oxidative stress, antioxidant, FRAP, DPPH, TPC, SOD, GSH

4.1 Introduction

Oxidative stress is caused by the presence of harmful free radicals, such as reactive oxygen species (ROS), nitrogen, or sulfur species, which can attack and damage biological molecules and contribute to the pathogenesis and pathophysiology of many diseases (Corrochano, Buckin, *et al.*, 2018; Kleekayai *et al.*, 2020). To protect against oxidative damage the human body uses different antioxidant mechanisms, including endogenous enzymatic methods (e.g. superoxide dismutase (SOD) and catalase (CAT)), non-enzymatic endogenous compounds (e.g. glutathione (GSH), and exogenous dietary compounds that have antioxidant properties (Kleekayai *et al.*, 2020). Infants are susceptible to oxidative stress for several reasons (Ozsurekci and Aykac, 2016), including the fact they have reduced antioxidant defence systems (Hanson *et al.*, 2016). Importantly, increasing evidence suggests that early exposure to oxidative stress is implicated in neonatal diseases such as bronchopulmonary dysplasia, retinopathy of prematurity, renal dysfunctions and necrotizing enterocolitis (Mutinati *et al.*, 2014). Nutrition-based strategies to reduce oxidative stress is of interest, and may provide a valid means of improving infant morbidity and promoting infant health (Perrone *et al.*, 2007).

Breast milk is the optimal food for infants, supplying adequate amounts of all nutrients to support growth and development (EFSA, 2014). In addition, breast milk contains various antioxidant (AO) compounds, including enzymes (e.g. glutathione peroxidase), vitamins C, E, and A, phytochemicals (e.g. β-carotene, isoflavones) and bioactive proteins and peptides (Aycicek *et al.*, 2006; Li *et al.*, 2009; Zarban *et al.*, 2009). While breastfeeding is highly recommended, it may not always be possible or solely adequate. Infant milk formula (IMF) is a breast milk substitute that aims to mimic, as much as possible, the nutritional profile of breast milk (Martin, Ling and Blackburn, 2016**).** Studies to date suggest that breast milk has superior antioxidant properties compared to IMF (Aycicek *et al.*, 2006; Tsopmo, 2018), however, manufacturers continue to try and improve formulations so that IMF may more closely resemble human milk in terms of composition and bioactive properties (Lonnerdal, 2016).

Key ingredients in IMF include bovine whey powder, skim milk powder (SMP), lactose and vegetable oils (Fenelon *et al.*, 2018). Whey powders, including whey protein isolate (WPI) and whey protein concentrate (WPC), have associated AO properties (Corrochano *et al*. 2018), attributable to intact proteins (Power-Grant *et al.*, 2015), bioactive peptides and free amino acids (FAAs) (Peng *et al.*, 2010; Kong *et al.*, 2012). It has also been reported that whey proteins can increase levels of intracellular AO compounds, such as

GSH and SOD (Xu *et al.*, 2011; Kong *et al.*, 2012; Piccolomini *et al.*, 2012; O'Keeffe and FitzGerald, 2014). α-Lactalbumin (α-lac) is the predominant whey protein in human milk (Joubran, Moscovici and Lesmes, 2015) and supplemented into IMF to boost essential amino acids (AAs) (Lonnerdal *et al.*, 2003), including AO tryptophan and cysteine (Hernández-Ledesma *et al.*, 2005; Kamau *et al.*, 2009). Of interest, it has also been shown that the addition of α-lac-enriched WPC to IMF, using specific manufacturing processes, can modify key lipid components, including cholesterol and complex lipids to levels that are comparable to human milk (Moloney *et al.*, 2018; Moloney, O'Connor and O'Regan, 2020).

The health benefits of milk lipids are well documented with reports demonstrating positive effects on neurodevelopment, gastro-intestinal and immune function (Delplanque *et al.*, 2015; Koletzko, 2017; Demmelmair and Koletzko, 2018). However, there are limited studies on the AO potential of milk lipids. MacGibbon and Taylor (2009) reported that milk phospholipids (PLs) may act as either pro-oxidants or antioxidants, with AO potential linked to their hydroxyl and nitrogen-containing amine groups (Saito and Ishihara, 1997). Interestingly, Huang *et al.* (2020) reported that while bovine milk PLs exhibited significant AO potential *in vitro*, they had limited cellular AO activity, possibly due to poor bioavailability.

It is difficult to demonstrate a direct relationship between the AO potential of a food and *in vivo* effects (Carlsen *et al.*, 2010). The need to consider the digestibility of complex food matrices, like IMF, is recognised as this will impact how AO compounds are released (Nguyen *et al.*, 2015a; Shani-Levi *et al.*, 2017; Su *et al.*, 2017). Currently, there is no single assay that is recommended for determining AO properties of foods. Protocols differ in their principles and chemistry; therefore, combining the results of different assays may provide an approximate total AO capacity of foods (Nwachukwu *et al.*, 2021). Chemical assays for measuring AO activity usually lack physiological relevance but are useful screening strategies to assess antioxidant potential. Cell-based AO assays are used to assess activity, uptake, metabolism and distribution of antioxidants at a cellular level and can be more representative of the target site of oxidative stress *in vivo* (Zhang, Liu and Tsao, 2016; Kleekayai *et al.*, 2020).

The current study aimed to investigate the AO properties of IMF, containing different protein and lipid profiles, pre and post *in vitro* digestion, and assess if there is a lipid protein profile associated with optimal AO properties.

4.2 Materials and methods

U937 cell line was obtained from the European Collection of Animal Cell Cultures (ECACC #85011440). Heat inactivated foetal bovine serum (FBS) and RPMI-1640 cell culture medium were purchased from Fisher Scientific Ireland; rabbit gastric extract (RGE) from Lipolytech, France. All other chemicals and reagents were purchased from Merck (Sigma-Aldrich, Ireland) unless otherwise stated.

4.2.1 Sample preparation

IMF prototypes were designed as described in Section 2.2.1 (Chapter 2). Briefly, five Group A (1A-5A) prototypes and five Group B (1B-5B) prototypes were designed to contain the same oil blends, but different whey protein sources, with demineralised whey powder (DWP) used in the preparation of Group A products, and α-lac enriched whey powder concentrate (αWPC) added to Group B products. Therefore, (1) prototypes within the same group contained the same whey protein source but different oil blend composition; (2) each Group A prototype had a corresponding Group B prototype that had the same oil blend.

Then a static gastrointestinal *in vitro* digestion (SGID) model simulating the digestive conditions of a full-term infant, was used to digest all ten IMF prototypes, as described in the Chapter 3 (Section 3.2.2). Briefly, this model included two consecutive phases: a gastric phase and an intestinal phase, which was established based on the model previously described by Menard et al (2018) with modifications.

4.2.1.1 Extraction of antioxidative compounds in IMF

Extraction of antioxidant compounds from IMF prototypes was performed following a previously described method (Li *et al,* 2009), where solution of HCl (1N)/95% ethanol (v/v, 15/85) was used for extraction. Briefly, 20 mL of solvent was added to 3.0 g of powder, before incubating in a shaking incubator set at 300rpm for 1 hour at 30°C. The resultant mixture was then centrifuged at 7800g at 5°C for 15 min. Extraction was carried out in triplicate, supernatants combined and stored at -20°C in the dark until further analysis for 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity, ferric reducing antioxidant power (FRAP) and total phenolic count (TPC).

4.2.2. Investigating antioxidant potential

To evaluate antioxidant capacity *in vitro* it is generally recommended to use more than one assay, as the chemical methods available are based on different antioxidant principles. In this study the antioxidant capacity of the IMF prototypes was investigated using *in vitro* chemical assays (FRAP, TPC and DPPH) and cellular assays (SOD and GSH).

4.2.2.1 Chemical based assays

Ferric Reducing Antioxidant Power (FRAP)

Reducing power and antioxidant potential were determined based on the FRAP method described by Benzie and Devaki (2018), adapted for a 96-well plate assay. In the FRAP assay, antioxidants reduce the colourless Fe^{3+}-TPTZ salt (2,4,6 tripyridyl-S-triazine) to a blue coloured Fe^{2+} -TPTZ form. Working FRAP reagent was freshly prepared by mixing acetate buffer (300 mmol/L), TPTZ solution (10 mmol/L in 40 mmol/L HCl) and ferric chloride solution (20 mmol/L) at the ratio 10:1:1. Samples (10 µl) were reacted in the dark with 300 µl FRAP reagent for 30 minutes at ambient temperature, after which absorbance was measured at 593 nm. To correct for background colour samples were mixed with acetate buffer and ferric chloride solution but no TPTZ reagent was added. Ferrous sulfate hepatahydrate as an Fe^{2+} standard was prepared at various concentrations (100 - 1000 µmol/L) to construct a calibration curve and results were expressed as µmol Fe^{2+} /100g powder.

DPPH Radical Scavenging Activity

DPPH radical scavenging activity (RSA) was determined according to a procedure described by Cheng *et al* (2006), optimised for 96 well plate assay. DPPH stock solution (0.625 mM) was prepared in methanol and stored in the dark at 4 °C for a maximum of one month. DPPH working solution (0.208 mM) was prepared fresh by further diluting stock solution in 50% ethanol. The stock solution of Trolox (25mM) was prepared with 50% ethanol and used to prepare a series of standards (10-50 µM). Reaction mixture in each well contained 100 µL sample or standard and 100 µL working DPPH solution (0.208 mM) and plates were incubated for 60 minutes at room temperature (in the dark). A blank with 200 µL of 50% ethanol and a DPPH control (100 µL 50% ethanol and 100 µL 0.208 mM DPPH) were also prepared. To correct for background 100 µL sample was mixed with 100 µL of 50% ethanol. Absorbance at 515 nm was measured and results expressed as µM Trolox equivalents (TE) using the standard curve.

Total Phenolic Content (TPC)

TPC was determined using the Folin–Ciocalteau method (Singleton *et al* 1998) with modifications. Briefly, 50 µl sample was mixed with 250 µl Folin–Ciocalteau reagent, vortexed and incubated for 4 minutes. Na_2CO_3 (500 µl at 20% w/v concentration) and 4.2 ml of water were then added, before incubation for 2 hours at 25 °C in the dark. Sample (50 µl) with water (4.95 ml) was used as a blank to correct for background colour

absorbance. Absorbance was measured at 765 nm. Gallic acid was used as a standard (10-50 mg/100 ml) and results expressed as gallic acid equivalents (GAE)/100g powder.

4.2.2.2 Cell-based assay

Cell viability investigations

A leukaemic monocytic lymphoma cell line, U937 cells, was used to investigate cellular antioxidant capacity of the IMF prototypes. This pro-monocytic, human myeloid leukaemia cell line exhibits many characteristics of monocytes, is easy to use and frequently employed to study the effects of various food compounds on the intracellular redox balance, anti-inflammatory, immunomodulatory (Verhoeckx *et al.*, 2015) and antioxidant effects (McCarthy *et al.*, 2014).

U937 cells were maintained at 37 °C in a 5 % CO_2 atmosphere in medium (RPMI-1640) supplemented with 10 % foetal bovine serum (FBS) and 1% penicillin-streptomycin. For experiments, reduced serum media was used (2.5% FBS).

To determine subtoxic concentrations of IMF prototypes for experiments the viability of U937 cells line was measured using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. For this, U937 cells ($1x10^5$ cells/ml) were exposed to various concentrations (0.1- 5% v/v) of digested prototypes for 24 hours at 37 °C. Following this, MTT solution (10 µl, 5 mg/ml) was added, and further incubation was allowed for 4 hours at 37 °C. Subsequently, 90 µl of dimethyl sulfoxide (DMSO) and 60 µl of 30 % sodium dodecyl sulfate (SDS) were added and absorbance measured at 570 nm. Blank wells contained culture media, MTT reagent and solvents only (no cells), and were used to correct for the background colour. Positive control wells included media with cells and % cell viability was determined as (absorbance of samples/absorbance of positive control) x 100. As cell viability was >80% for all prototypes at 0.5%, this was selected as test concentration for cellular antioxidant investigations.

Superoxide dismutase (SOD) and Glutathione (GSH) assays

U937 cells (1×10^5 cells/ml, 5 mL) were incubated with IMF digestates (0.5% v/v) for 24 h at 37 °C. Following incubation, cells were exposed to 200 µM H_2O_2 for 60 minutes. Cells were harvested, sonicated and centrifuged (1000 rpm, 10 minutes), and the supernatants were collected for the determination of antioxidant activity. SOD activity was measured using the SOD Assay Kit (Sigma-Aldrich, Ireland Ltd) in accordance with manufacturers guidelines. SOD catalyses the dismutation of superoxide (generated by aerobic respiration) to molecular oxygen and peroxide; its activity is determined

spectrophotometrically by measuring the decrease in superoxide anions generated by the enzyme xanthine oxidase. Data were expressed as a percentage of untreated control cells. GSH content of the cells was measured according to a method described by Hissin and Hilf (1976) with modifications. Briefly, 100 µL of test sample was mixed with 1.8 mL sodium phosphate-EDTA buffer and 100 µL σ-phthaldialdehyde (OPA). OPA is fluorescent and reacts with reduced glutathione (GSH) to form a fluorescent product which can be quantified. Fluorescence was measured at 350 nm (absorption) and 420 nm (emission) and GSH content of the samples was determined from a standard curve (0 - 25 µM GSH). Data were expressed as a percentage of untreated control cells.

4.2.3 Statistical analysis

All data represent the mean ± standard deviation (SD) of at least three independent experiments. Statistical analysis was carried out using the R Project for Statistical Computing. Data was analysed using one-way analysis of variance (ANOVA) followed by Tukey's honest significance test. Statistical significance was determined at $p < 0.05$.

4.3 Results

4.3.1 Antioxidant activity of the IMF prototypes

All IMF prototypes were assessed for antioxidant activity prior to and following *in vitro* digestion using three different chemical-based assays. All IMF prototypes demonstrated antioxidant activity pre-digestion and post *in vitro* digestion, as assessed by FRAP, DPPH and TPC assays. For the undigested prototypes, FRAP ranged from 88.9 to 103.12 (µmol $Fe^{+2}/100g_{powder}$) for Group A and from 74.81 to 98.68 (µmol $Fe^{+2}/100g_{powder}$) for the Group B prototypes (Table 4.1). Of interest, when the prototypes were digested, FRAP was significantly ($p<0.005$) reduced ranging from 54.85 to 61.68 (µmol $Fe^{+2}/100g_{powder}$) for the Group A prototypes and 56.37 to 61.10 (µmol $Fe^{+2}/100g_{powder}$) for the Group B prototypes.

For the undigested prototypes, within Group A there was a significant difference in FRAP activity between 1A (103.12 µmol $Fe^{2+}/100g_{powder}$) and 4A (88.9 µmol $Fe^{2+}/100g_{powder}$). There was no difference between any Group A prototypes post-digestion.

Within the Group B, prototype 3B had highest FRAP (98.68 µmol $Fe^{2+}/100g_{powder}$), which was significantly higher ($p<0.05$) than 2B (81.46 µmol $Fe^{2+}/100g_{powder}$) and 5B (74.81 µmol $Fe^{2+}/100g_{powder}$). This difference was maintained post-digestion, and digested prototype 3B also had significantly higher ($p<0.05$) FRAP compared to digested 3A (55.22 µmol $Fe^{2+}/100g_{powder}$).

For the undigested prototypes TPC ranged from 122.5 to 142.05 mg GAE/100g_{powder} for Group A prototypes and from 68.78 to 83.69 mg GAE/100g_{powder} for Group B prototypes (Table 4.1).

Table 4.1 Non cellular antioxidant potential of the IMF prototypes

	FRAP (µmol Fe^{2+}/100g)		TPC (mg GAE/100g)		RSA (µM TE)	
IMF Prototypes	**Undigested**	**Digested**	**Undigested**	**Digested**	**Undigested**	**Digested**
	Group A					
1A	103.12 ± 5.38[a]	61.68 ± 4.08[*]	126.31 ± 3.55	130.78 ± 4.39[b]	59.61 ± 2.21[*]	39.07 ± 1.02
2A	97.94 ± 8.65	60.24 ± 2.68[*]	142.05 ± 8.04	136.42 ± 6.2	60.1 ± 2.26[*]	47.52 ± 3.1
3A	97.37 ± 6.86	55.22 ± 1.78[b*]	127.85 ± 8.9	124.18 ± 5.43	58.72 ± 2.3[*]	46.58 ± 2.53
4A	88.9 ± 3.67[a]	54.85 ± 3.4[*]	136.9 ± 11.84	128.76 ± 5.77	58.95 ± 2.36[*]	46.02 ± 2.57
5A	95.71 ± 4.84	59.75 ± 1.15[*]	122.5 ± 7.58	133.1 ± 5.66	57.94 ± 2.58[*]	46.48 ± 2.48[b]
	Group B					
1B	81.31 ± 3.63	61.32 ± 3.09[*]	69.47 ± 3.94	153.22 ± 7.29[b*]	45 ± 3.95	44.64 ± 1.87
2B	81.46 ± 7.6	56.37 ± 2.34[a*]	68.78 ± 6.2	145.53 ±10.28[*]	45.46 ± 4.68	43.6 ± 1.2
3B	98.68 ± 4.05	68.1 ± 1.98[ab*]	76.87 ± 7.87	142.24 ± 9.01[*]	52.02 ± 2.66	45.36 ± 3.57
4B	89.4 ± 4.65	61.22 ± 2.63[*]	71.94 ± 4.71	143.7 ± 7.04[*]	47.36 ± 5.91	42.79 ± 4.26
5B	74.81 ± 3.4	57.73 ± 2.99[a*]	83.69 ± 8.5	133.8 ± 5.49[*]	50.47 ± 2.82[*]	40.96 ± 5.31[b]

FRAP, Ferric reducing antioxidant power; TPC, Total phenolic content; RSA, DPPH Radical Scavenging Activity. Data is presented as the mean ± standard deviation of three independent experiments. Statistical analysis by Anova Ttest. * Statistically significant difference between prototypes pre- and post- digestion (p value<0.05). [a] Statistically significant difference between prototypes within the same group. [b] Statistically significant difference between prototypes with the same oil blend

There was no significant ($p<0.05$) difference observed between digested and undigested Group A prototypes (1A-5A), however for the Group B prototypes (1B-5B) TPC increased significantly ($p<0.05$) post-digestion (133.84 to 153.22 mg GAE/100g_{powder}). While all Group B prototypes post-digestion had higher TPC compared to the corresponding Group A prototypes that had similar oil blends, only 1B had significantly higher ($p<0.05$) levels compared to 1A (153.22 mg and 130.78 mg GAE/100g_{powder} GAE/100g, respectively) (Figure 4.1 C).

Radical scavenging activity (RSA), measured by the DPPH assay, ranged from 57.94 to 60.10 (µM TE) for undigested Group A prototypes, which were significantly ($p<0.05$) higher than digested prototypes (39.07 to 47.52 µM TE). No significant difference in DPPH radical scavenging activity was observed for Group B prototypes pre- (45 to 52.02 µM TE) and post-digestion (40.96 to 45.36 µM TE), except for prototype 5B, which once digested had lower RSA (40.96 µM TE) than 5A (50.47 µM TE). No statistically

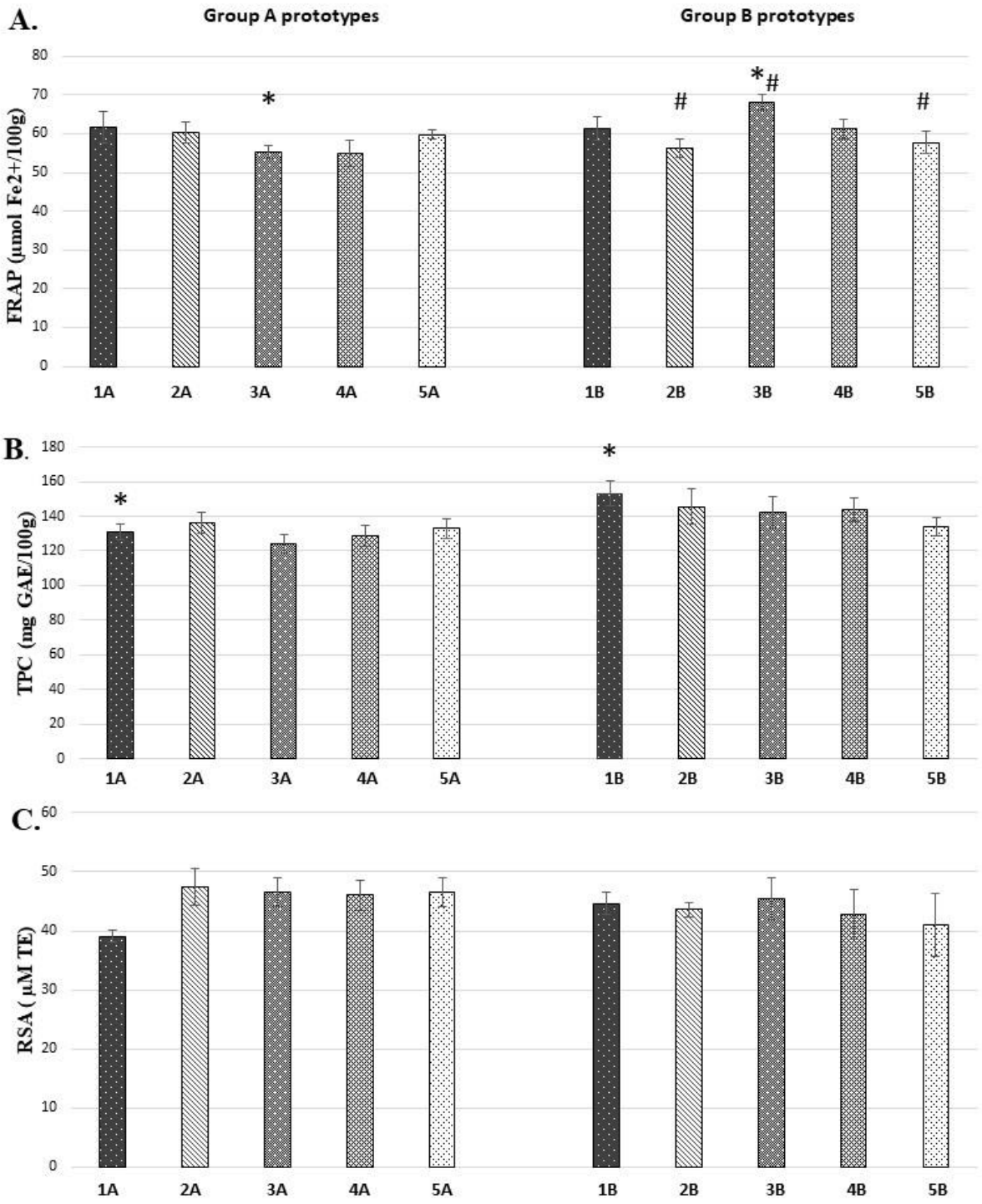

Figure 4.1. Antioxidant Activity of IMF prototypes 1A-5A (Group A) and 1B-5B (Group B) following *in vitro* simulated digestion. **A.** Ferric reducing antioxidant power (FRAP), **B.** Total phenolic content (TPC), **C.** DPPH Radical Scavenging Activity (RSA). Data is presented as the mean ± standard deviation of three independent experiments. Statistical analysis by Anova Ttest. * Statistically significant difference between the prototypes with the same oil blend (p value<0.05). # Statistically significant difference between the prototypes within the same line (p value<0.05)

significant difference in DPPH radical scavenging activity was observed between prototypes within the Group A, whether digested (Table 4.1) or undigested (Figure 4.1C).

4.3.2 Cellular antioxidant activity following SGID

H_2O_2 treatment of the U937 cells reduced SOD activity by 24% confirming that oxidative stress was induced (Figure 4.2). Significant ($p<0.05$) protection in SOD depletion of H_2O_2 stressed cells was observed for IMF prototypes 1A (122.58%), 3A (111.84%), 4A (142.72%) and 5A (119.61%) as well as Group B prototypes 1B (111.71%) and 2B (147 %). In addition, prototypes 4A and 2B significantly increased ($p < 0.05$) SOD activity above that of the untreated control cells. Within Group A prototypes, made with different oil blends, there was no significant difference between AO activity of samples. However, enriching the IMF with αWPC significantly modified the AO properties of some of the IMF. Significantly higher SOD activity was observed for prototype 2B (147 %) compared to 2A (107.18%), while prototype 4B (97.82%) had a significantly lower SOD activity compared to 4A (142.72%). SOD activity of prototype 2A was also significantly higher than all other Group B prototypes.

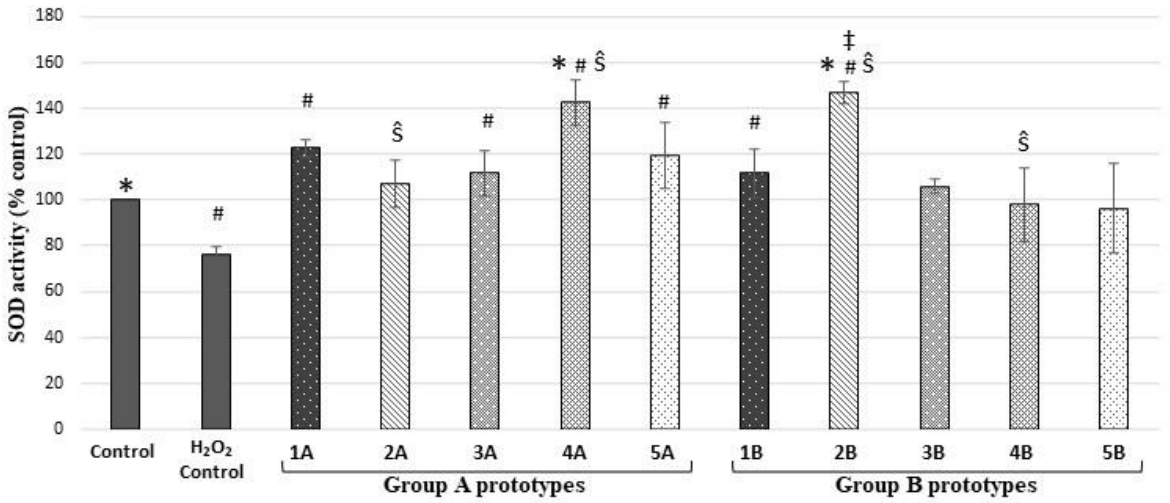

Figure 4.2. Cellular antioxidant potential of IMF prototypes, as measured by their ability to protect the cells against oxidative stress induced by hydrogen peroxide (H_2O_2), using superoxide dismutase (SOD) assay. Data expressed as a percentage of untreated, control cells. Data represents the mean ±standard deviation of at least three independent experiments. Statistical analysis by T-test. * Statistically significant difference between the sample and control ($p<0.05$). # Statistically significant difference between the samples and H_2O_2 control ($p <0.05$). § Statistically significant difference between the samples with the same oil blend ($p <0.05$). ‡ Statistically significantly different from all other samples within the same line ($p <0.05$).

Following H_2O_2 treatment, intracellular GSH content was reduced to 19.38 % confirming that oxidative stress was induced (Figure 4.3). Four prototypes, 5A (116.44%), 1B (109.22%), 4B (121.32%) and 5B (113.78%), significantly ($p<0.05$) protected against

H_2O_2-induced GSH depletion. Enriching 1A and 4A with αWPC, significantly improved the AO properties of the IMF. Of interest, prototypes containing OB5 (5A and 5B) were equally able to significantly protect the cells against oxidative stress, despite differing WPC sources None of the prototypes significantly improved intracellular GSH content compared to the untreated control cells.

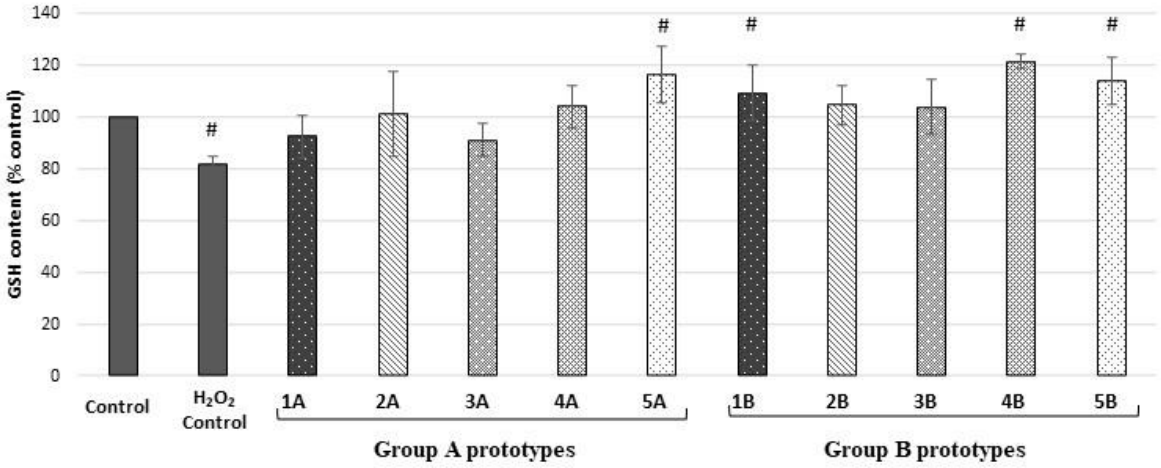

Figure 4.3. Cellular antioxidant potential of IMF prototypes, as measured by their ability to protect the cells against oxidative stress induced by hydrogen peroxide (H_2O_2), using glutathione (GSH) assay. Data expressed as a percentage of untreated, control cells. Data represents the mean ±standard deviation of at least three independent experiments. Statistical analysis by T-test. # Statistically significant difference between the samples and H_2O_2 control ($p < 0.05$).

4.4 Discussion

The AO properties of a food are influenced by a range of factors including nutrient composition, the food matrix, and the digestion process (Cömert and Gökmen, 2020). It has also been demonstrated that combinations of bioactive compounds in food sources can increase AO activity by providing a synergistic effect (Zou and Akoh, 2015; Cömert and Gökmen, 2020). The nutritional strategy of the current study was to design IMF with different oil blends and protein sources and investigate if certain formulations had improved AO properties.

All IMF prototypes (undigested and digested) exhibited AO activity, which varied across different assays. For prototypes designed with DWP, digestion significantly decreased the FRAP and radical (DPPH) scavenging activity, but there were no differences observed for TPC. Prototypes enriched with αWPC had significantly higher TPC when digested, however FRAP and DPPH were significantly lower. The structure of milk components can also change due to processing-induced modifications, which can affect the digestion of proteins and lipids, and therefore their bioactivity (Bavaro *et al.*, 2021).

Following ingestion, antioxidants are released from a food matrix via mechanical digestion, exposure to gastric acid, and enzyme action in the GI tract to become available for intestinal absorption. Therefore, assessing AO activity of IMF post-digestion is more biologically relevant (Cömert and Gökmen, 2020).

While limited studies are available that compare the AO activity of IMF pre- and post-digestion, it has been previously demonstrated that the AO properties of individual bovine milk components increases after hydrolysis or SGID, as demonstrated for WPC (Power-Grant *et al.*, 2015), WPI (Corrochano et al. 2019) and α-lac (Hernández-Ledesma *et al.*, 2005; Joubran *et al.*, 2017; Corrochano *et al.*, 2019). However, varying conditions and AO assays used in these studies do not facilitate a direct comparison with results of the current study. For example, Joubran et al (2015; 2017) have used semi-dynamic SGID infant model, that more closely resembles *in vivo* conditions, whereas Corrochano et al (2019) used an adult SGID model.

It is important to recognise that AO activity of individual compounds may not directly translate to a complex food matrix, such as IMF, that contains this compound and that is digested, as the release of bioactive peptides and AAs and therefore their activity may be affected by digestion conditions as well as food matrix composition. For instance, Power-Grant et al. (2015) have shown that whey proteins with different AA profiles have various effects on AO activity, measured by the ORAC assay, suggesting that activity may be

reduced following SGID if a substrate contains a high proportion of hydrophobic AA residues, which are further degraded by the gastrointestinal enzymes resulting in the decrease in AO activity. However, as IMF is a complex food matrix due to its protein, fat, and carbohydrates content, therefore chemical structures and the disposition of antioxidant molecules in the food matrix (i.e., free, glycosylated, polymerised or chemically bound to other food components) may affect their AO activity (Pellegrini *et al.*, 2020), and therefore the differences in the activity prior to and following digestion .

Following SGID, it was found that the radical scavenging properties of all prototypes were comparable, while assessment of their reducing power highlighted differences between prototypes. There was no significant difference observed between the prototypes designed with DWP with respect to their reducing power, possibly suggesting that the oil blends did not alter antioxidant potential. However, αWPC enrichment of prototypes improved the AO properties for prototypes containing OB3 with AMF (3B), and OB1 with coconut, soy, and high-oleic sunflower vegetable oils (1B). Corrochano *et al* (2019) demonstrated that α-lac had higher antioxidant activity, both as an intact protein and post digestion, compared to other major milk proteins, such as β-lactoglobulin, lactoferrin, and bovine serum albumin. Therefore, higher levels of α-lac in these prototypes may account for the higher AO activity observed. Of interest, while prototype 3B had the lowest levels of α-lac and total PLs compared to other αWPC enriched prototypes, it had highest levels of β-lactoglobulin. Enzymatic hydrolysis of β-lactoglobulin does release bioactive peptides with antioxidant properties (Conway, Gauthier and Pouliot, 2013; Power *et al.*, 2014; Bamdad *et al.*, 2017). Therefore, it is possible that the combination of αWPC with AMF modified the protein-lipid profile to generate a prototype with improved reducing power.

The lipid profile of prototype 1B indicated it had higher PLs than its corresponding prototype 1A, including phosphatidylethanolamine, phosphatidylcholine, and sphingomyelin (Table 2.1, Chapter 2), which are associated with antioxidant properties (Saito and Ishihara, 1997). Other studies have demonstrated that TPC in IMF changes, based on the ingredients used (Li *et al.*, 2009; Sánchez-Hernández *et al.*, 2021). In the current study 1B had highest total PLs and α-lac levels, thus it may be that this combination influenced TPC, but this would need further investigation.

It was hypothesised that αWPC supplementation would improve GSH content and SOD activity of the IMF prototypes. To date, studies have reported that whey proteins and WPC can modify GSH levels *in vitro* (Tseng *et al.*, 2006; Xu *et al.*, 2011; Pyo *et al.*, 2016). *In vivo* studies have also confirmed suggested a positive correlation between whey protein

supplementation in the diet and cellular and plasma GSH and SOD levels (Zavorsky *et al.*, 2007; Haraguchi *et al.*, 2011; Sheikholeslami Vatani and Ahmadi Kani Golzar, 2012; Garg *et al.*, 2020). Also, previously it has been reported that intracellular SOD activity increased in rats fed with the diet enriched in α-lac (Eliwa *et al.*, 2014; Mansour *et al.*, 2015).

In this study prototypes containing oil blends OB1 and OB3 significantly protected intestinal cells from oxidant-induced depletion of SOD, regardless of the protein source in the IMF. However, αWPC enrichment of prototypes with oil blend OB2 (soybean, high oleic sunflower, coconut and palm oils) did significantly protect cells against oxidative stress. Interestingly, while the SOD activity of this prototype (2B) was significantly higher than other enriched products, its α-lac and PL content was not distinctively different from other enriched prototypes. These data suggest that intracellular SOD activity associated with the IMF prototypes may not be solely attributed to a higher levels of α-lac or PLs but warrants further investigation. Furthermore, AO properties of α-lac may be cell specific, as recent studies demonstrated that α-lac improved SOD activity in Caco-2 cells but this effect was not observed in HT-29 cells (Corrochano, Arranz, *et al.*, 2018). Of interest, the A4 prototype, which was designed with a novel oil blend (OB4, containing specific sn-2 fat 1) was significantly ($p<0.05$) better at protecting cells from oxidant-induced depletion in SOD activity. However, enriching with αWPC significantly decreased its AO properties, by reducing SOD activity.

αWPC enriched prototypes designed with OB1 and OB4 significantly protected cells from oxidant-induced depletion of GSH even though there was no significant difference between these prototypes and their corresponding prototypes. Of interest, both prototypes designed with OB5 significantly protected against oxidative stress, regardless of the protein source. These data suggest that altering the protein and lipid profiles did impact the AO properties of the IMF. In general, αWPC enrichment of prototypes improved GSH content. Other *in vivo* have reported that α-lac enriched diets can have a positive effect on GSH levels (Mariotti *et al.*, 2004; Mansour *et al.*, 2015). On the contrary, it was recently reported that PLs, have low cellular AO activity possibly due to their poor bioavailability (Huang, 2020). Therefore, the cellular effects on GSH levels in this study may be attributed more to α-lac supplementation than to PLs. PLs are associated with several AO mechanisms, such as chelation of prooxidative metals, formation of AO Maillard reaction products, or regeneration of primary antioxidants (Cui and Decker, 2016). However,

EFSA have reported that protective effects of PL against oxidative stress have not yet been sufficiently investigated or proven (EFSA, 2009).

Overall, variability of the results presented in this work may be explained by the different mechanisms through which α-lac enriched WPC and its combination with different oils affect each of the AO assays performed. The methods used in this study to assess AO activity do not identify single antioxidant compounds and the mechanisms involved vary for each assay. While these *in vitro* studies do not reflect the complexity of processes occurring *in vivo* they are useful in providing preliminary data. In conclusion, no single oil blend has shown superior AO activity, however cellular protection against oxidative stress was more associated with oil blends containing soybean, high oleic sunflower, and coconut oils; and a blend of same oils in addition of novel sn-2 fat 2. While enrichment of prototypes with αWPC did not improve AO activity of all prototypes, combination of αWPC with soybean, high oleic sunflower, and coconut oils blend and those with added novel sn-2 fat l may have a potential to protect against oxidative stress.

Conclusions

The focus of the present study was to compare AO activity across IMF prototypes with different oil blends and protein sources. Data suggests that altering the protein and lipid profiles of IMF prototypes did impact their AO properties. A single optimal combination of oil blend and protein source that significantly improved AO activity across chemical and cell-based assays could not be identified, however, some prototypes demonstrated greater antioxidant potential. In most instances, AO potentials were maintained or enhanced following SGID.

In summary, the study highlights that combining αWPC with specific oil blends has the potential to protect against cellular oxidative stress and thereby be a strategy to promote infant health. However, further investigations based on bioavailability studies may be warranted to identify an optimal combination of oil blends and protein source.

Chapter 5. General discussion

Breast milk is the best form of nutrition for infants, but infant milk formula (IMF) is an effective milk substitute when breast-feeding is inadequate or not possible (Ahern *et al.*, 2019). Manufacturers of IMF strive to produce formulae that have a similar composition and nutritional profile to HM, so that IMF-fed newborns and growing infants are provided with optimum nutrition for growth and development (Kent *et al.*, 2015). However, it is still evident from epidemiological studies that differences remain between breastfed infants and formula-fed infants with regard to both short-term (e.g. susceptibility to infections, cognitive development) and long-term (e.g. development of chronic diseases such as obesity, diabetes, cardiovascular disease) health outcomes (Ip *et al.*, 2009; Montagne *et al.*, 2009; Lonnerdal, 2016). As it is challenging for IMF manufacturers to exactly mimic HM composition, recent studies have focused on developing IMF to contain ingredients that will have similar health benefits (Koletzko *et al.*, 2011). Specific bioactive components identified in HM can be isolated and supplemented into IMF thus potentially improving the health of formula fed infants (Ahern *et al.*, 2019). Several studies have reported on the benefits of adding multifunctional bioactive ingredients to IMF, including prebiotics (e.g. human milk oligosaccharides), probiotics (e.g., *Bifidobacterium lactis, Lactobacillus rhamnosus, Bifidobacterium longum*), novel protein fractions (e.g., lactoferrin, osteopontin and α-lac) and specialised lipid fractions (e.g., LC-PUFAS, MFGM and sn-2 palmitate) (Montagne *et al.*, 2009; Ahern *et al.*, 2019; Yu *et al.*, 2019).

The focus of the presented research was to produce IMF prototypes with different lipid and protein profiles, which was achieved by using different oil blends, including some novel fats, and different protein sources, one of them being α-lactalbumin enriched whey powder (αWPC). It was hypothesized that addition of αWPC and its combination with different oil blends could potentially improve protein quality of the IMF prototypes as well as important nutrients such phospholipids content. The aim was to determine whether different oil blends and their combination with αWPC would affect bioactivities of the IMF prototypes, such as anti-infective and antioxidant properties. Ten different IMF recipes were developed and produced and subjected to *in vitro* simulated gastrointestinal digestion as well as antioxidant and antimicrobial assays, which included cellular bioactivity assays. Cell culture models used to assess bioactivity are effective and economical alternative to animal or human trials. For this research U937 (a human monocytic blood cell line), and HT-29 clone 34 (human colorectal adenocarcinoma) cells were chosen to study potential antioxidant and anti-adhesion effects respectively.

This study demonstrated that αWPC supplementation in IMF allows for IMF formulations production with higher levels of both PLs and α-lac compared with prototypes designed with demineralised whey powder. Levels of PLs and α-lac were also found to be closer to those found in HM with a concomitant decrease in allergenic β-lactoglobulin.

While this study has shown that there is no bactericidal activity or anti-adhesion effect exhibited by IMF prototypes in their intact form nor following digestion against tested bacterial strains, the lack of antimicrobial activity observed in this study may be due to low concentrations of antimicrobial compounds present in the tested samples. A limitation of this study is the limited number of bacterial strains used. Further analysis may be warranted to test the IMF prototypes for their potential bactericidal activity and anti-adhesion effect using other bacterial strains of the same species. Moreover, another cell line may be employed for cellular assays, such as CaCo-2 cells (human colon adenocarcinoma).

Several the tested oil blends have demonstrated the ability to protect against oxidative damage induced by hydrogen peroxide (H_2O_2), by increasing intracellular SOD and GSH levels in U937 cells. Cellular protection against oxidative stress was observed to be promising for the oil blends containing soybean, high oleic sunflower, and coconut oils; and a blend of same oils in addition of novel sn-2 fat 2. While enrichment of prototypes with αWPC did not improve AO activity of all prototypes, combination of αWPC with soybean, high oleic sunflower, and coconut oils blend and the same oil blend with added novel sn-2 fat l have shown potential to protect against oxidative stress. However, since complex *in vivo* conditions cannot be replicated *in vitro,* human trials are necessary for the substantiation of health claims relating to IMF.

To date, research on antioxidant and antimicrobial activity has been focused on individual ingredients, i.e., phospholipids and whey proteins, such as α-lactalbumin, that are proposed for the addition to IMF. Limited studies focus on the health-promoting properties of the IMF or HM digesta. This study appears to be the first one to investigate potential health effect of digested IMF. This novel data on the *in vitro* bioactivity of digested IMF progresses this area of research. Presented findings show that the addition of αWPC to IMFs and modification of various oil blends, can result in IMF with selective bioactive potential and protein and lipid profiles closer to these in HM. Some oil blends in addition to αWPC used in this study could be further investigated for other bioactivities, which would facilitate identification of optimal combination of oils blends and whey protein source that would promote infant growth and development.

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