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Te Mana Kounga Kai – Ahitereiria me Aotearoa

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Supporting document 1

Risk, benefit and technical assessment – Application A1253

A1253 – Bovine lactoferrin in infant formula products

Executive summary

Food Standards Australia New Zealand (FSANZ) has assessed an Application from Synlait Milk Ltd. (the Applicant) to amend the Australia New Zealand Food Standards Code (the Code) to permit the voluntary addition of bovine lactoferrin (bLf) as a nutritive substance to infant formula products (IFP).

The Applicant is proposing to add bLf to infant formula, follow-on formula and infant formula for special dietary use up to a maximum permitted amount of 40 mg/100 kJ, equivalent to ~ 1 g/L. The Application states the purpose for adding bLf to IFP is to more closely reflect the lactoferrin (Lf) content in human milk, and to provide a reduced risk of infection in formula-fed infants compared with those receiving standard IFP not fortified with bLf.

FSANZ has undertaken an assessment of the food technology aspects, safety, nutritional impact and beneficial health effects of the addition of bLf to IFP.

bLf is a protein naturally present at low levels in cow's milk. It shares 69% amino acid sequence homology with human lactoferrin (hLf), found in human milk. Information reviewed in the food technology assessment demonstrates that bLf is sufficiently characterised, and confirms its stability in IFP. Identity and purity specifications specifically related to bLf have been proposed for inclusion in Schedule 3 of the Code, with which bLf would have to comply.

The safety assessment concluded there are no toxicological safety concerns from the addition of bLf to IFP at the proposed concentrations.

bLf is subject to partial hydrolysis in the stomach and small intestine, but a proportion resists digestion and is excreted in the faeces. Some fragments produced by partial hydrolysis also resist further digestion and are excreted in the faeces. In addition, a small proportion of intact bLf and its fragments is absorbed into the systemic circulation and excreted via the urine.

bLf is of low acute toxicity, with no adverse effects observed following oral administration to rats up to 2000 mg/kg bw. It was not mutagenic *in vitro*. No adverse effects were observed in a 13-week oral gavage toxicity study in rats at doses up to 2000 mg/kg bw/day, the highest dose tested.

No adverse effects of bLf have been reported in multiple intervention studies in infants, including the highly vulnerable group of preterm and very low birth weight infants. bLf concentrations up to 1000 mg/L formula were tested in the studies in term infants while the

doses tested in preterm and very low birth weight infants ranged from 100 – 300 mg/kg bw/day. These doses were estimated as being equivalent to bLf concentrations ranging from 370 – 3704 mg/L.

The first bLf-fortified IFP were released for sale overseas in 1986 and to the best of FSANZ's knowledge there have been no adverse events related to consumption of these products in markets where they are available. The Applicant has also indicated that its post-marketing surveillance overseas, and that of international formula brand owners it supplies, has not identified any complaints or adverse events related to the addition of bLf.

Based on the maximum permitted amount proposed by the Applicant, the estimated mean and 90th percentile (P90) intakes of bLf from infant formula and follow-on formula range between 0.59 and 1.8 g/day (equivalent to 70 – 270 mg/kg bw/day). These intakes are less than the estimated mean and P90 intakes of hLf from human milk of 0.7 to 5.0 g/day, and approximately 10 – 30-fold lower than the no observed adverse effect level of 2000 mg/kg bw/day from the 13-week toxicity study of bLf in rats.

bLf is derived from cow's milk which is a major food allergen. Some individuals with cow's milk allergy have immunoglobulin E (IgE) antibodies to bLf indicating sensitisation, but the clinical significance of this has not been confirmed and bLf is not currently listed as a cow's milk allergen by the World Health Organisation and International Union of Immunological Societies (WHO/IUIS). The limited available evidence however is insufficient to conclude that bLf does not pose a food allergy risk to consumers with cow's milk allergy.

No additional microbiological safety risks arise from addition of bLf to powdered infant formula products and its preparation and consumption beyond those encountered with IFP that is not supplemented with bLf.

Several double-blind, randomised controlled trials (RCTs) have investigated the potential for bLf to affect infant growth and development. Differences in weight gain between bLf and control formula groups were less than the clinically relevant threshold of 3 g/day. It is concluded that consumption of infant formula with added bLf, at up to 1 g/L (equivalent to 40 mg/100 kJ), is unlikely to adversely affect infant growth and development. Infant iron status, investigated in one of these RCTs, was unaffected by bLf addition to infant formula.

In terms of beneficial effects, the weight of evidence suggests a plausible mechanism by which bLf can reduce the risk of bacterial and viral infection. bLf has been shown to reduce the severity and duration of infection in relevant animal infection models. The few relevant human studies provided weak but consistent support for the proposed beneficial effect.

1 Introduction

Food Standard Australia New Zealand (FSANZ) received an Application from Synlait Milk Ltd (Synlait) to amend the Australia New Zealand Food Standards Code (the Code) to permit the voluntary addition of bovine lactoferrin (bLf) as a nutritive substance to infant formula products (IFP).

Lactoferrin is also present in human milk and milk from other mammalian species. The focus of the present Application is solely on bLf.

The Applicant is proposing to add bLf to infant formula, follow-on formula and infant formula for special dietary use up to a maximum permitted amount of 40 mg/100 kJ. The stated purpose for adding bLf to IFP is to more closely reflect the lactoferrin content in human milk, and to provide a reduced risk of infection in formula-fed infants compared with those receiving standard IFP not fortified with bLf.

The objectives of this assessment were to assess the food technology aspects, safety, nutritional impact and beneficial health effects of the addition of bLf to IFP.

2 Food technology assessment

2.1 Objectives of the food technology assessment

This assessment reviewed the Applicant's bLf ingredient from a food technology perspective. As such, the assessment provides information on the identity and properties of the bLf; its use and stability in IFP; and includes a proposed specification for inclusion in Schedule 3 – Identity and purity of the Code. The assessment also considered the manufacturing process and the ability of analytical methods to detect/quantify the bLf in IFP.

2.2 Assessment of the bLf ingredient

2.2.1 Identity of the bLf ingredient

Lactoferrins are glycoproteins, and have a molecular weight of about 80 kDa with 670-690 amino acid residues. bLf occurs naturally in the whey fraction of cow's milk. Lf is naturally present in human milk.

As stated in the Application (section 2.2), lactoferrin is a non-haem iron-binding protein. It is a member of the transferrin family of iron-binding proteins, which is characterised by the capacity to reversibly bind ferric iron with high affinity.

bLf has a molecular weight of approximately 77 kDa and consists of a single amino acid chain of 689 amino acids. Each lactoferrin macromolecule is composed of two lobes (a N- and a C-lobe (representing the N and C-terminals of the molecule, respectively). The lobes are unevenly glycosylated, the C-lobe typically containing more N-linked glycosylation sites. In the presence of either bicarbonate or carbonate, ferric iron binds to lactoferrin such that one Lf molecule is able to bind 2 ferric irons. The amino acid sequence homology of bLf with hLf is 69% (Latorre et al, 2012).

As described in the Application, bLf has 5 potential glycosylation sites, whereas hLf has 3 potential glycosylation sites. bLf contains N-glycosidically-linked glycans possessing N-acetylneuraminic acid, galactose, mannose, fucose, N-acetylglucosamine, and N-acetylgalactosamine.

Lf with less than 5% iron saturation is called apolactoferrin, while the iron-saturated Lf is called hololactoferrin. The bLf subject to this Application contains a maximum of 15mg Fe/100g bLf (i.e., at the time of addition to IFP). As noted in the Application (section 2.2), this equates to a maximum iron saturation of 10.7%.

The Chemical Abstracts Service (CAS) Registry Number for bovine lactoferrin is CAS Reg. No.146897-68-9.

2.2.2 Physical properties of the bLf ingredient

Table 2-3 in the Application summarises the physical and chemical properties of bLf. The bLf ingredient is a pink to reddish brown coloured, free-flowing powder. It is sold in 5kg commercial packs and does not need to be refrigerated.

2.2.3 Use and stability in IFP

IFP are usually sold in the form of powder, for reconstitution with water. The bLf ingredient is intended to be incorporated into IFP (sold in powder form) via dry-blending technology, to protect bLf from denaturation due to heat exposure and therefore to protect its bioavailability.

The Applicant's bLf ingredient has a shelf life of three years as a raw material.

Additional information was provided to demonstrate stability in IFP. The longest ambient shelf-life study with a product containing bLf was carried out over a period of 30 months. The results support the stability of bLf over this period of time, in IFP. Stability is ensured by achieving appropriate water activity for finished products (close to 0.2 as much as possible) for optimum product preservation and by using packaging material with excellent barrier properties (metal can). The exchange of moisture and oxygen and the influx of light are not possible with this packaging material, thus providing more assurance on the stability of product throughout its shelf-life.

2.3 Manufacturing process

2.3.1 Manufacture of the bLf ingredient

Bovine milk is naturally low in bLf. Dairy technology including ion exchange and ultrafiltration are used to separate, isolate and concentrate the bLf from skim milk.

The applicant has provided general details of the manufacturing process in section 2.2.4 of the Application (and shown diagrammatically in Figure 2-6). In summary, raw milk is separated to provide the skim milk stream that is concentrated via an ion exchange column, and is subsequently subjected to ultrafiltration steps.

The ultra-filtrate solution is pasteurised and further concentrated, prior to evaporation and spray drying, to produce the bLf in an isolated and pure form. The applicant provided additional information confirming that pasteurisation and spray drying of isolated bLf has virtually no impact on the structure and consequently on the bioactivity of bLf.

The processing aids used are listed in Table 2-7 of the Application. All processing aids are approved in S18. The 'Sepharose™ Big Beads' ion exchange resin was approved under Application A1120 – Agarose Ion Exchange Resin as a Processing Aid for Lactoferrin

Production.[1] The permission for use of the ion exchange resin in the production of lactoferrin from bovine milk is listed in the table to subsection S18—9(3), under 'Sulphonate agarose ion exchange resin' (as defined in subsection S18—9(2)).

2.3.2 Information on impurities

Section 2.2.3 of the Application provides information on impurities. The applicant's bLf has a minimum protein content of 95%, of which more than 95% is lactoferrin, and up to 5% other proteins. These are not quantified in the Application, however endotoxin levels (including lipopolysaccharide) are addressed.

The Applicant provided certificates of analysis for 8 non-consecutive batches (Table 2-5 in the Application), which demonstrate endotoxins are effectively absent, with levels less than 0.1 endotoxin units (EU)/mg bLf.

2.4 Specification for the bLf ingredient

Since there are no specifications for the bLf in any of the monographs in Schedule 3 (subsections S3—2 and S3—3), a new specification will be written into Schedule 3. The applicant's bLf pure and isolated ingredient is sold in powder form, and does not contain any carriers or anticaking agents. The Applicant has provided a specification with information relevant to a new specification. Table 1 below shows the specification proposed by FSANZ, for inclusion in Schedule 3 of the Code.

The specification parameters comprise physical appearance, purity, total bLf levels, moisture, ash, fat, and iron, as well as limits for potential chemical and microbiological impurities, and contaminants. Solubility is included as a parameter, as this indicates the degree of denaturation-induced insolubility (Wang et al 2017).

The Applicant has provided manufacturing specifications and testing methods for a number of parameters for bLf (Table 2-8 of the Application). The applicant also provided analytical results for five non-consecutive batches, shown in Table 2-9 of the Application, confirming production of the bLf ingredient meets the manufacturing specification parameters.

FSANZ assessed the manufacturing specification and analytical results and has proposed the specification in Table 1 for inclusion in Schedule 3 of the Code. The microbiological parameters are limited to safety parameters. Limits for heavy metals and arsenic are required, as the default values in S3—4 for substances are not consistent with the Applicant's specification values for the bLf ingredient.

Table 1 Proposed specification parameters for the bLf ingredient

[1] https://www.foodstandards.gov.au/code/applications/Pages/A1120AgaroseIonExchangeResinPA.aspx

Physical and Chemical Properties	Specification	Unit
Name	Bovine lactoferrin	
Molecular formula	$C_{141}H_{224}N_{46}O_{29}S_3$	
CAS number	146897-68-9	
Description	Pink to reddish brown coloured, free-flowing powder	-
Protein (N x 6.38)	> 95.0%	
bLf (purity)(on a protein basis)	>95.0%	
Moisture	< 4.5	g/100g
Ash	≤1.3	g/100g
Fat	≤1	g/100g
Iron	≤15	mg/100g
pH (10% solution)	5.2 – 7.2	
Solubility transmittance (2% solution, 20°C)	transparent	
Microbiological Properties		
Salmonella spp	absent	per 25 g
Listeria monocytogenes	absent	per 25 g
Cronobacter spp.	absent	per 10 g
Contaminants		
Lead	≤ 0.02	mg/kg
Cadmium	≤0.10	mg/kg
Mercury	≤0.10	mg/kg
Arsenic	≤0.02	mg/kg
Melamine	Not detected	
Aluminium	≤4.8	mg/kg
Aflatoxin M1	≤0.05	µg/kg
Nitrate	≤50	mg/kg
Nitrite	≤2.0	mg/kg

IFP are required to comply with the microbiological and contaminant limits set in the Code. Manufacturers may also refer to FSANZ's Compendium of Microbiological Criteria for Food which provides guidance on appropriate process hygiene criteria for IFP.

2.5 Analytical method of detection

A method is described under section 2.2.7 of the Application. The method measures bLf content and purity. Additional information was provided as CCI.

2.6 Food Technology conclusion

The food technology assessment concludes that the data demonstrates that bLf is sufficiently characterised, and stable in IFP.

Specifications have been proposed for inclusion in Schedule 3 of the Code.

3 Safety assessment

3.1 Toxicology assessment

3.1.1 Absorption, distribution, metabolism and excretion studies

In vitro studies

In vitro digestibility studies suggest that bLf is hydrolysed in conditions mimicking the stomach and/or small intestine, with a small proportion remaining intact, depending on the pH and duration of digestion (Brock et al. 1976; Lönnerdal et al. 2011; Grosvenor et al. 2014). Several bLf fragments have been detected following digestion, including fragments of ~ 52 kDa and 32 kDa (Brock et al. 1976; Lönnerdal et al. 2011). One study reported that 6% intact iron-saturated bLf and 4% intact iron-free bLf (apolactoferrin) remained after 3 h digestion with trypsin (Brock et al. 1976), while another found that native bLf (partially iron-saturated) was more resistant to pepsin digestion at pH 4 than iron saturated bLf, but both were digested by pepsin at pH 2 (Lönnerdal et al. 2011).

Evidence suggests that hLf and bLf are more resistant to digestion in pre-term or neonatal infants compared to older infants. In studies using gastric fluid obtained from preterm or neonatal infants, hLf was resistant to digestion at pH values of 4 and above, and partially resistant at pH 3.5 (Britton and Koldovský 1989; Chatterton et al. 2004). The prevailing postprandial pH in the gastric fluid samples collected in the study by Britton and Koldovsky was 5.5 – 6, and there was negligible evidence of degradation in gastric fluid with pH of 5.8. At pH 3.5, hLf degradation products of 34 and 42 kDa were observed in addition to residual intact hLf (Britton and Koldovsky 1989). Following incubation of bovine whey protein concentrate in gastric juice, bLf was not hydrolysed at pH 5 or 6.5, whereas hydrolysis was observed at lower pH values (Chatterton et al. 2004). Incubation of partially or completely iron-saturated bLf in gastric or duodenal secretions (pH not reported) obtained from 1 – 3 month old infants found no evidence of hydrolysis by gastric secretions and only partial hydrolysis by duodenal secretions (Spik et al. 1982).

Binding of bLf to brush-border membrane vesicles (BBMVs) from the small intestine of human foetuses was very low compared with binding of hLf. No inhibition of hLf binding to BBMVs was observed with excess bLf, suggesting that the limited binding of bLf to BBMVs, and any subsequent absorption, was likely to be non-specific rather than receptor-mediated (Kawakami and Lönnerdal 1991). Species specificity of lactoferrin binding to small intestine BBMVs from piglets has been reported, with no binding of bLf or hLf observed (Gislason et al. 1993). A study with the human intestinal epithelial cell line Caco 2 indicated cell binding and uptake of native and iron-saturated hLf and bLf, although lower binding was observed for bLf compared with hLf. The study authors concluded that hLf, bLf and CbLf were all bound and taken up by Caco-2 cells (Lönnerdal et al. 2011).

Animal studies

Low levels of intact lactoferrin were detected by enzyme-linked immunosorbent assay (ELISA) in the stomach, intestine and peripheral blood of female BALB/c mice (age 10 – 15 weeks) prior to administration of bLf, suggesting detection of endogenous mouse lactoferrin. Concentrations of intact lactoferrin in blood, liver, gall bladder, kidneys, spleen and brain were substantially increased 10 – 20 minutes following oral gavage administration of bLf compared with baseline levels. The highest tissue concentrations were found in the liver. Serum and tissue bLf levels were approximately 50% lower in mice exposed daily for 4 weeks compared with those given only a single dose (Fischer et al. 2007).

In adult male Wistar Imamuchi rats, bLf was detected in the thoracic lymph fluid following intraduodenal administration. Increased plasma bLf concentrations were not observed in rats from whom thoracic lymph fluid was collected, but significantly increased plasma concentrations were found in rats whose thoracic lymph fluid was not collected, suggesting bLf was transported into the circulation via the lymphatic pathway. Binding of bLf to BBMVs in the small and large intestine was also observed, with a 2-fold higher binding affinity (Kd 23.8 ± 2.8 versus 48.9 ± 4.8 μM, respectively) in the small intestine compared to the large intestine (Takeuchi et al. 2004).

Intact bLf was not detected in the small intestine of adult F344Crj rats following oral gavage, but bLf fragments with molecular masses of 42, 36, 33 and 29 kDa were detected between 20 and 180 minutes following administration. It was concluded that functional fragments of bLf are resistant to proteolytic degradation in the gastrointestinal tract (Kuwata et al. 2001).

In suckling miniature piglets, digestibility of ^{15}N-labelled bLf in the stomach and small intestine was 49.8 ± 4.85% and 82.3 ± 4.8%, respectively, similar to that of porcine lactoferrin and substantially lower than that of bovine casein. Based on immunoblotting, 1.1% of bLf was intact in the last third of the small intestine and 3.4% was partially digested, with a molecular weight of ~40 kDa. In adult pigs, digestibility of bLf was much higher and similar to that of bovine casein (Drescher et al. 1999).

bLf has been detected in the serum of neonatal piglets following oral gavage, with plasma concentrations peaking 2 h following administration and declining thereafter (Harada et al. 1999a, 1999b). bLf was also detected in bile 30 minutes after oral administration, peaking at 12 h. Intraduodenal administration of bile collected from orally exposed piglets resulted in increased bLf concentrations in plasma, suggesting a potential for enterohepatic circulation. Immunohistochemistry suggested that bLf was transported by endocytosis via epithelial cells (Harada et al. 1999a). Another study found bLf in the serum and cerebrospinal fluid following administration into the intestinal lumen of neonatal piglets (Harada et al. 2002). Based on immunohistochemistry of the small intestine, Kitagawa et al. (2003) concluded that bLf is predominantly transported to the systemic circulation via the lymphatics, with a smaller proportion transferred by the portal vein.

Faecal excretion of intact bLf following dietary administration to neonatal piglets has also been reported (Reznikov et al. 2014).

Studies in infants

Limited studies are available in humans investigating the pharmacokinetics of bLf, with the majority of studies focusing on hLf.

A comparison of peptides present in human milk and gastric aspirates from three mother-infant dyads (term infants aged 4 – 12 days) found a range of peptides released from hLf in the gastric samples that were not present in the milk, suggesting extensive proteolysis of hLf occurs in the term infant stomach (Dallas et al. 2014).

The absorption and excretion of bLf by preterm infants was assessed by Itell et al. (2021). Very low birth weight (<1500 g) infants (gestational age 24.1 – 34 weeks; starting from day 2-15 of life) were administered 100 (n=10), 200 (n=10) or 300 (n=11) mg/kg bw/day bLf enterally for 30 days. Infants were fed expressed or donor human milk during the study. Infant saliva, blood, urine and stool samples were collected prior to the first bLf supplement (study day 0), 22 days into supplementation (study day 22) and 7 days after the last administration of bLf (study day 37). Samples were assayed for bLf and hLf by ELISA. During treatment bLf was detected in the saliva, plasma, urine and stool. bLf levels did not vary significantly with dose, although a trend towards higher saliva and plasma levels at 200 and

300 mg/kg bw/day was observed on day 22. Levels of bLf in the saliva and stool began to decline within 12 h after dosing, and bLf was undetectable in all samples 1 week after treatment was terminated. The concentrations of hLf exceeded those of bLf across sample types and time points. The presence of intact bLf rather than immunologically reactive fragments was not confirmed in this study.

Intact and fragmented hLf have been detected in the urine of infants fed human milk (Hutchens et al. 1991a, 1991b, Goldman et al. 1990). Hutchens et al. (1991b) detected intact hLf (78 kDa) and hLf fragments (51 and 39 kDa) while intact hLf and a 'nicked' but apparently intact hLf (both 78 kDa) have been detected in the urine in another study. The 'nicked' form was shown to comprise two fragments that were tightly associated *in vivo*. One fragment was identified as the N terminus of the N-lobe (residues 3 – 283), while the other started with serine 284 and included the α-helical structures at the C terminus of the N-lobe and the entire C-lobe (Hutchens et al. 1991a). The most prominent hLf fragments detected by Goldman et al. (1990) were of approximately 44, 38, 34 and 32 kDa. These fragments were of a similar size to those produced by *in vitro* hydrolysis, suggesting they were produced by hydrolysis in the gastrointestinal tract.

Faecal samples from infants (age 3 days – 2 months) fed cow's milk formula supplemented with partially or fully iron-saturated hLf or bLf were shown to contain intact hLf or bLf, respectively. Protein bands of approximately 34 or 42 kDa were also detected in infants given hLf or bLf, respectively, suggesting partial hydrolysis had taken place. Both hLf and bLf isolated from faeces were shown to be able to bind iron (Spik et al. 1982).

Several other studies have reported the presence of intact hLf in the faeces of breastfed infants (Davidson and Lönnerdal 1987; Goldman et al. 1990; Mastromarino et al. 2014). Goldman et al. also detected fragments of the same size as those detected in urine from the same infants. Faecal hLf concentrations in term infants were significantly higher at 1 month compared with the concentration in meconium at birth, and higher faecal hLf concentrations were observed in preterm infants compared with term infants at both time points (Mastromarino et al. 2014). Davidson and Lonnerdal reported that approximately 2 – 6% of the hLf consumed was excreted in the faeces of term infants during the first week of life, with the proportion slowly declining with age to 0.4 – 1.6% at age 3 – 4 months.

Intact hLf has been found in the faeces of breastfed infants. Approximately 2 – 6% of the hLf consumed was excreted during the first week of life, with the proportion slowly declining with age to 0.4 – 1.6% at age 3 – 4 months (Davidson and Lönnerdal 1987).

Summary

The available evidence indicates that bLf is subject to partial hydrolysis in the stomach and small intestine, however a proportion of bLf resists digestion, persists throughout the gastrointestinal tract and is excreted in the faeces. Some fragments produced by partial hydrolysis also resist further digestion and are excreted in the faeces. *In vitro* studies, as well as studies in experimental animals and pre-term or term infants, suggest that hLf and bLf are more resistant to digestion in pre-term or neonatal infants compared to older infants. Animal and infant studies have shown that a small proportion of intact bLf and its fragments is absorbed into the systemic circulation and excreted via the urine. Experimental animal studies suggest that the primary route of absorption may be via the lymphatic system, while some bLf may also be excreted in the bile and subject to enterohepatic circulation.

3.1.2 Toxicology studies

The test item used in the key toxicology studies of bLf was produced by Morinaga Milk Industry Co. Ltd. The Applicant provided a comparison of analytical data for five batches of

Synlait's bLf and six batches of Morinaga's bLf, demonstrating the similarity in composition of these substances. In addition, the GRAS notice for Synlait's bLf (GRN669, 2016) includes a comparison of the specifications of bLf produced by Synlait and Morinaga, as well as the European specification for bLf, further demonstrating the equivalence of these substances. Synlait's bLf is spray dried whereas Morinaga's product is freeze dried, however this is not considered to be a significant difference, and the European specification for bLf indicates that both freeze-drying and spray-drying are permitted production technologies.

Based on the available data, it is considered that the toxicology studies with Morinaga's bLf are suitable for the safety assessment of bLf produced by Synlait.

Acute and short-term toxicity

Limited details of unpublished acute and 4-week toxicity studies of bLf, unavailable to FSANZ for review, are provided in a publication by Yamauchi et al. (2000a) as well as in the GRAS notice for Morinaga's bLf (GRN 465, 2013).

In the acute toxicity study, male and female Crj:CD(SD) SPF rats were administered single doses of 1000 or 2000 mg/kg bw standard bLf or iron-saturated bLf by oral gavage. Clinical signs were monitored for 14 days and body weights were measured periodically. At the end of the study animals were killed and organs were examined for any macroscopic abnormalities. No adverse effects were reported.

In the 4-week study, male and female Sprague Dawley rats (age 4 weeks) were administered 0, 200, 600 or 2000 mg/kg bw/day bLf by oral gavage for 28 days. The vehicle control was water. The summary available states that there were no deaths and no treatment-related adverse effects on body weight, feed consumption, organ weight, ophthalmology, haematology, clinical chemistry, gross pathology and histopathology examinations. The no observed adverse effect level (NOAEL) in this study was reported to be 2000 mg/kg bw/day, the highest dose tested.

13-week repeated dose oral toxicity study of bLf in rats (Yamauchi et al. 2000a) Regulatory status: Non-GLP; Protocol broadly consistent with OECD Test Guideline 408

Sprague Dawley rats (age 6 weeks; 12/sex/group) were administered 0, 200, 600 or 2000 mg/kg bw/day bLf (Morinaga Milk Industry Co. Ltd; iron content 14.7 mg/100 g) by oral gavage for 13 weeks. The vehicle control was water. Clinical signs were monitored daily, body weight and food consumption were recorded twice weekly. An ophthalmological examination was performed on 6 males and 6 females in each group in the final week of treatment. Urine samples were collected from all animals in weeks 6 and 13. At the end of the study blood was collected from all animals for haematology and clinical chemistry analysis, then animals were necropsied and examined for external abnormalities. Organs and tissues were subjected to gross examination, organs were weighed, and histopathological investigations were performed on organs and tissues from all animals in the control and high dose groups, any animals that died, and on the pancreas of male rats in the low and mid dose groups.

One male in the low dose group died in week 10 due to an intubation error. One female in the high dose group died in week 13, which was attributed to malignant lymphoma following gross and histopathological examination. There were no findings in the histopathology or peripheral blood profile indicative of lymphoma in any of the other animals, so this death was not considered to be treatment-related.

No treatment-related clinical signs were observed in any of the surviving animals, and there were no treatment-related adverse effects on body weight, ophthalomology, haematology

and clinical chemistry observations. Urinalysis indicated lower pH in high dose males and females compared with controls, but the degree of change was slight and there were no accompanying changes in other urinalysis parameters, clinical chemistry or kidney histopathology so this change was not considered to represent an adverse effect. Absolute and relative (to bw) thyroid weights were observed in high dose females, but this was considered incidental as the decrease was slight, observed in only one sex and not accompanied by correlated morphological changes. No other changes in absolute or relative organ weights were observed. No treatment-related adverse macroscopic or histopathologic changes were observed. Slight or mild islet fibrosis in the pancreas was observed in 3/12 males in the control group and 7/12, 6/12 and 6/12 males in the 200, 600 and 2000 mg/kg bw/day groups. However pancreatic islet fibrosis is known to be an age-related lesion in the male Sprague Dawley rat, and the incidence in males of a similar age fed a standard diet has previously been reported as 14/20 and 7/15 in 18 and 20 week old rats, respectively (Imaoka et al 2007; Molon-Noblot et al 2001). As a result, the islet fibrosis was not considered to be treatment-related.

It was concluded that the NOAEL in this study was 2000 mg/kg bw/day, the highest dose tested.

13-week repeated dose oral toxicity study of milk basic protein containing bLf in rats (Kruger et al. 2007) Regulatory status: Non-GLP; conducted in accordance with Japanese Ministry of Health and Welfare guidelines

Crj:CD(SD) IGS rats (10/sex/group; age not reported) were administered milk basic protein (MBP) derived from pasteurised skimmed milk (54% bLf; manufactured by Snow Brand Milk Products Co. Ltd., Japan) by oral gavage for 13 weeks. Doses administered were 0, 200 or 2000 mg/kg bw/day MBP, equal to 0, 108 or 1080 mg/kg bw/day bLf, respectively. The vehicle control was water. Clinical signs were monitored daily and body weight and food consumption recorded regularly. Ophthalmologic examination was performed in week 13 and urine samples were collected for urinalysis. Blood was collected for haematology and clinical chemistry analysis prior to necropsy. Gross observations were performed at necropsy and organ weights were recorded. Histopathological examinations were performed on all animals in the control and high dose groups.

All animals survived to the end of the study and no adverse clinical signs were observed. There were no treatment-related adverse effects on body weight, body weight gain, feed consumption and feed efficiency, ophthalmology, haematology, clinical chemistry or urinalysis parameters. No adverse treatment-related changes were observed at necropsy and there were no adverse effects on organ weights (absolute and relative) or histopathologic observations.

It was concluded that the NOAEL in this study was 2000 mg/kg bw/day MBP, equal to 1080 mg/kg bw/day bLf.

Long-term toxicity and carcinogenicity

Chronic dietary toxicity study with bLf in rats (Tamano et al. 2008) Regulatory status: Non-GLP, non-guideline

A summary of two chronic feeding studies with bLf sourced from Morinaga Milk Industry Co. Ltd is available in a publication by Tamano et al. These studies were not performed in accordance with OECD test guidelines and only limited details are reported, so they are of limited value for regulatory purposes but have been summarised as supporting information.

In experiment 1, groups of 15 male F344/DuCrj rats (age 6 weeks) were given diet containing

0 or 0.2% bLf for 40 weeks. Clinical signs were monitored daily and body weight and feed consumption recorded at regular intervals. At the end of the study, blood samples were collected for analysis of the following clinical chemistry parameters: aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyltranspeptidase (γ-GTP), alkaline phosphatase (ALP), blood urea nitrogen (BUN), creatinine, glucose, total cholesterol, triglyceride, total protein, albumin and serum iron. Gross inspection for any lesions were made at autopsy and the liver, kidneys and spleen were weighed. In experiment 2, groups of 17-week old male and 11-week old female F344/DuCrj rats were fed diets containing 0, 0.02, 0.2, 2.0 or 5.0% bLf for 60 weeks in males and 65 weeks in females. The control and high dose groups comprised 25 rats/sex while the other groups comprised 10 rats/sex. Additional rats (10/sex/group) were fed diets containing 2.0% bLf hydrosylate generated by acid-pepsin hydrolysis or lactoferricin. Clinical signs, body weights and feed consumption were monitored. Gross examinations were performed at autopsy and liver, kidney, spleen, adrenal and pituitary weights recorded. A range of organs and tissues were examined histopathologically.

In experiment 1, no treatment related clinical signs or deaths or effects on body weight were observed. The authors noted that slight but significantly decreased relative liver weights (but not absolute weights) were found in the bLf-treated group, but additional details were not reported. AST, ALT, ALP, BUN and TG were significantly lower in the 0.2% bLf group. No treatment-related macroscopic or histopathological changes were observed.

In experiment 2, no treatment-related clinical signs or deaths were recorded. There were no treatment-related adverse effects on body weight, feed and water consumption, organ weights, gross observations or histopathology.

The study authors concluded that the NOAEL for bLf in these studies was 5.0% in the diet, equivalent to approximately 3000 mg/kg bw/day (calculated by FSANZ based on default conversion factors [WHO 2016]).

Genotoxicity

Bacterial reverse mutation assay with bLf (Yamauchi et al. 2000b) Regulatory status: non-GLP; Protocol consistent with OECD TG 471

The potential mutagenicity of bLf (Morinaga Milk Industry Co. Ltd; iron content 14.7 mg/100 g) in bacteria was assessed in the presence and absence of metabolic activation (S9 mix) following the pre-incubation method. Test systems for this assay were *Salmonella* Typhimurium strains TA1535, TA100, TA1537 and TA98 and *Escherichia coli* strain WP2 *uvr*A. Saline was used as the vehicle and negative control. Positive controls in the absence of S9 were sodium azide (TA 1535), 9-aminoacridine (TA1537) and furfurylamide (TA100, WP2 *uvr*A and TA98). In the presence of S9 positive controls were benzo[a]pyrene (TA100, TA98 and TA1537) and 2-aminoanthracene (TA1535 and WP2 *uvr*A). Based on a dose range finding test concentrations ranging from 0.16 – 5.0 mg/plate were tested. The positive and negative controls were plated in triplicate while duplicate plates were used for the test items. The assay was conducted twice.

bLf is reported to have antimicrobial properties but no evidence of growth inhibition was observed during the study. No significant increases in the number of revertant colonies were observed following exposure to bLf in the presence or absence of S9 mix. The vehicle and positive controls produced numbers of revertant colonies consistent with the laboratory's historical control data, confirming the validity of the assay.

It was concluded that bLf was not mutagenic under the conditions of this assay.

Other studies

Behavioural effects of bLf during postnatal development of rats (Shumake et al. 2014; Regulatory status: Non-GLP, non-guideline

The effect of oral administration of bLf (98% purity, iron content 120 mg/kg) to Holtzman albino rats during postnatal development on behavioural responses to stress was assessed in two experiments.

In experiment 1, 750 mg/kg bw/day bLf was administered orally in the form of a vanilla wafer paste suspension in drinking water (10% w/v vanilla wafer, 10% w/v sucrose) to rats from postnatal day (PND) 16 for 18 days. Nine males and nine females were administered bLf and 8 males and 8 females received the vehicle control. Following weaning on PND 23 rats were assessed for general motor activity and performance in a range of behavioural tests over several days: open field (forced exploration of risky environment), light–dark emergence (voluntary exploration of risky environment), baited holeboard (working and reference memory), food neophobia (preference for familiar versus novel food), forced swim (test for antidepressant efficacy), and shuttle-box escape (learning to escape footshock).

In experiment 2, 0, 500, 1000 or 2000 mg/kg bw/day bLf in the same vehicle was administered to rats (7-8 males and 6-7 females per group) from PND 16, with bLf administration continued for 2 weeks after weaning on PND 23 (i.e. up to PND 37). Behavioural testing began on PND 37 and consisted of light-dark emergence, food neophobia, escape-swim test, passive avoidance and shuttle-box escape.

No adverse effects of bLf on general motor activity, behaviour and or learning were observed in either study. In experiment 1, bLf-supplemented rats showed less exploration of the risky environment, greater preference for a familiar food odour, and faster escape responses. In experiment 2, males receiving 1000 or 2000 mg/kg bw/day bLf mastered the water-escape task 20-25% sooner than those receiving the vehicle or 500 mg/kg bw/day. A similar effect was not observed in females.

The study authors concluded that bLf supplementation during development may improve subsequent cognitive performance during stress in rats.

3.1.3 Human tolerance studies

A number of studies of the administration of bLf to term, pre-term and/or very low birth weight infants include details of safety and tolerance. bLf was administered via IFP or directly to the infants. These studies are summarised in Table 2. In these studies bLf was well tolerated with no adverse events related to treatment reported. bLf concentrations up to 1000 mg/L formula were tested in the studies in term infants, while the doses tested in preterm and very low birth weight infants ranged from 100 – 300 mg/kg bw/day. These doses were estimated as being equivalent to bLf concentrations ranging from 370 – 3704 mg/L.

In addition, a recent Cochrane review of the use of enteral lactoferrin supplementation (human or bovine) for prevention of sepsis and necrotizing enterocolitis in preterm infants found that no adverse effects were reported in 12 studies involving 5425 preterm babies (Pammi & Gautham 2020).

Table 2 Intervention studies reporting on safety and tolerance of bLf-supplemented IFP in term and pre-term infants

Reference	Study design	Country	bLf source	Study population	Study groups and intervention	Findings related to safety
Studies in term infants						
Hernell and Lönnerdal (2002)	Single-blind intervention	Sweden	SMR, Sweden bLf was saturated with iron by researchers (1.24 mg/g protein)	Healthy term infants; age 4 ± 2 weeks	Breastfed infants (n=16) Infant formula (IF) with 4 mg/L iron as $FeSO_4$ (n=11) IF with 1.8 mg Fe/L, 1.3 mg from bLf + 0.5 mg as $FeSO_4$. bLf concentration 1000 mg/L (n=10) IF with 2.2 mg Fe/L from $FeSO_4$ + fortified with nucleotides as monophosphates (n=10) IF with 1.6 mg Fe/L as $FeSO_4$ (n=12) Intervention duration: until 6 months of age	All formulas were well tolerated.
King et al. (2007)	Double-blind, randomised controlled trial	USA	DMV International (Friesland Campina) Iron content 0.120 mg/g bLf	Healthy formula-fed infants; ≥34 weeks gestation, ≤ 4 weeks of age	IF supplemented with bLf (850 mg/L; n=26) Control IF (bLf content 102 mg/L; potentially denatured during formula production) Intervention duration: 12 months	bLf-supplemented formula was well-tolerated. Equal numbers of serious adverse events (hospitalisations) in bLf and control groups.
Johnston et al. (2015)	Multi-centre, double-blind randomised controlled trial	USA	DMV International (Friesland Campina)	Healthy term infants 12-16 days old	Control IF (n=155) IF containing 600 mg/L bLf	Study formulas were well-tolerated. Parent-reported gassiness and fussiness were similar among groups. Between days 30-180 the control group had more infants with a formed stool and fewer infants with an unformed or

Reference	Study design	Country	bLf source	Study population	Study groups and intervention	Findings related to safety
					IF containing 1000 mg/L bLf Investigational formulas contained a prebiotic blend of polydextrose and galactooligosaccharides and reduced arachidonic acid compared with control. Intervention period: until 1 year of age	seedy stool consistency compared with bLf groups; no significant differences by day 275. No significant difference in formula discontinuation (related or unrelated to the study formula) between groups. No difference in the number of participants with at least 1 medically confirmed adverse event. The incidence of serious adverse events was similar between groups, and all but one in the control group were deemed unrelated to study formula.
Chen et al. (2016)	Multicentre, double-blind randomised controlled trial	China	bLf-fortifed formula produced by Beingmate Baby and Child Food Co.	Healthy term infants aged 4-6 months	bLf-fortified infant formula (380 mg/kg powder; n=115) Unfortified infant formula (n=98) Breastfed reference group (n=103) Intervention duration: 3 months	No adverse effects related to bLf reported.
Li et al. (2019)	Multi-centre, double-blind randomised controlled trial	China	Friesland Campina DMV	Healthy term infants age 10-14 days	Control IF (n=228) IF + 600 mg/L bLf + 5000 mg/L milk fat globule membrane Intervention duration: 1 year	Fussiness and amount of gas similar between groups. No differences in mean stool frequency or stool consistency. No serious adverse events reported; no difference in the number of participants with at least one medically-confirmed adverse event. Overall incidence of adverse events in the respiratory and gastrointestinal system was lower in the bLf group

Reference	Study design	Country	bLf source	Study population	Study groups and intervention	Findings related to safety
						compared with controls; no other differences in adverse events reported.
Björmsö et al. (2021)	Double-blind randomised controlled trial	Sweden	Hilmar Ingredients	Healthy term infants aged 6 ± 2 weeks.	Breastfed reference group (n=72) IF containing 2 mg/L iron, no bLf IF containing 2 mg/L iron + 1000 mg/L bLf IF containing 8 mg/L iron, no bLf (control formula) Intervention duration: until 6 months of age	No adverse effects on gastrointestinal parameters observed in the bLf group. The number of stools/day and soft stools/day was lower in the bLf group than in breastfed infants, with no difference in watery stools, hard stools or days with abdominal pain. Use of simeticone (used to relieve symptoms of extra gas) was higher in formula-fed groups (2 mg/L Fe + bLf: 20%; 2 mg/L Fe – bLf: 15.9%; 8 mg/L Fe: 18.8%) than breastfed infants (2.9%).
Chen et al. (2021)	Multicentre, double-blind randomised controlled trial	China	Hilmar Cheese Company	Infants with anaemia aged 6-9 months	bLf-fortified infant formula (380 mg/kg; n=33) bLf-fortified infant formula (760 mg/kg; n=28) Control formula (n=35) Intervention duration: 3 months	No serious adverse events observed in either bLf-supplemented group.
Björmsö et al. (2022)	Double-blind, randomised controlled trial	Sweden	Hilmar Ingredients	Healthy term infants aged 6±2 weeks	Low iron infant formula (2 mg/L) containing bLf (1000 mg/L; n=72) Low iron formula without bLf (n=71)	No adverse effects were observed. No significant differences in serum cytokines observed between groups.

Reference	Study design	Country	bLf source	Study population	Study groups and intervention	Findings related to safety
					Standard formula (8 mg/L iron) without bLf (n=33) Breastfed reference group (n=71) Intervention duration: Until 6 months of age	
Studies in pre-term or very low birth weight infants						
Manzoni et al. (2009)	Multicentre, double-blind randomised controlled trial	Italy	Dicofarm	Very low birth weight (VLBW; < 1500g) neonates age < 3 days	Orally administered bLf (100 mg/day; n=153) bLf (100 mg/day) + *Lactobacillus rhamnosus* GG ($6x10^9$ colony forming units/day; n=151) Equivalent bLf concentration calculated as 370 – 1316 mg/L[2] Placebo (n=168) Intervention duration: from birth until day 30 of life (day 45 for neonates < 1000 g at birth)	No intolerance or adverse effects related to bLf were recorded. Administration was not discontinued due to presumed adverse effects, intolerance or potentially dangerous interactions with other drugs. No infants displayed signs of hepatotoxicity or cholestasis.
Manzoni et al. (2014)	Multicentre, double-blind randomised controlled trial	Italy and New Zealand	Dicofarm	VLBW infants age 3 days	Orally administered bLf (100 mg/day; n=247) bLf (100 mg/day) + *Lactobacillus*	No adverse effects or treatment intolerance occurred.

[2] Calculated by the Applicant based on the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) proposed range of milk feeding for preterm infants of 150-180 mL/kg bw/day.

Reference	Study design	Country	bLf source	Study population	Study groups and intervention	Findings related to safety
	(continuation of the study by Manzoni et al 2009)				*rhamnosus* GG ($6x10^9$ colony forming units/day; n=238) Equivalent bLf concentration calculated as 370 – 1316 mg/L[2] Placebo (n=258) Intervention duration: from birth until day 30 of life (day 45 for neonates < 1000 g at birth)	
Akin et al. (2014)	Double-blind randomised controlled trial	Turkey	Dicofarm	Infants either VLBW or born before 32 weeks	Orally administered bLf (200 mg/day; n=25) Equivalent bLf concentration calculated as 593 – 1961 mg/L[2] Placebo (n=25) Intervention duration: from when the baby reached 20 mL/kg bw/day feeding volume until the end of hospitalisation	Treatment was well tolerated and no adverse effects were reported.
Ochoa et al. (2015)	Multicentre, double-blind randomised controlled trial	Peru	Tatua Co-operative Dairy Co	Nenoates with birth weight 500-2500 g born in or referred to neonatal units in the first 72 hours of life	Orally administered bLf (200 mg/kg bw/day; n=95) Equivalent bLf concentration calculated as 1111 – 1316 mg/L[2]	No signs of allergy or treatment intolerance in 99.7% of observed days, with only 3 episodes of vomiting in the intervention periods. None of the severe adverse events recorded were attributable to the intervention.

Reference	Study design	Country	bLf source	Study population	Study groups and intervention	Findings related to safety
					Placebo (n=95) Intervention duration: from enrolment for 4 weeks	
Kaur and Gathwala (2015)	Double-blind randomised controlled trial	India	Not reported	Neonates with a birth weight < 2000 g	Orally administered bLf (n=63): Birth weight 1000-1249 g: 100 mg (80-100 mg/kg bw/day) Equivalent bLf concentration calculated as 444 – 667 mg/L[2] Birth weight 1250-1499 g: 150 mg (100-120 mg/kg bw/day) Equivalent bLf concentration calculated as 556 – 798 mg/L[2] Birth weight 1500-1749 g: 200 mg (114-133 mg/kg bw/day) Equivalent bLf concentration calculated as 635 – 885 mg/L[2] Birth weight 1750-1999 g: 250 mg (125-142 mg/kg bw/day) Equivalent bLf concentration calculated as 694 – 954 mg/L[2] Placebo (n=67)	No discontinuations of treatment due to intolerance. No adverse effects were recorded.

Reference	Study design	Country	bLf source	Study population	Study groups and intervention	Findings related to safety
					Intervention duration: 4 weeks from 1st day of life	
Barrington et al. (2016)	Single-centre masked randomised pilot trial	Canada	AOR	Infants born <31 weeks gestation.	Milk (maternal human milk or preterm formula) with 100 mg/day bLf (n=40) Equivalent bLf concentration calculated as 556 – 667 mg/L[2] Milk without bLf (n=39) Intervention duration: up to 36 weeks postmenstrual age	bLf was well tolerated and no adverse events related to the study intervention were reported. No effect of bLf on feeding tolerance. Mortality, late-onset sepsis and other complications of prematurity were no different between groups.
ELFIN trial investigators group (2019)	Randomised controlled trial	UK	Tatua Co-operative Dairy Co	Very preterm infants <32 weeks gestation	Orally administered bLf (150 mg/kg bw/day; maximum 300 mg/day; n=1093) Equivalent bLf concentration calculated as 833 – 987 mg/L[2] Placebo (n=1089) Intervention duration: up to 34 weeks postmenstrual age	16 serious adverse events (1.5%) in the bLf group and 10 (0.9%) in the sucrose group. 2 serious adverse events (1 blood in stool, resolved spontaneously; 1 death after intestinal perforation) assessed as possibly related to the trial intervention. All other serious adverse events considered to be unrelated to the trial intervention.
Tarnow-Mordi et al. (2020)	Multicentre, double-blind randomised controlled trial	Australia and New Zealand	Australia's Own	VLBW infants aged < 8 days.	Pasteurised bLf (200 mg/kg bw/day) in human milk or formula milk (n=771)	No safety concerns reported. Similar incidence of death/morbidity in each group.

Reference	Study design	Country	bLf source	Study population	Study groups and intervention	Findings related to safety
					Equivalent bLf concentration calculated as 1109 – 1331 mg/L[2] Milk without added bLf (n=771) Intervention duration: until 34 weeks post-menstrual age (or for 2 weeks if longer) or until discharge from study hospital	
Ochoa et al. (2020)	Multicentre, double-blind randomised controlled trial	Peru	Friesland Campina	Neonates with birth weight 500-2000 g	bLf dissolved in human milk or infant formula (200 mg/kg bw/day; n=209) Equivalent bLf concentration calculated as 1111 – 1316 mg/L[2] Placebo dissolved in human milk or infant formula (n=205) Intervention duration: 8 weeks	Signs or symptoms of allergic reactions or intolerance were closely monitored: no significant differences in the incidence of vomiting, diarrhoea and abdominal circumference were observed between groups. No serious adverse events were attributed to the intervention. Neurodevelopmental outcomes were similar in each group.
Kaufman et al. (2021)	Dose escalation safety study	USA	Glanbia Nutritionals	Preterm neonates (< 32 weeks), birth weight <1500 g	Enteral bLf (100 [n=10], 200 [n=10] or 300 [n=11] mg/kg bw/day) Equivalent bLf concentration calculated as 383 - 1124 mg/L (100 mg/kg bw/day); 752 – 1563 mg/L (200 mg/kg	All infants tolerated the study solution. No adverse events related to the study solution were recorded. There were no adverse effects on hepatic, renal or haematological function.

Reference	Study design	Country	bLf source	Study population	Study groups and intervention	Findings related to safety
					bw/day); or 1128 – 3704 mg/L (300 mg/kg bw/day)[3] Intervention duration: 30 days	

[3] Calculated by FSANZ based on the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) proposed range of milk feeding for preterm infants of 150-180 mL/kg bw/day.

3.1.4 Post-marketing surveillance

The applicant has been manufacturing IFP containing bLf for the Chinese market since 2011, and using its own internally manufactured bLf since 2014 (GRN669, 2016). It has a system in place to record and investigate any customer complaints and concerns. No complaints related to the presence of bLf in IFP have been recorded to date.

The applicant has also been selling bLf as an ingredient to large international infant formula brand owners for us in their IFP for many years, and has indicated that none of these companies have reported any adverse events related to addition of bLf.

The first IFP containing bLf were released in Japan in 1986. In a GRAS notification to the US FDA in 2011, Morinaga stated that over a million infants and toddlers had consumed bLf-fortified IFP since 1986 with no significant health problems associated with products based on post-marketing surveillance (GRN465, 2013).

FSANZ is unaware of any overseas recalls of products related to the presence of bLf.

3.1.5 Allergenicity

bLf is derived from cow's milk, which is a major food allergen. Several studies have demonstrated that some individuals with cow's milk allergy have IgE antibodies to bLf leading to the suggestion that bLf may be a cow's milk food allergen (Natale et al. 2004; Wal et al. 1995, 1998; Gaudin et al. 2008). To date, the clinical significance of sensitisation to bLf in these individuals has not been confirmed by positive oral challenge tests with bLf (Gaudin et al. 2008; Taylor et al. 2004), and it is not currently listed as a cow's milk allergen in the World Health Organization/International Union of Immunological Societies (WHO/IUIS) allergen nomenclature database.

FSANZ considers the available evidence is insufficient to conclude that bLf does not pose a food allergy risk to consumers with cow's milk allergy.

3.1.6 Safety assessments by other agencies

The European Food Safety Authority (EFSA) Panel on Dietetic Products, Nutrition and Allergies (NDA) published a scientific opinion on the use of bLf produced by Friesland Campina as an ingredient in infant and follow-on formula, food supplements, dietetic food for special medical purposes and sport nutrition, and for a variety of foods (EFSA 2012). The toxicity studies conducted with Morinaga's bLf were used to inform the evaluation. EFSA concluded that bLf is safe under the proposed uses and use levels.

Subsequent to the approval of Friesland Campina's bLf in the EU, several companies have been granted 'substantial equivalence' following an assessment by a Competent Authority within the EU, enabling them to place bLf on the European market. Synlait did not apply for substantial equivalence in the EU, because as of 2018 this mechanism was replaced by an updated regulation[4] allowing any bLf that meets the EU specification for bLf as listed in the European Union list of authorised novel foods[5] to be used within the EU.

In the USA, bLf has been the subject of several GRAS Notices submitted to the US Food and Drug Administration, including notices from Synlait and Morinaga relating to use of bLf in IFP (GRN 669 and GRN 465, respectively). The US FDA has issued 'no questions' letters to both of these notices, although this does not constitute an independent safety evaluation by the

[4] https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32015R2283
[5] http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32017R2470&from=EN

US FDA.

3.1.7 Discussion of toxicological assessment

bLf is subject to partial hydrolysis in the stomach and small intestine, however a proportion of bLf resists digestion, persists throughout the gastrointestinal tract and is excreted in the faeces. Some fragments produced by partial hydrolysis also resist further digestion and are excreted in the faeces. In addition, a small proportion of intact bLf and its fragments is absorbed into the systemic circulation and excreted via the urine. The evidence suggests that hLf and bLf are more resistant to digestion in pre-term or neonatal infants compared to older infants.

bLf is of low acute toxicity, with no adverse effects observed following oral administration to rats up to 2000 mg/kg bw.

Short-term oral gavage toxicity studies in rats with bLf produced by Morinaga are available in the published literature. The test item in these studies is representative of the bLf produced by the Applicant. No adverse effects were observed in 4-week and 13-week toxicity studies at doses up to 2000 mg/kg bw/day, the highest dose tested.

bLf was not mutagenic in a bacterial reverse mutation assay. Reports of two long-term dietary toxicity studies with bLf administered to rats for 40 weeks or 60-65 weeks are available. No evidence of toxicity or carcinogenicity were reported in these studies and the study authors concluded that the NOAEL in these studies was 5% in the diet, equivalent to approximately 3000 mg/kg bw/day, the highest concentration tested. The long-term studies provide supporting evidence of safety but have limited value for regulatory purposes given the limited details available in the literature. Additional long-term toxicity/carcinogenicity studies are not considered to be required, however, noting that bLf is a protein naturally present in cow's milk, it was not mutagenic and no lesions that might progress to neoplasia by non-genotoxic mechanisms were observed in subchronic studies. No adverse effects on general motor activity, behaviour or learning were observed in a study in which bLf was administered orally to rats from PND 16 – 34.

bLf was well tolerated with no adverse effects reported in multiple intervention studies in infants, including the highly vulnerable group of preterm and very low birth weight infants.

The first bLf-fortified IFP were released for sale in Japan in 1986 and FSANZ is not aware of any adverse events related to consumption of these products in markets where they are available. The applicant has indicated that its post-marketing surveillance, and that of international formula brand owners it supplies, has not identified any complaints or adverse events related to the addition of bLf.

bLf is derived from cow's milk which is a major food allergen. Some individuals with cow's milk allergy have IgE antibodies to bLf indicating sensitisation, but the clinical significance of this has not been confirmed and bLf is not currently listed as a cow's milk allergen by the WHO/IUIS. The limited available evidence however is insufficient to conclude that bLf does not pose a food allergy risk to consumers with cow's milk allergy.

3.2 Microbiology assessment

The applicant produces bLf by ion-exchange chromatography from clarified and filtered skim milk that has been cooled to below 8°C to prevent microbial growth. The eluate is concentrated and desalted by ultrafiltration before pasteurisation at 73.5°C for 18 seconds, which is sufficient to inactivate pathogenic microorganisms. Subsequent microfiltration, evaporation, spray drying and packaging steps are conducted under HACCP controls, and

introduce no unacceptable risk of microbial contamination. Each batch is tested against suitable microbiological specifications, including zero tolerance for the key pathogens associated with infant formula products, *Salmonella* spp. and *Cronobacter* spp.

Arnold et al. (1980) demonstrated an inducible reduction in susceptibility of a strain of *Streptococcus pneumoniae* to hLf after serial passage of the bacteria five times through mice. This apparent resistance to hLf was lost after subsequent serial passage in broth medium. They also observed varying degrees of resistance in strains of other streptococci and *E. coli*. The authors concluded that resistance was likely related to cell surface components (intrinsic or inducible virulence factors) which reduced the ability of lactoferrin to access and disrupt the peptidoglycan cell wall.

This effect is not considered to constitute a significant risk of the spread of antimicrobial resistance determinants between resistant pathogenic bacterial species. bLf has been shown to inhibit acquisition of antibiotic resistance genes by *S. pneumoniae* in *in vitro* transformation assays (Angulo-Zamudio et al., 2019). This is most likely related to its metal-dependent deoxyribonuclease catalytic activity (Zhao and Hutchens, 1994; Babina et al., 2004; Soboleva et al., 2018).

No additional microbiological safety risks arise from addition of bLf to powdered infant formula products and its preparation and consumption beyond those encountered with IFP that is not supplemented with bLf.

3.3 Dietary intake assessment

3.3.1 Objective

The objective of this dietary intake assessment is to estimate the dietary intake of bLf from the proposed addition to infant formula, follow-on formula and infant formula for special dietary use. Infant formula is specified in the Code as being applicable for infants 0-6 months and follow-on formula from 6-12 months.

3.3.2 Approach to estimating dietary intakes of bLf

Dietary intake assessments require data on the concentrations of the chemical of interest in the foods requested, including any naturally occurring sources and any current permissions for additions to food; and consumption data for the foods which are usually collected through a national nutrition survey. The dietary intakes of bLf for this assessment were estimated using: (1) the proposed maximum permitted amount of bLf in infant formula and follow-on formula; and (2) model diets for infants aged 3 months and 9 months.

Dietary intakes of hLf from human milk and bLf from bovine milk were also estimated for comparative purposes.

A dietary intake assessment for iron was not undertaken. This was because of the low iron content of the lactoferrin, and because there is a maximum permitted amount of iron specified in the Code for IFP, therefore infants would not be expected to have higher iron intakes from formula containing bLf compared to other IFP.

A summary of the general FSANZ approach to conducting the dietary intake assessment for this Application is in Appendix 1. A detailed discussion of the FSANZ methodology and approach to conducting dietary intake assessments is set out in Principles and Practices of

Dietary Exposure Assessment for Food Regulatory Purposes[6] (Food Standards Australia New Zealand 2009).

3.3.2.1 Consumption data used

The hazard identification and characterisation did not identify any population sub-groups for which there were specific safety considerations in relation to the intake of bLf. The population groups that were used for the dietary intake assessment are:

- Infants aged 3 months – representing exclusively formula-fed / breastfed infants
- Infants aged 9 months – representing infants who consume food as well as follow-on formula or human milk.

Model diets were used for the population groups 3 months and 9 months, to represent the consumption of infant formula or follow-on formula (where appropriate) for these groups. This was because food consumption data for individuals in this age group were not included in the most recent nationally representative nutrition surveys in Australia and New Zealand. How the model diets were constructed is outlined in Appendix 1.

3.3.2.2 Concentrations of Lactoferrin

3.3.2.2.1 Proposed concentrations of bLf in infant formula and follow-on formula, from the Application

The food categories requested in the Application to contain bLf and the proposed maximum permitted amount (in mg/100 kJ as requested by the applicant and the converted values in mg/L) are listed in Table 3.

Table 3 Proposed maximum permitted amount of bLf in foods, from the Application

Food	Maximum permitted amount (mg/100 kJ)[1]	Maximum permitted amount (mg/L)[2]
Infant formula (as prepared or ready-to-feed)	40	1109
Follow-on formula (as prepared or ready-to-feed)	40	1109
Infant formula for special dietary use (as prepared or ready-to-feed)	40	1109

[1] As requested by the applicant.
[2] The value is calculated using the proposed concentration of bLf by applicant (40 mg/100 kJ), the energy content of infant formula, 6-12 months, prepared with water (264 kJ/100 g) (FSANZ 2016) and the density of infant formula (1 L prepared infant/follow on formula is equivalent to 1,050 g).

3.3.2.2.2 Concentrations of hLf in mature human milk

The applicant reported a range of concentrations of hLf in human milk from published Australian and international studies, noting that concentrations vary depending on a number of factors, primarily the stage of lactation and geography. In particular, the applicant highlighted the mean hLf concentrations in the human milk of Australian Aboriginal and Caucasian women, which were categorised by the author (Houghton et al. 1985) as per the number of days post-partum, and by the percentage of the mother's weight for height. For Australian women with a weight for height ≥ 90%, mean hLf in mature milk (≥ 15 days post-partum) was reported as 1230 mg/L for Aboriginal women (n=8) and 1420 mg/L for Caucasian women (n=16) (Houghton et al. 1985).

[6] https://www.foodstandards.gov.au/publications/Pages/Principles-and-Practices-of-Dietary.aspx

In addition to the studies cited by the applicant, FSANZ conducted a search of the literature and confirmed a range of mean hLf values in mature human milk (>15 days and ≤12 months) between 1230 mg/L and 3390 mg/L (Czosnykowska-Lukacka et al. 2019; Goldsmith et al.1983; Houghton et al.1985; Hirai et al. 1990; Lien et al. 2004; Mastromarino et al. 2014; Rai et al. 2014; Montagne et al. 2001; Liu et al. 2022). To reflect this range of values, FSANZ used the mid-point of the two means presented by Houghton et al. (1985) as a representative lower concentration (1325 mg/L) and the mean concentration of 3390 mg/L reported by Czosnykowska-Lukacka (2019) as a representative higher concentration in mature milk to estimate the dietary intake of hLf from human milk. The concentrations of hLf considered in this assessment are greater than the proposed maximum permitted amount of bLf proposed in the Application (refer to Table 4).

3.3.2.2.3 Concentrations of bLf in domestic mammalian milks

Infant formula and follow-on formula are made with domestic mammalian milk bases, particularly cow's milk and goat's milk. The milk itself and other foods made from cow's, sheep's and goat's milk could be consumed by Australian and New Zealand infants. Consequently, the sources of naturally occurring bLf from domestic mammalian milks were investigated.

Several authors reported the mean concentration of bLf in mature cow's milk between 80-177 mg/L and in goat's and sheep's milk between 17-166 mg/L (Chen and Mao 2004, Cheng et al. 2008; El-Hawiet 2017, Hagiwara et al. 2003; Hiss et al. 2008, Nisbet et al. 2013, Rainard et al. 1982, and Wang et al. 2018). The applicant noted that bLf concentrations can vary depending on the animal and stage of lactation, and cited a typical concentration value of 100 mg/L which falls in the ranges reported in the literature. The applicant also estimated the content of bLf in standard, made-up not-fortified infant formula to be 10-27 mg/L (refer to Table 4).

In the risk assessment for Application A1155, the mean consumption of cow's milk and all of its products (e.g. cheese), expressed in milk equivalents, for 2-3 year old Australian children in the 2011-12 National Nutrition and Physical Activity Survey (2011-12 NNPAS) was 707 g/day (FSANZ 2019). Although it is not recommended for infants to consume cow's milk as a drink until after 12 months of age, infants over the age of 6 months may consume cow's milk in products such as full-fat yoghurt, cheese and custard (NHMRC 2012). In a conservative approach, assuming 9 month old infants consume a mean of 707 g of cow's milk as cow's milk equivalents (not including infant formula), the estimated mean dietary intake of bLf would be approximately 70 mg/day[7]. As 9 month old infants should not be consuming milk as a beverage, this is likely an overestimation of intake.

As included in the risk assessment for A1155 (FSANZ 2019), no child aged 2-3 years specifically reported eating goat's cheese or goat's milk either on its own or as an ingredient in mixed foods (e.g. salad) in the 2011-12 NNPAS. Therefore the contribution of goat's milk foods to naturally occurring bLf dietary intakes is likely to be minimal.

Table 4 Comparison of mean Lf concentrations of mature cow's milk, goat's milk and human milk and the proposed permissions in infant formula and follow-on formula

[7] Assuming bLf concentration in cow's milk of 100 mg/L and the density of cow's milk is equivalent to 1,030 g.

	Mean lactoferrin concentration (mg/L)	References
Cow's Milk (mature)	80-177	Chen and Mao, 2004; Cheng et al. 2008; Hagiwara et al. 2003; Rainard et al. 1982
Goat's Milk and Sheep Milk (mature)	17-166	Chen and Mao, 2004; Hiss et al. 2008; El-Hawiet 2017; Nisbet et al. 2013; Wang et al. 2018
Infant formula / follow on formula (unfortified)	10-27	From the Application
Infant formula / follow on formula (proposed)	1109	Calculated from the proposed maximum permitted amount in the Application
Human milk (mature)	1230-3390	Czosnykowska-Lukacka et al. 2019; Goldsmith et al.1983; Hirai et al. 1990; Houghton et al.1985; Lien et al. 2004; Mastromarino et al. 2014; Rai et al. 2014; Montagne et al. 2001; Liu et al. 2022

3.3.2.3 Assumptions and limitations of the dietary intake assessment

The aim of the dietary intake assessment was to make the most realistic estimation of dietary intakes of bLf as possible. However, where significant uncertainties in the data existed, conservative assumptions were generally used to ensure that the estimated dietary intake was not an underestimate of intake.

Assumptions made in the dietary intake assessment included:

- unless otherwise specified, all foods within a category contain bLf at the concentrations specified in Table 3 for infant formula and follow-on formula
- 1 litre of infant formula and follow-on formula equals 1,050 g
- 1 litre of human milk equals 1,040 g
- there is 100% market penetration of the infant formula and follow-on formula containing bLf
- infants aged 3 months are exclusively infant formula fed
- infants aged 9 months consume follow-on formula
- consumption of foods as outlined in the model diets represent current food consumption amounts for Australian and New Zealand children aged 3 months and 9 months
- there is no contribution to bLf intakes through foods and beverages other than from infant formula and follow-on formula
- there is no contribution to bLf intakes through the use of or complementary or other medicines.

In addition to the specific assumptions made in relation to this dietary intake assessment, there are a number of limitations associated with the nutrition surveys from which the food consumption data used for the assessment are based. A discussion of these limitations is included in Section 6 of the Principles and Practices of Dietary Exposure Assessment for Food Regulatory Purposes[6] (Food Standards Australia New Zealand 2009).

3.3.3 Estimated dietary intakes

3.3.3.1 Estimated dietary intake of hLf from human milk

When it is assumed that infants aged <12 months are consuming mature human milk (and no infant formula or follow-on formula), the estimated mean and 90th percentile (P90) intakes of hLF from human milk are 975-2494 mg/day and 1949-4987 mg/day respectively for 3 month old infants and 656-1679 mg/day and 1312-3357 mg/day respectively for 9 month old infants.

On a grams per kilogram body weight per day basis, the estimated mean and P90 dietary intakes of hLf from mature human milk are 152-390 mg/kg bw/day and 305-779 mg/kg bw/day respectively for 3 month old infants and 74-189 mg/kg bw/day and 147-377 mg/kg bw/day respectively for 9 month old infants.

Further details are presented in Table 5.

Table 5 Estimated dietary intakes of hLf for infants aged 3 months and 9 months consuming mature human milk based on 2 different hLf concentrations

	Unit	3 months	9 months
Recommended energy intake[1]	kJ/kg bw/day	343	330
P50 body weight[2]	kg	6.4	8.9
Recommended energy intake	kJ/day	2195	2937
Amount of human milk required to meet 100% energy requirements[3]	g/day	765	n/a
Amount of human milk required to meet 50% energy requirements[3]	g/day	n/a	515
Mean dietary intake of hLf from human milk (low concentration)[4]	mg/day	975	656
	mg/kg bw/day	152	74
P90 dietary intake hLF from human milk (low concentration)[4]	mg/day	1949	1312
	mg/kg bw/day	305	147
Mean dietary intake of hLf from human milk (high concentration)[5]	mg/day	2494	1679
	mg/kg bw/day	390	189
P90 dietary intake hLF from human milk (high concentration)[5]	mg/day	4987	3357
	mg/kg bw/day	779	377

[1] United Nations University et al. 2004.
[2] World Health Organization 2006.
[3] Energy content of human milk is 286 kJ/100 g (FSANZ 2016).
[4] Minimum concentration of hLf used in the calculation is 1325 mg/L and 1 L of human milk is equivalent to 1,040 g.
[5] Maximum concentration of hLf used in the calculation is 3390 mg/L and 1 L of human milk is equivalent to 1,040 g.

3.3.3.2 Estimated dietary intake of bLf from infant formula

The estimated mean and P90 intakes of bLf from infant formula are 878 mg/day and 1756 mg/day respectively for 3 month old infants, and from follow-on formula are 587 mg/day and 1175 mg/day respectively for 9 month old infants.

On a grams per kilogram body weight per day basis, the estimated mean and P90 dietary intakes of bLf from infant formula are 137 mg/kg bw/day and 274 mg/kg bw/day respectively for 3 month old infants, and from follow-on formula are 66 mg/kg bw/day and 132 mg/kg bw/day respectively for 9 month old infants (see Table 6).

Table 1 Estimated mean dietary intake of bLf in infant and follow-on formula based on the maximum permitted amount proposed by the applicant

	Unit	3 months	9 months
Recommended energy intake[1]	kJ/kg bw/day	343	330
P50 body weight[2]	kg	6.4	8.9
Recommended energy intake	kJ/day	2195	2937
100% energy requirements[3]	kJ/day	2195	na
50% energy requirements[3]	kJ/day	na	1469
Mean dietary intake of bLf from infant/follow-on formula[4]	mg/day	878	587
	mg/kg bw/day	137	66
P90 dietary intake bLF from infant/follow-on formula[4]	mg/day	1756	1175
	mg/kg bw/day	274	132

[1] United Nations University et al. 2004.
[2] World Health Organization 2006.
[3] Energy content of infant/follow-on formula is 264 kJ/100 g (FSANZ 2016).
[4] The maximum concentration of bLf in infant formula and follow-on formula is 40 mg/100 kJ.

3.3.4 Conclusion

Based on the maximum permitted amount proposed by the Applicant, the estimated mean and P90 intakes of bLf from infant formula and follow-on formula range between 587 and 1756 mg/day. These intakes are less than the estimated mean and P90 intakes of hLf from mature human milk of 656 to 4987 mg/day. Assuming 9 month old infants consume 707 g cow's milk per day (as cow's milk equivalents), this would add 70 mg/day of bLf to estimated intakes in addition to that from IFP.

4 Nutrition assessment

4.1 Background

4.1.1 Lactoferrin function

hLf, found in colostrum and mature milk, may have a role during neonatal development (Manzoni et al. 2018). bLf shares 69% amino acid sequence homology with hLf, and bLf has five potential glycosylation sites whereas hLf has three (section 2.2.1). *In vitro* and rat studies indicate that the degree of iron saturation may affect lactoferrin function, and binding of other metal ions (e.g. manganese) may also have a functional role (Majka et al. 2020). The iron saturation of bLf preparations are reported to vary widely, e.g. 8.7% (mean) for the Application's spray-dried bLf powder (Application Table 2-9, p. 48), and up to ~90% (Hernell & Lönnerdal 2002). The Application proposes a maximum limit of 15 mg iron/100 g bLf powder (equivalent to 10.7%; see section 2.2.1) and does not propose a minimum limit.

4.1.2 Data requirements to assess nutrition-related outcomes

In this assessment, we consider the effects of consumption of bLf-supplemented IFP (bLf-IFP) on nutrition-related outcomes, specifically infant growth and development. The most directly relevant trials in infants would be those using bLf conforming to the Application's specifications, added to IFP that are consistent with the compositional requirements of Standard 2.9.1 and Schedule 29 of the Code.

Data requirements for the assessment of compositional changes to IFP are outlined in FSANZ's Application Handbook. The criteria for study selection were formulated based on these requirements, as prescribed by section 3.6.2 A.3.1 (b) of the FSANZ Application Handbook. These are referenced in the footnotes to Table A.1 (Appendix 2). We also set

parameters for the iron content of IFP tested in infant studies (iron minimum and maximum levels of 0.2 and 0.5 mg/100 kJ, respectively, as prescribed by the Code), and the iron saturation of the bLf based on alignment with the Application's product. Details are provided in Appendix 2.

4.2 Effect on growth and development

4.2.1 Objectives

The objective of this assessment was to determine the effect (if any) on infant growth and development of consuming IFP with bLf added up to the proposed maximum permitted amount (40 mg/100 kJ) compared to consuming human milk, or consuming IFP without added bLf.

4.2.2 Methods and results

We reviewed the literature published up to June 2022. We identified and screened the literature using the strategy and study selection criteria described in sections A2.1.1, for the comparison with human milk consumption, and A2.2.1 for the comparison with non-bLf IFP consumption. No studies met the pre-specified inclusion criteria (see Table 7 for examples). Details are provided in Appendix 2.

Table 7 Reasons for exclusion of selected studies[1].

Author (year)	Reasons for exclusion[2]
Objective: compare the effect of consuming bLf-IFP at the proposed maximum permitted amount, with the consumption of both human milk and IFP without added bLf.	
Lönnerdal & Hernell (1994)	• bLf content: not stated. • Iron content: 31% lower than minimum level prescribed in Code. • Iron saturation of bLf: not stated. • Infant age at enrolment: 6 ± 2 weeks. • Exploratory or hypothesis generating study. • Inadequately small sample size: ~10 per intervention group.
Hernell & Lönnerdal (2002)	• Iron content: bLf-IFP was 67% lower, and non-bLf-IFP was 71% lower, than minimum level prescribed in Code. • Iron saturation of bLf: 88.6% (i.e. higher than the Application's specifications and that of human milk). • Infant age at enrolment: 4 ± 2 weeks. • Exploratory or hypothesis generating study. • Inadequately small sample size: 10 per intervention group.
Björmsjö et al. (2021)	• Iron content: bLf-IFP and non-bLf-IFP was 64% lower than minimum level prescribed in Code. • Iron saturation of bLf: not stated. • Infant age at enrolment: 6 ± 2 weeks.
Objective: compare the effect of consuming of bLf-IFP at the proposed maximum permitted amount, with the consumption of IFP without added bLf.	
King et al. (2007)	• bLf content of intervention group: 23% less bLf than that specified by the Application. • bLf content of comparator group: 102 mg bLf/L instead of 0 mg bLf/L for a meaningful comparison. • Iron content: bLf-IFP and low-bLf-IFP was 45% lower than minimum level prescribed in Code. • Iron saturation of bLf: not stated for comparator (low-bLf-IFP).

	• Pilot study (i.e. exploratory or hypothesis generating study). • Inadequately small sample size: 26 per intervention group
Johnston et al. (2015)	• Iron content: all IFP contained 67% lower iron than minimum level prescribed in Code. • Iron saturation of bLf: not stated. • Two intervention IFP contained an added blend of polydextrose and galactooligosaccharides (4 g/L) that was not added to the control IFP.
Li et al. (2019)	• bLf content of intervention group: 45% less bLf than that specified by the Application. • Iron content: both IFP contained 82% lower iron than minimum level prescribed in Code. • Iron saturation of bLf: not stated. • The intervention IFP contained added bovine milk fat globule membrane (MFGM; 5 g/L) that was not added to the control IFP.

[1]Note: studies have additional limitations that affect the quality of the evidence, beyond the reasons for exclusion stated in Table 7. For example, one study reported a high drop-out rate of approximately one-third and additionally did not conduct an intention-to-treat analysis (King et al. 2019).
[2]Some studies' IFP differed to the compositional requirements for the Code for other nutrients. For example, the copper content of the IFP used by Lönnerdal & Hernell (1994) was 82% lower than the minimum level prescribed in Code. Other micronutrient levels of IFP are not reported because this did not form part of our study selection criteria (see Appendix 2, section A2.1.1).

A number of studies shared reasons for exclusion, as discussed below.

Studies with small sample sizes (Lönnerdal & Hernell 1994; Hernell & Lönnerdal 2002; Björmsjö et al. 2021; King et al. 2019) should be interpreted with caution. The ability to detect outcome differences between groups (the study power) is low in these studies (Jarrold et al. 2020). For comparison, in studies on human milk oligosaccharides, ~200-300 infants were enrolled to detect a pre-determined difference in bodyweight gain of 3 g/day or greater (Marriage et al. 2015; Puccio et al. 2017), which is considered to be a clinically relevant difference (AAP 1988).

The age at enrolment of some infants in three studies (Lönnerdal & Hernell 1994; Hernell & Lönnerdal 2002; Björmsjö et al. 2021) exceeded the recommendations (see Appendix 2).

The iron saturation of bLf in almost all studies was either not stated or differed substantially to that proposed by the Application. Differences in iron saturation may affect bLf's function, and these studies' findings may not be transferable to the Application's bLf product.

The iron content of all IFP tested was substantially lower (up to 82% lower) than the minimum level prescribed in the Code.

4.2.3 Selected excluded studies' findings

We report below a selection of excluded studies that either represent the closest alignment to the study selection criteria or are studies provided by the Application. This includes: two studies for the comparison with human milk consumption (Hernell & Lönnerdal 2002; Björmsjö et al. 2021); and five studies for the comparison with non-bLf IFP consumption (Hernell & Lönnerdal 2002; Björmsjö et al. 2021; King et al. 2007; Johnston et al. 2015; Li et al. 2019). Selected study characteristics and outcomes are reported in Table 8 (additional study design characteristics and results are provided in section 3.1.3, Table 2).

Table 8 Summary of selected studies

Author (year) Country	Age at enrolment Study duration Sample size	IFP composition[1]	Mean difference in body weight gain (g/day)
Hernell & Lönnerdal (2002) Sweden	Age: 4 ± 2 weeks Study duration: ~5 mo (from age 4 ± 2 weeks to 6 mo) Human milk: n=16 bLf-IFP: n=10 non-bLf-IFP: n=12	*bLf-IFP* bLf: 1 g/L[2] Iron saturation of bLf: 88.6% (calculated from 1.24 mg iron/g protein)[3] Iron: 1.8 mg/L (1.3 g from bLf + 0.5 mg as $FeSO_4$)[4] *Non-bLf-IFP* bLf: 0 g/L (not analysed)[5] Iron saturation of naturally occurring bLf (if present): not stated[3] Iron: 1.6 mg/L (as $FeSO_4$)[4]	Weight gain (g/day from 1 mo to 4 mo) calculated using reported mean weight. bLf-IFP gained 10.4 g/day more, relative to human milk. bLf-IFP gained 7.2 g/day more, relative to non-bLf-IFP. Between group differences from 1 mo to 4 mo were not statistically analysed.
Björmsjö et al. (2021) Sweden	Age: 6 ± 2 weeks Study duration: ~4.5 mo (from age 6 ± 2 weeks to 6 mo) Human milk: n=72 enrolled, 71 analysed at 4 mo, 70 analysed at 6 mo bLf-IFP: n=72 non-bLf-IFP: n=72 enrolled, 71 analysed at 4 and 6 mo	*bLf-IFP* bLf: 1 g/L Iron saturation of bLf: not stated[3] Iron: 2 mg/L[4] *Non-bLf-IFP* bLf: 0 g/L[6] Iron saturation of naturally occurring bLf (if present): not stated[3] Iron: 2 mg/L[4]	Weight gain (g/day from 6 weeks to 4 mo) calculated using reported weight gain (mean; g/day). bLf-IFP gained 1.8 g/day more, relative to human milk. bLf-IFP gained 0.9 g/day less, relative to non-bLf-IFP. Between group differences from 1 mo to 4 mo were not statistically analysed. Authors report that from 6 weeks to 6 mo, the bLf-IFP gained significantly more weight relative to human milk (2.6 g/day more, $P<0.05$); and weight gain of the bLf-IFP was NSD to that of non-bLf-IFP.

King et al. (2007) USA	Age: ≤4 weeks Study duration: ~11 mo (from age ≤4 weeks to 12 mo) bLf-IFP: n=39 enrolled, 13 dropouts, 26 analysed Low-bLf-IFP: n=40 enrolled, 14 dropouts, 26 analysed	*Moderate-bLf-IFP* bLf: 0.85 g/L Iron saturation of bLf: 8.6% (calculated from 120 µg Fe/g bLf)[3] Iron: 3 mg/L[4] *Low-bLf-IFP* bLf: 0.102 g/L[7] Iron saturation of bLf: not stated[3] Iron: 3 mg/L[4]	Weight gain (g/day from 1 mo to 4 mo[8]) calculated using reported mean weight. Moderate-bLf-IFP gained 2.0 g/day more, relative to low-bLf-IFP. Between group differences from 1 mo to 4 mo were not statistically analysed. From birth to 6 mo, there was a trend toward greater weight gain over time for the bLf-IFP group (*P*=0.06).
Johnston et al. (2015) USA	Age: 12-16 days Study duration: until 1 year of age Females: n= 51 (non-bLf-IFP), 80 (low-bLf-IFP), and 63 (bLf-IFP) at 120 days of age. Males: n= 69 (non-bLf-IFP), 58 (low-bLf-IFP), and 55 (bLf-IFP) at 120 days of age.	*Non-bLf-IFP* control bLf: 0 g/L Iron saturation of naturally occurring bLf (if present): not stated[3] Iron: 1.8 mg/L[4] *Low-bLf-IFP* bLf: 0.6 g/L Iron saturation of bLf: not stated[3] Iron: 1.8 mg/L[4] *bLf-IFP* bLf: 1 g/L Iron saturation of bLf: not stated[3] Iron: 1.8 mg/L[4] Investigational formulas contained a prebiotic blend of polydextrose and galactooligosaccharides (total 4 g/L) and reduced arachidonic acid compared with control.	There were no statistically significant differences between groups in weight gain from 14–120 days of age. For males, at 120 days, the 1000 mg bLf/L gained 0.1 g/day more than the control and 600 mg bLf/L group. For females, at 120 days, the 1000 mg bLf/L gained 0.1 g/day more than the 600 mg bLf/L group. For females, at 120 days, the 1000 mg bLf/L gained 0.7 g/day less than the control group.

Li et al. (2019) China	Age: 10-14 days Study duration: 1 year Females: n= 77 (non-bLf-IFP), and 76 (low-bLf-IFP) at 120 days of age. Males: n= 111 (non-bLf-IFP), and 111 (low-bLf-IFP) at 120 days of age.	*Non-bLf-IFP* bLf: 0 g/L Iron saturation of naturally occurring bLf (if present): not stated[3] Iron: 1.0 mg/L[4] *Low-bLf-IFP* bLf: 0.6 g/L + 5000 mg/L milk fat globule membrane Iron saturation of bLf: not stated[3] Iron: 1.0 mg/L[4]	From days 14 to 120, weight gain in the bLf + MFGM group was 0.6 g/day lower in males and 1.1 g/day higher in females, however the differences were not statistically significant.

mo, months; NSD, not significantly different ($P \geq 0.05$).

[1]Other micronutrients levels of IFP are not reported because this did not form part of our study selection criteria (see Appendix 2, section A2.1.1).

[2]Calculated from details provided by the publication: 1.24 mg iron/g protein and bLf providing 1.3 mg iron/L.

[3]The Application's bLf product has a mean iron saturation of ~8.7% and proposes a maximum specification of 15 mg iron/100 g bLf (equivalent to 10.7%). In comparison, the iron saturation of bLf of tested bLf-IFP varies widely, from 8.6% (King et al. 2007) to 88.6% (Hernell & Lönnerdal 2002). We note the iron saturation is not reported for the bLf-IFP tested by Björmsjö et al. (2021), the low-bLf-IFP tested by King et al. (2007), nor for any potentially naturally occurring bLf present in the non-bLf-IFP (Hernell & Lönnerdal 2002 and Björmsjö et al. 2021).

[4]The compositional requirements of Standard 2.9.1 and Schedule 29 of the Food Standards Code list a minimum and maximum iron level of 0.2 and 0.5 mg/100 kJ. Relative to the minimum level (0.2 mg/100 kJ equivalent to 5.5 mg/L based on 264 kJ/100 g and 1050 g/L), the iron content of IFP is 67% lower (bLf-IFP; Hernell & Lönnerdal 2002), 71% lower (non-bLf-IFP; Hernell & Lönnerdal 2002), 64% lower (bLf- and non-bLf-IFP; Björmsjö et al. 2021), 45% lower (bLf- and low-bLf-IFP; King et al. 2007), 67% lower (for all IFP; Johnston et al. 2015), and 82% lower (for both IFP; Li et al. 2019).

[5]IFP was a whey-predominant (60:40) formula, but the study does not report if any naturally occurring bLf was present in the existing non-fortified IFP. We assume that if small amounts of bLf were present, this same amount would also be present in the bLf-IFP (in addition to the reported values).

[6]IFP is reported to contain 0 g bLf/L in the supplementary Table S1 (Björmsjö et al. 2021), however, it is unclear whether this is based on analytical results or inferred from the lack of bLf being added to this IFP. We assume that if small amounts of bLf were present, this same amount would also be present in the bLf-IFP (in addition to the reported values).

[7]We interpret this value as representing the naturally occurring bLf was present in the existing non-fortified bLf IFP (commercial cow-milk based formula).

[8]We calculated body weight gain using the weight when the trial started (1 mo not birth weight).

Hernell & Lönnerdal (2002) measured weight and height (mean ± SD) at one, four, and six months of age. For the comparison between bLf-IFP and human milk consumption: infant weight at enrolment (one month of age) was lower in the bLf-IFP intervention group (n=10) than the human milk-fed comparator (n=16): 4117 ± 445 g versus 4607 ± 757 g. Infant weight at four months of age was higher in the intervention (bLf) group than the human milk-fed comparator: 7298 ± 980 g versus 6852 ± 830 g. These differences did not reach statistical significance ($P \geq 0.05$). Infants consuming bLf-IFP grew faster than infants consuming non-bLf-IFP. They gained a mean of 10.4 g/day more, relative to human milk, between the age of one month and four months (see Table 8), however this difference in weight gain between groups was not detected statistically despite being much greater than the 2.6 g/day difference (Table 8) detected in a subsequent trial, discussed below (Björmsjö et al. 2021). The lack of detection is likely due to the much larger variances in body weight of infants studied by Hernell & Lönnerdal (2002) relative to the mean body weight. A similar pattern was observed for infant height; lower in the intervention (bLf) group at one month, higher at four months, but not statistically significant ($P \geq 0.05$).

For the comparison between bLf-IFP and non-bLf-IFP consumption: infants were heavier in the bLf-IFP intervention group (n=10) than the non-bLf-IFP comparator (n=12) at all time points; for example, 8524 ± 1315 g versus 7721 ± 625 g, respectively, at six months. Infant height was greater in the bLf-IFP intervention group (n=10) than the non-bLf-IFP comparator (n=12) at four and six months of age. These weight and height differences (within an age group) did not reach statistical significance ($P \geq 0.05$). Infants consuming bLf-IFP grew faster than infants consuming non-bLf-IFP. They gained a mean of 7.2 g/day more, relative to non-bLf-IFP, between the ages of one and four months (see Table 8).

Björmsjö et al. (2021) reported nine anthropometric measures: weight; length; head circumference; weight SD score (age-adjusted standard deviation scores); length SD score; head circumference SD score; weight gain (g/day from six weeks to six months only); length gain (mm/day from six weeks to six months); and, head circumference gain (mm/day from six weeks to six months). For the comparison between bLf-IFP and human milk consumption: at four months of age, anthropometric data did not differ between the bLf intervention group (n=72) and the human milk-fed comparator (n=70). However, differences between groups over time, for the weight gain, length gain, and head circumference gain were not analysed at four months. We are unsure about the extent to which complementary feeding contributed energy intake and whether there were differences between groups. To avoid this potential confounder, we considered growth up to four months. Greater weight gain of 2.6 g/day was detected statistically, in infants consuming bLf-IFP compared to human milk between six weeks and six months. For the comparison between bLf-IFP and non-bLf-IFP consumption: at four and six months of age, anthropometric data did not differ between the bLf-IFP intervention group (n=72) and the non-bLf-IFP comparator (n=71) for any measure.

A pilot study by King et al. (2007) reported weight, height and head circumference of infants consuming IFP with a moderate bLf level (850 mg/L) versus a low bLf level (102 mg/L). There were no statistically significant differences in any anthropometric outcome between the groups, at any time point (birth, and one, two, four, and six months of age). There was a trend toward greater weight over time for the moderate-bLf-IFP group for the first six months ($P=0.06$). Weight (kg; mean ± SD) at six months was: 8.24 ± 1.10 kg versus 7.95 ± 1.02 kg for the moderate- and low-bLf-IFP group (both n=26), respectively. The moderate-bLf-IFP group gained 2 g/day more weight compared to the low-bLf-IFP, from one to four months of age. However, this was not statistically analysed.

In the study by Johnston et al. (2015), the two test formulas (containing bLf at 0.6 and 1.0 g/L) also contained an added blend of polydextrose and galactooligosaccharides (4 g/L) that was not added to the control formula. The sample size (~160/group) was chosen to

detect a clinically relevant difference of 3 g/day in weight gain. There were no statistically significant differences between groups in weight gain from 14–120 days of age.

In the study by Li et al. (2019) the test formula contained added bLf (0.6 g/L) and bovine milk fat globule membrane (MFGM; 5 g/L). There were ~190 infants in both the control and test groups by day 120 of the study. From days 14 to 120, weight gain in the bLf + MFGM group was 0.6 g/day lower in males and 1.1 g/day higher in females, however the differences were not statistically significant.

4.3 Bioavailability of bovine versus human lactoferrin

4.3.1 Objective

The objective of this assessment is to determine the extent to which bovine and human lactoferrin are equivalent in terms of their bioavailability.

4.3.2 Methods and results

We reviewed the literature published since date of inception to June 2022. No studies meeting the pre-specified inclusion criteria were identified. Details are provided in Appendix 2.

4.3.3 Selected excluded studies' findings

The Application provided a study by Kawakami & Lönnerdal (1991) which was not retrieved by our search because it is an *in vitro* study. Kawakami & Lönnerdal (1991) investigated the binding of hLf and bLf to brush-border membrane vesicles, taken from small intestines of human foetuses aborted at 22-24 weeks of gestation, to estimate the ability of hLf to deliver iron to infants. The publication states that the preparation of the Lf allowed for 90-100% retention of its iron-binding ability. A substantially lower binding of bLf to brush-border membrane vesicles was observed in comparison to hLf, suggesting a lower relative efficiency of bLf to deliver iron than hLf. To explore the specificity of binding, a competitive binding assay of ^{125}I-labelled hLf to the brush-border membrane vesicles was conducted. Increasing amounts of unlabelled hLf and bLf were used as inhibitors. Unlabelled hLf inhibited the binding of labelled hLf to the brush-border membrane vesicles, but increasingly excess bLf exhibited no inhibition. This demonstrates that the binding of hLf to the intestinal brush-border membrane vesicles is specific. Bovine Lf did not compete with hLf for binding. The authors suggest that bLf binding is probably non-specific and that human intestinal brush-border member lacks a receptor that is specific to bLf.

4.4 The effect of bovine versus human lactoferrin on nutrient bioavailability

4.4.1 Objective

The objective of this assessment is to determine the effect (if any) of consuming IFP with added bLf at the proposed maximum permitted amount (40 mg/100 kJ) compared to consuming human milk on nutrient bioavailability.

4.4.2 Methods and results

We reviewed the literature published since date of inception to June 2022. No studies meeting the pre-specified inclusion criteria were identified. Details are provided in

Appendix 2.

4.4.3 Selected excluded studies' findings

In lieu of relevant evidence, we have reported on a selection of the excluded studies that either represent the closest alignment to the study selection criteria or are studies provided by the Application. This includes: Kawakami & Lönnerdal (1991); Björmsjö et al. (2021); Hernell & Lönnerdal (2002); Chierici et al. (1992); and, Schulz-Lell et al. (1991).

Kawakami & Lönnerdal (1991), discussed above, hypothesised a higher bioavailability of iron bound to hLf compared to bLf.

Björmsjö et al. (2021) found statistically significant differences between the intervention (bLf-IFP) group (n=63-69) and the human milk-fed group (n=65-69) at four months of age in mean cell volume (higher in intervention) and ferritin (lower in intervention). At six months of age, hepcidin was higher in the intervention group (n=65-67; $P<0.05$) compared to the human milk-fed group (n=65-67). Other parameters did not significantly differ, including haemoglobin, iron, transferrin, transferrin saturation, transferrin receptor, iron depletion, iron deficiency, iron deficiency anaemia, mean cell volume and ferritin (at six months of age), and hepcidin (at four months of age only).

Chierici et al. (1992) measured haemoglobin, haematocrit, serum iron, serum ferritin, and serum zinc at multiple time points (at 0, 7, 30, 90 and 150 days of age). Of the two bLf-IFP intervention groups, we report the results of the group with the higher bLf concentration (1 g bLf/L). Serum iron was higher in the bLf-IFP intervention group (n=14) compared to the human milk-fed group (n=10) at day 30 ($P=0.041$). Median values were 22 and 17 µmol/L, respectively. Serum ferritin was lower in the bLf-IFP intervention group compared to the human milk-fed comparator at day 30 ($P<0.05$; values are provided in a figure and were not extracted for the current assessment). In addition to the limitations described in Appendix 2, section A2.4.1, the trial by Chierici et al. (1992) was not randomised, which increases the level of uncertainty in its findings.

Hernell & Lönnerdal (2002) reported no significant differences in haemoglobin, mean corpuscular volume, serum iron, total-iron-binding capacity, serum ferritin, serum transferrin receptor, serum zinc or serum copper at four and six months of age, after correction for initial differences at one month of age, between the bLf-IFP intervention group (n=10) and the human milk-fed comparator (n=16).

Schulz-Lell et al. (1991) conducted iron balance studies in infants from their third to 17th week of life. Infants consuming either a bLf-IFP containing 100 mg bLf/100 mL and 1060 µg iron/L (n=7) or a non-bLf-IFP containing 770 µg iron/L (n=9). The bLf-IFP group received 169 µg iron/kg body weight (BW)/day and retained 63 µg iron/kg BW/day. The non-bLf-IFP group received 118 µg iron/kg BW/day and retained 43 µg iron/kg BW/day. The mean percentage retention of iron in the bLf-IFP and non-bLf-IFP groups were 36% and 28%, respectively, however this was not statistically significant.

4.5 Discussion and conclusion

The Application describes five trials investigating the growth and development of healthy term or near-term infants consuming IFP with added bLf at various levels. As discussed in section 4.2, these trials had a number of limitations. FSANZ did not locate studies in the published literature that did not have one or more of these limitations.

King et al. (2007) was a double-blind, randomised, controlled trial (RCT) with 26 infants in each of the bLf and control formula groups. Bovine Lf concentrations in the test and control

formulas were 820 and 102 mg/L, respectively. Mean body weight gain from one to four months (i.e. before the introduction of complementary feeding) was 2.0 g/day greater in the bLf group, however statistical significance testing was not conducted.

In a double-blind RCT described by Johnston et al. (2015), the two test formulas (containing bLf at 0.6 and 1.0 g/L) also contained an added blend of polydextrose and galactooligosaccharides (4 g/L) that was not added to the control formula. The sample size (~160/group) was chosen to detect a clinically relevant difference of 3 g/day in weight gain. There were no statistically significant differences between groups in weight gain from 14–120 days of age.

Li et al. (2019) describe a double-blind RCT in which the test formula contained added bLf (0.6 g/L) and bovine milk fat globule membrane (MFGM; 5 g/L). There were ~190 infants in both the control and test groups by day 120 of the study. From days 14 to 120, weight gain in the bLf + MFGM group was 0.6 g/day lower in males and 1.1 g/day higher in females, however the differences were not statistically significant.

Two trials included a human milk-fed reference group (Hernell & Lönnerdal 2002; Björmsjö et al. 2021). The desired growth trajectory for formula-fed infants is one similar to that of a concurrent human milk-fed reference group. Compositional differences between human milk and infant formula, including temporal changes in human milk composition, contribute to differences in growth trajectories observed in trials.

Hernell & Lönnerdal (2002) describe a small single-blind study with 10 to 12 infants in the formula groups and 16 in the breastfed group. Mean bodyweight gain from one month to four months was 7.2 and 10.4 g/day greater in the bLf group compared to the non-bLf and human milk-fed groups, respectively. These differences were not statistically significant.

Björmsjö et al. (2021) describe a double blind RCT investigating the growth of infants (n=33−72/group) fed formula containing: (i) bLf (1 g/L) + iron (2 mg/L); (ii) no added bLf + iron (2 mg/L); or, (iii) no added bLf + iron (8 mg/L). A group of 70 infants served as a breastfed reference. Mean body weight gain from six weeks to four months was 1.8 g/day greater in the bLf group compared to the human milk-fed group, but was 0.9 and 1.8 g/day lower relative to groups (ii) and (iii). Statistical significance testing was only reported relative to the human milk-fed group, with no differences evident. There were no statistically significant differences in iron status indicators.

Despite limitations in the available studies, the observed differences in weight gain in four of the five studies were less than 3 g/day, a value considered to be the clinically relevant threshold (AAP 1988). It is therefore concluded that consumption of infant formula with added bLf, at up to 1 g/L (equivalent to 40 mg/100 kJ), is unlikely to adversely affect infant growth and development.

5 Beneficial health effects assessment

The applicant proposes that bLf added to infant formula is likely to have a beneficial role in the growth and development of infants similar to that carried out by human lactoferrin present in the diet of breastfeeding infants. In particular, they suggest that bLf will have the specific health outcome of reducing the risk of infection through:

- antibacterial and/or bacteriostatic effects
- an anti-viral effect
- an immunomodulatory effect
- reducing the severity and duration of infection.

This assessment considers the weight of evidence derived from *in vitro*, *ex vivo* and animal studies, which provide evidence about possible mechanisms by which the beneficial effect might be achieved, as well as evidence generated through human intervention and observational studies.

5.1 Antibacterial and/or bacteriostatic effects

Lactoferrins are known to exert antimicrobial effects by at least two distinct mechanisms: a bacteriostatic effect related to sequestration of iron; and a bactericidal effect mediated through binding to cell surface molecules and causing damage to cell membranes.
Iron sequestration leading to inhibition of bacterial growth was first demonstrated by Bullen et al. (1972). They demonstrated that inhibition of the growth of a strain of enteropathogenic *Escherichia coli* 0111 by human lactoferrin was negatively correlated with the degree of iron saturation, and could be abolished by addition of free ferric ions. They also reported that bovine colostrum—known to have high levels of lactoferrin (up to 2 mg/mL: Tsuji et al., 1990)—exerted a similar bacteriostatic effect, although they did not directly demonstrate a role for bLf. Similar Fe-sensitive bacteriostatic effects of hLf and/or bLf have been reported for a variety of microorganisms, including *Staphylococcus aureus*, clostridia and Enterobacteriaceae, (Aguila et al., 2001; Teraguchi et al., 1993;1995; Lonnerdahl et al., 2020).

A direct bactericidal effect of apo-hLf against a wide variety of microorganisms was demonstrated by Arnold et al. (1980). Micromolar concentrations of hLf typically resulted in greater than a 3 log reduction of viable colony forming units within 1 hour of exposure to apo-hLf. Lactoferrin sensitivity was observed for Gram-positive and Gram-negative bacteria; rods and cocci; aerotolerant anaerobes, facultative anaerobes and strict aerobes; and *Candida albicans*.

Ellison et al. (1988; 1990) showed that hLf causes the release of lipopolysaccharide (LPS) from the outer membrane of Gram-negative bacteria (*E. coli* and *Salmonella* Typhimurium) in a dose-dependent manner and at a level comparable to that caused by the chelating agent, EDTA. The release of LPS was blocked by addition of Fe^{3+} ions and by low levels of the divalent cations Ca^{2+} and Mg^{2+}, which are known to stabilise the bacterial outer membrane by binding to LPS. Damage to the bacterial membrane was demonstrated by the concomitant increase in the antibacterial effect of an otherwise sub-inhibitory concentration of the antibiotic rifampicin, which is normally excluded from cell entry by the outer membrane.

While also able to be blocked by addition of free ferric ions, the bactericidal effect was shown to be independent of the iron sequestration function of lactoferrin, and is related to direct interaction of the N-terminal domain of the protein with cell surface structures.
Applemelk et al. (1994) demonstrated that hLf bound to the lipid A region of LPS from a range of human pathogens with an affinity constant of around 2 nM. Elass-Rochard et al (1995) subsequently showed that the bactericidal effect of hLf was mediated by this direct binding interaction between the N-terminal domain of hLf and LPS. The N-terminal domain bound LPS with a binding constant of around 3.6 nM. They also demonstrated that there was a second, low-affinity site, in the C-terminal domain, which had a binding constant around 390 nM. They further demonstrated that bLf also contained two LPS-binding sites, with similar affinities to those of hLf, and showed that an N-terminal fragment of bLf inhibited the binding of hLf to LPS.

It should also be noted that N-terminal fragments of bLf, termed lactoferricin and lactoferrampin—which can be generated through peptic digestion in the stomach—have been described as having similar or greater antibacterial and/or antiviral activity to the parent protein both *in vitro* and *in vivo* (Gifford et al., 2005; van der kraan et al., 2005; Vogel 2012).

The relevance of these *in vitro* effects of lactoferrins to the potential for bLf to inhibit colonisation and/or infection by human intestinal pathogens *in vivo* can be seen in studies by Teraguchi and colleagues on populations of faecal Enterobacteriaceae in mice fed a diet of either bovine milk or ordinary solid commercial pellets (Teraguchi et al., 1993; 1995).

Across a four week period, the number of faecal Enterobacteriaceae (expressed as CFU/g faeces) remained fairly constant for mice on the commercial pellet diet, regardless of whether or not it was supplemented with bLf (iron saturation 14.5%). However, on a bovine milk diet, levels of faecal Enterobacteriaceae increased considerably. Supplementation of the milk diet with 5% bLf between days 8 to 21 reduced the levels of Enterobacteriaceae almost to baseline, but they rose again quickly on cessation of supplementation after day 21. In contrast to other reports of the iron-sensitivity of the antibacterial properties of lactoferrins, there was little effect of the degree of iron saturation (between 2.3% to 97.6%) on the ability of bLf to suppress the growth of Enterobacteriaceae in milk-fed mice (Teraguchi et al., 1993).

In subsequent experiments, Teraguchi et al. (1995) investigated the effect of bLf and a pepsin hydrolysate of bLf (bLfH) on milk-fed mice that had been orally inoculated with strains of *Clostridium* spp., including human strains of *C. difficile* and *C. perfringens*. Initial *in vitro* studies determined that bLfH had at least an 8-fold lower minimal inhibitory concentration than bLf for many of the clostridial strains, in line with some other studies on lactoferricin and lactoferrampin (see above). Intestinal colonisation by a strain of *C. ramosum* administered at a dose of 10^5 or 10^7 CFU persisted for the 14 days of the experiment in milk-fed mice, but only transient residence of the strain was observed in pellet-fed mice, 1 day after inoculation. Administration of 2% bLf in the milk-fed diet after day 7 resulted in a reduction in faecal levels of *C. ramosum* and some commensals, while levels of Bifidobacteria did not change. In a separate experiment, the diet of milk fed mice was supplemented with 2% bLf for seven days prior to, and 7 days after inoculation with six clostridial strains (separately). Compared to the unsupplemented milk diet, bLf significantly reduced the levels and/or incidence of four of the six strains in faeces tested seven days post-inoculation. The authors conclude that these results demonstrate a bacteriostatic effect of bLf supplementation, although it is not clear that they could not also be explained by a direct bactericidal effect of bLf *in vivo*.

5.2 Anti-viral effect

As with the antibacterial effects discussed above, the evidence for an antiviral effect of bLf is mainly derived from *in vitro* studies.

An indirect protective effect of subcutaneous injection of hLf against the severity of viral disease in mice inoculated with the polycythemia-inducing strain of the Friend virus complex was reported by Lu et al. (1987; 1991). Subsequently, Hasegawa et al. (1994) examined the effect of hLf and bLf on infection and replication of the human herpes viruses, herpes simplex virus-1 (HSV1) and cytomegalovirus (CMV), in human embryo lung cell tissue culture. They demonstrated that pre-incubation of cell cultures with 0.5–1 mg/mL lactoferrin resulted in 3- and 6-log reduction in viral titres of HSV-1 and CMV, respectively, ten days after infection. This effect was shown to be related to the protein component of the lactoferrins, and not due to iron or to sialic acid residues of their glycan moieties. It was also shown that the inhibitory effect of lactoferrin was mediated by a cell-binding interaction, rather than by viral binding, leading to inhibition of viral adsorption and/or penetration of the host cells.

Superti and colleagues investigated the ability of bLf to inhibit rotarial infection of a human colon enterocyte cell line, HT-29 (Superti et al., 1997; 2001). They showed dose-dependent inhibition of viral attachment to cellular receptors mediated by bLf binding to viral particles. bLf also inhibited viral antigen synthesis and viral yield when added during or shortly after viral infection, implying a further intracellular role for bLf in its antiviral activity. The antiviral

activity was shown to be independent of the state of iron saturation of bLf, and was not due to iron or to sialic acid residues, in agreement with the results reported by Hasegawa et al. (1994).

Other *in vitro* studies have also demonstrated that the antiviral activity of hLf and/or bLf is mediated by binding to either cell- or virus-surface structures and interfering with viral attachment and uptake into cells (Portelli et al., 1998; Clarke and May, 2000; Arnold et al., 2002; Pietrantoni et al., 2003; Yamamoto et al., 2010). Several studies have identified lactoferrin binding to cell-surface glycosaminoglycan viral-recognition and/or receptor molecules as a critical step in inhibition of viral binding (Pietrantoni et al., 2003; Berlutti et al., 2011; Denani et al., 2021). In cases where comparisons have been made, bLf is usually shown to have stronger antiviral activity than hLf (Hasegawa et al. 1994; Arnold et al., 2002; Berlutti et al., 2011).

5.3 Immunomodulatory effect

Cell culture experiments and studies conducted in mammalian cells or organs *ex vivo* demonstrate that ingested bLf has a complex array of effects on the host innate and adaptive immune responses in the gut lumen beyond the generation of a specific antibody response to the protein. There is compelling evidence that these effects are mediated by internalisation of bLf through interactions with specific gut epithelial cell surface receptor molecules.

Receptors for lactoferrins have been reported in the small intestines of many mammalian species (Suzuki et al., 2005). Cross-species receptor binding and internalisation has been observed in many instances, presumably enabled by the evolutionary conservation of lactoferrin protein sequences and structure (Demmelmair et al., 2017). This has enabled the study of immune responses to dietary bLf in a number of species, including mice, rats and pigs. It should also be noted that bacterial LPS, which strongly binds to bLf, interferes with many of its receptor-mediated functions, most likely through steric hindrance of receptor binding (Miyazawa et al., 1991).

The immunomodulatory effects of bLf in animal and cell culture studies is observed as a variety of effects on cellular proliferation, antibody production, and cytokine gene expression, production and/or secretion.

Induction of antibody production and increased antibody levels in the gut lumen were described by Debbabi et al. (1998) in mice fed bLf daily for four weeks at two dose levels, with water as a control. In mice given bLf, specific anti-bLf IgA and IgG were observed in intestinal fluids and serum. However, increases in total immunoglobulin levels were only observed in intestinal fluids and spleen, but not serum, implying a separate activation of the mucosal immune system by bLf. IgA has a key role in prevention of pathogen attachment to the gut epithelium, as well as in prevention of inflammatory damage and maintenance of intestinal homeostasis (Murphy et al., 2008).

Similarly, Arciniega-Martinez et al. (2015) observed increased levels of IgA and IgM in the distal small intestine in mice fed bLf at a single dose for four weeks, compared to water as control. This effect was accompanied by up-regulation of IgA^+ and IgM^+ plasma cells in mucosal immune inductor (eg Peyer's patches) and effector (eg lamina propria) regions. Analysis of cytokine responses indicated time- and site-dependency, with the proportion of $CD3^+/CD4^+$ T cells producing various pro-inflammatory or IgA-inducing cytokines rising and falling across the 28 days of bLf administration. Depending on the cytokine, changes in Peyer's patches and the lamina propria were either reinforcing or opposite in effect at any particular time point. The impact of this on the efficacy of an immune response to challenge by a pathogen was not assessed.

These waves of pro-inflammatory and inhibitory cytokine production, allied with a bLf induced increase in IgA production, were also observed in follow-up experiments reported by Ynga-Durand et al. (2021).These effects, were modulated by various cytokine-producing cells. They show complex dynamics across experimental timeframes, and differ in detail and scale between the proximal and distal small gut, and between mucosal immune inductor (e.g. Peyer's patches) and effector (e.g. lamina propria) regions.

Other cell culture and animal studies have demonstrated similar dual effects of bLf on pro-inflammatory and inhibitory cytokine production in the mucosal immune system.

For example, Takakura et al. (2006), observed enhanced production of interferon gamma (IFN-γ) and interleukin-10 (IL-10) by T cell populations isolated from mice after three days of oral administration of bLf (compared to bovine serum albumin as control). Intestinal intra-epithelial lymphocytes and mesenteric lymph node cells, isolated 24 hours after the last bLf dose, were assayed for cytokine production with or without T-cell receptor stimulation. IFN-γ and IL-10 are considered to be inflammatory (T helper type 1; T_H1) and anti-inflammatory (T helper type 2; T_H2) cytokines, respectively.

Lonnerdahl et al. (2011; 2020) observed similar, apparently antagonistic immunomodulatory effects of bLf in Caco-2 cell culture experiments. They observed enhanced secretion of the inflammatory cytokine, IL-18, on treatment with iron-saturated (holo-) bLf, while partially saturated (native-) bLf induced expression of transforming growth factor β1 (TGF-β1), an inhibitory cytokine (Lonnerdahl et al., 2011). Both these effects were also seen in an analysis of the function of a number of commercially available samples of bLf (all partially iron-saturated), including that produced by the applicant (Lonnerdahl et al., 2020). Interestingly, Takakura et al. (2006) noted that the inhibitory cytokine IL-10, in the presence of IL-18, can also enhance inflammatory response indicators such as the cytotoxic response, natural killer cell proliferation and production of IFN-γ.

These studies demonstrate that the immunomodulatory effects of bLf are complex, with time- and site-dependency observed, but without complete explanation for causes or consequences of these dynamics. In addition, studies vary in the degree to which they control for confounding factors, such as the prior or concurrent exposure of test animals to pathogens or other immune-stimulatory factors. While the effects point to some immunomodulatory mechanisms by which bLf might have a beneficial health effect—such as inducing an increase in IgA levels in the gut lumen—no clear conclusions can be drawn from these studies as to the potential for bLf to reduce the risk of infection in infants consuming bLf supplemented formula.

5.4 Reduced severity and duration of infection

Several animal studies have investigated the potential for prevention of gastrointestinal and other infections by dietary/oral lactoferrins, including bLf.

Kawasaki et al. (2000) investigated the binding of two strains of enterotoxigenic *E. coli* (ETEC) to the intestinal tract of mice provided a 10 mg/mL solution of bLf *ad libitum* in place of drinking water. On day seven of bLf exposure, ETEC was administered by oral gavage (dose unknown). Periodic assessment of ETEC bound to colon, jejunum/ileum and duodenum over the following 30 days consistently showed 1-2 log lower counts for the bLf--treated animals.

These results were consistent with the results of *in vitro* experiments assessing the effect of bLf on the adherence of several strains of ETEC and enteropathogenic *E. coli* (EPEC) to a human epithelial cell line, JTC-17 (Kawasaki et al., 2000). Partially iron-saturated (30%) bLf at 16mg/mL showed 87–100% inhibition of binding by eight strains of *E. coli* to the cell line.

Further analysis of the inhibition of binding of one ETEC strain showed 50% inhibition at around 1-2 mg/mL bLF, and even stronger inhibition by holo-bLf, in contrast to other studies showing that iron binding reduced the antibacterial effects of lactoferrins.

Mosquito et al. (2010) examined the protective effect of bLf (15% iron saturated) on mice exposed to $3x10^6$ CFU *Salmonella* Typhimurium C52 by oral gavage two hours after *ad libitum* access to 10 mg/mL bLf in drinking water. bLf administration continued for 7 days post-infection, before animals were sacrificed for histopathologic analysis. Compared to controls, bLf group animals showed reduced weight loss, mortality and clinical signs of infection through the seven days post-infection, and had lower rates of bacteraemia and fewer histopathological abnormalities.

Similar results were reported by Drago-Serrano et al. (2010) for mice treated with 5 mg or 100 mg bLf daily for seven days preceding, and 21 days after, infection with either 10^4 CFU (sub-lethal dose) or 10^8 CFU (lethal dose) of *Salmonella* Typhimurium intragastrically. Both bLf doses decreased mortality to a similar extent in the lethal dose experiment (80% survival at day 14 post-injection, cf 40% for control animals). For the sub-lethal dose experiment, both bLf doses showed antibacterial and immunomodulatory effects. Antibacterial effects included reduced bacterial shedding in faeces and in intestinal fluids; reduced colonization of Peyer's patches; and reduced translocation of *Salmonella* to liver and spleen. Immune effects included an increased antibody response, with higher serum levels of total IgG and IgM, and higher levels of IgA in intestinal secretions.

Chen et al. (2008) investigated the effect of porcine lactoferrin (pLf), expressed in the milk of transgenic mice, on suckling mice infected with human enterovirus 71 (EV71) on the 4^{th} day of life. Compared to mice ingesting non-transgenic milk, pLf-ingesting mice showed higher body weights and increased rates of survival, implying a direct effect of pLf in inhibiting EV71 infection.

There are few reports demonstrating anti-infectious activity of lactoferrins in organs besides the intestinal tract.

Bhimani et al. (1999) showed a weak anti-staphylococcal activity of apo-hLf and apo-bLf *in vivo*. They observed that both intravenous and oral bLf administered one day before injection of a sub-lethal dose of *S. aureus* reduced bacterial loads in infected kidneys by about 1 log CFU, as well as reducing the incidence of kidney infection. However, Shin et al. (2005) showed very little effect of oral bLf on lung infection in a mouse model of influenza, with no effect on viral titre in bronchoalveolar fluid, and a small reduction in infiltration of inflammatory cells into lung tissue.

Reznikov et al (2018) compared the effects of bLf versus whey protein control in formula-fed piglets, as well as any effect of added *Bifidobacterium infantis* as probiotic. Piglets received 4 g/day bLf or whey, ± 10^9 CFU/day *B. infantis* by oral gavage. On day 7, they were injected intravenously with *S. aureus*, 10^5 CFU/kg body weight, and assessed for a further 5 days. The bLf cohort showed increased weight gain and lower *S. aureus* counts in the kidney, with a tendency to lower counts in heart and lung. There was no synergistic effect of *B. infantis* with bLf. Immunological effects included reduced circulating B cells and monocytes; reduced kidney IL-10, and increased IFN-γ mRNA in lung tissue, indicating a pro-inflammatory response to bLf.

5.5 Human intervention studies

Four human intervention studies were identified assessing the effects of bLf on risk of infection in healthy term infants.

In a small study in the USA (52 infants across treatment and control groups), King et al. (2007) compared control formula naturally containing 10.2 mg bLf per 100 mL with formula supplemented with 85 mg bLf per 100 mL. Infants were enrolled at 0-4 weeks of age and followed for the first year of life. They observed similar rates of adverse events and dropouts between bLf and control groups, and found significantly lower incidence of lower respiratory tract infections in the bLf-fed infants. The study did not include a breastfed reference group.

In a larger study in China, Chen et al. (2016) considered the effect of the addition of 38 mg bLf per 100g (ca 57mg/L made up formula) infant formula powder (n=115), compared to formula without bLf (n=98), and with a breastfed reference group (n=103). Infants had been breastfed to 4-6 months of age, then weaned before starting on the formula diet for 3 months. Breastfed and bLf-containing formula-fed infants had similar health outcomes, and both groups had significantly fewer respiratory and diarrhoea-related illnesses, and shorter duration of those illnesses, compared to the control formula group. A smaller follow up study by the same group (Chen et al. 2021), which was provided by the applicant, has not been considered, as infants were anaemic; had been breastfed to 6-9 months of age, then weaned before starting on the formula diet for 3 months; and the study lacked a breastfed reference group.

A study in Sweden by Björmso et al. (2022) investigated the effect of the addition of 1000 mg bLf per litre formula (n=72), compared to low- (n=71) and high-iron (n=33) formulas without added bLf, and with a breastfed reference group (n=70). Infants were enrolled at 4-8 weeks of age and followed until 6 months of age. No significant effects of bLf supplementation on infection-related morbidity were reported. Cytokine levels in venous blood were also assessed at inclusion and at 4-monthly intervals until 12 months of age. No significant differences between groups were reported in levels of cytokines.

5.6 Conclusions of beneficial health effects assessment

Evidence from *in vitro* studies demonstrate that bLf and other lactoferrins exhibit strong bacteriostatic, bactericidal and antiviral effects. The bacteriostatic effect is principally mediated through iron-sequestration, denying bacteria access to a necessary mineral for growth. Direct bactericidal effects are mediated through binding to cell surface molecules, leading to membrane disruption and leakage. Antiviral effects are mainly mediated through binding to viral particles and/or receptors on target cells, hindering viral adsorption and/or internalisation.

These *in vitro* effects are supported by animal studies demonstrating the ability of bLf to interfere with pathogen adherence to intestinal epithelia, and reductions in bacterial loads and disease severity in experimentally-infected animals. These effects go beyond the gastrointestinal tract, with effects seen on bacterial load in kidney infections due to inoculation of mice with *S. aureus*.

A large body of evidence from *in vitro* studies also supports an immunomodulatory effect of bLf, particularly on the mucosal immune system. These effects follow internalisation of bLf, mediated by interactions with specific gut epithelial cell surface receptor molecules, and include cellular proliferation; antibody production; and cytokine gene expression, production and secretion. However, while they imply immunomodulatory mechanisms by which bLf might function to reduce infection—such as inducing an increase in IgA levels in the gut lumen—the studies do not demonstrate clear links to that outcome.

Evidence from human studies of the ability of bLf to reduce infection in healthy, full-term infants is limited, of low quality, and does not provide obvious links to the mechanistic evidence from *in vitro* and animal studies. However, the studies do demonstrate a tendency for bLf-supplemented formula to reduce reported gastrointestinal and respiratory illnesses

compared to control formula.

Considering all these lines of evidence together, FSANZ concludes that there is a strong evidence base from *in vitro* and animal studies providing a mechanism by which bLf can reduce the risk of bacterial and viral infection through direct (e.g. binding interactions) and indirect (e.g. through immune stimulation) effects. bLf has been shown to reduce the severity and duration of infection in relevant animal models of infection. FSANZ identified no human studies that could provide strong evidence in support of the proposed beneficial effect, but results of the few studies considered are consistent with a reduction in risk of infection.

6 Conclusions from the risk and technical assessment

FSANZ has undertaken an assessment of the food technology aspects, safety, nutritional impact and beneficial health effects of the addition of bLf to IFP.

bLf is a protein naturally present at low levels in cow's milk. It shares 69% amino acid sequence homology with hLf, found in human milk. Information reviewed in the food technology assessment demonstrates that bLf is sufficiently characterised, and confirms its stability in IFP. Specifications have been proposed for inclusion in Schedule 3 of the Code.

The safety assessment concluded there are no toxicological safety concerns from the addition of bLf to IFP at the proposed concentrations.

bLf is subject to partial hydrolysis in the stomach and small intestine, but a proportion resists digestion and is excreted in the faeces. Some fragments produced by partial hydrolysis also resist further digestion and are excreted in the faeces. In addition, a small proportion of intact bLf and its fragments is absorbed into the systemic circulation and excreted via the urine.

bLf is of low acute toxicity, with no adverse effects observed following oral administration to rats up to 2000 mg/kg bw. It was not mutagenic *in vitro*. No adverse effects were observed in a 13-week oral gavage toxicity study in rats at doses up to 2000 mg/kg bw/day, the highest dose tested.

No adverse effects of bLf have been reported in multiple intervention studies in infants, including the highly vulnerable group of preterm and very low birth weight infants. bLf concentrations up to 1000 mg/L formula were tested in the studies in term infants, while the doses tested in preterm and very low birth weight infants ranged from 100 – 300 mg/kg bw/day. These doses were estimated as being equivalent to bLf concentrations ranging from 370 – 3704 mg/L.

The first bLf-fortified IFP were released for sale overseas in 1986 and FSANZ is not aware of any adverse events related to consumption of these products in markets where they are available. The Applicant has also indicated that its post-marketing surveillance overseas, and that of international formula brand owners it supplies, has not identified any complaints or adverse events related to the addition of bLf.

Based on the maximum permitted amount proposed by the Applicant, the estimated mean and P90 intakes of bLf from infant formula and follow-on formula range between 0.59 and 1.8 g/day (rounded DEA estimates; equal to 70 – 270 mg/kg bw/day). These intakes are less than the estimated mean and P90 intakes of hLf from human milk of 0.7 to 5.0 g/day and approximately 10 – 30-fold lower than the no observed adverse effect level of 2000 mg/kg bw/day from the 13-week toxicity study of bLf in rats.

bLf is derived from cow's milk which is a major food allergen. Some individuals with cow's milk allergy have IgE antibodies to bLf indicating sensitisation, but the clinical significance of this has not been confirmed and bLf is not currently listed as a cow's milk allergen by the WHO/IUIS. The limited available evidence however is insufficient to conclude that bLf does not pose a food allergy risk to consumers with cow's milk allergy.

No additional microbiological safety risks arise from addition of bLf to powdered infant formula products and its preparation and consumption beyond those encountered with IFP that is not supplemented with bLf.

Several double-blind RCTs have investigated the potential for bLf to affect infant growth and development. Despite limitations in the available studies, observed differences in weight gain were less than the clinically relevant threshold of 3 g/day. It is concluded that consumption of infant formula with added bLf, at up to 1 g/L (equivalent to 40 mg/100 kJ), is unlikely to adversely affect infant growth and development. Infant iron status, investigated in one of these RCTs, was unaffected by bLf addition to infant formula.

In terms of beneficial effects, the weight of evidence suggests a plausible mechanism by which bLf can reduce the risk of bacterial and viral infection. bLf has been shown to reduce the severity and duration of infection in relevant animal infection models. The few relevant human studies provided weak but consistent support for the proposed beneficial effect.

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Appendix 1: How the infant model diets were constructed

Children aged 3 and 9 months

As there are no data available from the 2011-12 Australian National Nutrition and Physical Activity Survey or the 2002 New Zealand National Children's Nutrition Survey for children less than two years of age, model diets were constructed to estimate the bLf and hLf intakes for children aged 3 months and 9 months. The same model diets were used for Australia and New Zealand.

As the 3 month and 9 month old infant model diets are based on mean food consumption amounts only, a distribution of food consumption was not available, and hence, a distribution of the intake of bLf and/or hLf was not able to be produced. Therefore, the 90th percentile dietary intakes were estimated using the calculation shown in Equation 1.

Equation 1: 90th percentile dietary exposure calculation for the 3 month and 9 month old infant model diets

$$90^{th} \text{ percentile exposure} = \text{mean exposure} \times 2^{*}$$

* (World Health Organization et al.,1985)

The energy content of human milk and infant formula is required for the calculation of the dietary intake of hLf and bLf in the model diets for 3 month and 9 month old infants. AUSNUT 2011-13 (the nutrient dataset for the 2011-12 National Nutrition and Physical Activity Survey (NNPAS)) is the latest survey specific nutrient data set published for Australian foods. In this dataset, the energy content of *Milk, human/breast, mature, fluid* is 286 kJ/100 g and for *Infant formula, 6-12 months, prepared with water* is 264 kJ/100 g (FSANZ, 2016). This assessment examined a range of infant formula and follow-on formula products currently available on the market and found the energy content to be in the range of 252-295 kJ/100g. A set of model diets were developed using the AUSNUT energy contents for human milk and infant formula in the calculation of the dietary intake of hLf and bLf for 3 month and 9 month old infants.

A set of model diets was not established for infants consuming infant formula products for special dietary uses as the energy and/or fluid requirements can vary depending on the medical conditions of the infant. Additionally, the energy content of the various infant formula products for special dietary uses can be variable. The assessment of A1155 included an examination of products, including formulas for premature infants, formulas for use by infants with inborn errors of metabolism, and formulas for use by infants with severe food allergies, which found the range of energy contents was 269 – 415 kJ/100 g. If an infant consuming infant formula products for special dietary uses has similar energy requirements to those used in the model infant diets and their specific formula has a similar energy content to that used in the model diets, then their intake of bLf is anticipated to be similar to that outlined in the assessment for this application. If an infant consuming infant formula products for special dietary uses has similar energy requirements to those used in the model infant diets and their specific formula has a higher energy content to that used in the model diets, then their intake of bLf is anticipated to be similar to or lower than that outlined in this assessment.

Infants aged 3 months

The recommended energy intake for a three-month-old boy (343 kJ/kg bw/day) (United Nations University et al. 2004) and the 50th percentile weight (6.4 kg) (World Health Organisation 2006) for the same age and sex were used as the basis for the model diet. Boys' weights were used because boys tend to be heavier than girls at the same age and therefore have higher overall energy and food requirements. The entire energy requirement in the 3 month old infant diet is derived from infant formula or human milk, depending on the assessment. The body weight of 6.4 kg was used to estimate dietary intakes for 3 month old infants on a body weight basis.

Infants aged 9 months

By the age of 9 months, infants are consuming a mixed diet of solids and follow-on formula/ human milk. The model diet was constructed based on recommended energy intakes, mean body weight and the proportion of milk and solid foods in the diet for a 9 month old infant. The recommended energy intake for a 9 month old boy (330 kJ/kg bw/day) (United Nations University et al. 2004) and the 50th percentile weight (8.9 kg) (World Health Organisation 2006) for the same age and sex was used as the basis for the model diet. The body weight of 8.9 kg was used to estimate dietary intakes for 9 month old infants on a body weight basis.

It was assumed that 50% of energy intake was derived from follow-on formula/human milk and 50% from solids and other fluids (Butte et al, 2004; Hitchcock 1986; Pan American Health Organization, 2003). As noted in the section discussing concentrations of bLf in other dairy based foods, the concentrations are minimal compared to human milk or infant formula if permitted at the proposed maximum permitted amount. An estimated intake of bLf from cows' milk based on mean consumption as reported for 2-3 year old children in the risk assessment for A1555 was used for 9 month old infants.

Appendix 2: Nutrition assessment literature screening

A2.1 Effect on growth and development: comparison to human-milk fed infants

A2.1.1 Methods and results

We reviewed the literature published since date of inception to June 2022. On 6th July 2022, we searched PubMed using ("infan*"[All Fields] AND ("food, formulated"[MeSH Terms] OR ("food"[All Fields] AND "formulated"[All Fields]) OR "formulated food"[All Fields] OR "formula"[All Fields] OR "formula s"[All Fields] OR "formulae"[All Fields] OR "formulas"[All Fields]) AND ("lactoferrin"[MeSH Terms] OR "lactoferrin"[All Fields] OR "lactoferrins"[All Fields] OR "lactoferrin s"[All Fields])) and filtered results by studies in humans, published in English, available in full text, and article type (clinical trial, controlled clinical trial, meta-analysis, randomised controlled trial, review, and systematic review). We identified primary research by screening individual publications against inclusion criteria (Table A.1) and included moderate or high quality reviews of such primary research. For time efficiency, we did not check studies' non-compliance with any of the compositional requirements of Standard 2.9.1 other than iron, or the pH of IFP (which may affect bLf's ability to bind iron). As we did not exclude studies on this basis, included studies may have used non-compliant IFP.

Majka et al. (2020) do not report the impact of varying iron saturation on lactoferrin's ability to affect nutritional, growth and development outcomes in infants. it is possible that differences in the saturation of lactoferrin (with iron or other metals) affect nutrient bioavailability and potentially, infants' nutritional status. For example, Troost et al. (2001) found that the amount of intact bLf entering the small intestine of adult humans (as a percentage of the amount intragastrically administered) was higher, with bLf of a higher iron saturation (79% versus 62% after consumption of the 100% and ~20% iron-saturated bLf, respectively). The holo-bLf tended to be more resistant to degradation than the apo-bLf, although this difference did not reach statistical significance (P=0.09). This supports the plausibility that the iron saturation or iron-binding capacity of bLf may affect nutrition-related outcomes. This informs the 'intervention' criteria below (Table A.1).

Table A.1 PICOTS criteria for study selection

Population	All apparently healthy term infants aged one month or less at enrolment, and followed up for up to 12 months.[1]
Intervention	Consumption of a bLf-supplemented IFP (exclusively BMS fed at enrolment).[2,3]
Comparator	Exclusive consumption of human milk.[3]
Outcome	Any growth and development outcome[4] (other than those considered in the toxicological and microbiological assessments; sections 3.1 and 3.2, respectively).
Time	Minimum three to four months duration for growth and development outcomes.[1,5]
Study design	Controlled trials or moderate or high quality[6] rapid or systematic reviews or meta-analyses of controlled trials.

BMS, breast milk substitute.

[1]FSANZ's Application Handbook section 3.6.2 A.3.1 (b) (i) requires participants are no older than one month when beginning participation in the study. Essential criteria for clinical trials of BMSs, determined by Jarrold et al. (2020) includes: (i) the age range at enrolment is sufficiently narrow for treatment effects to be comparable across the trial population; and, (ii) the age at start and end of the intervention period is appropriate for the trial objectives. With regards to the latter, Jarrold et al. (2020) did not specify a particular age but noted that the US FDA requires that growth trials must enrol infants at age 14 days or younger, with an intervention period that lasts for 15 weeks or more.

[2]FSANZ's Application Handbook section 3.6.2 A.3.1 (b) (i) requires studies demonstrate the effects (if any) on growth and

development of “infant formula products containing the substance at the proposed level”. The proposed maximum permitted amount is 40 mg bLf/100 kJ or ~1100 mg bLf/L. As we anticipated insufficient data using the proposed level, we widened the inclusion criteria to include at the proposed maximum permitted amount or higher, as if no adverse effects are demonstrated at a higher dose level it is likely that adverse effects would not be present at a lower dose level.
Essential criteria determined by Jarrold et al. (2020) includes: (i) participants should be exclusively BMS fed at enrolment; and, (ii) the BMS group/s meet legally required compositional standards. The latter, corresponds to (a) IFP with an iron content of 0.2 to 0.5 mg/100 kJ (the minimum and maximum iron level that forms the compositional requirements of Standard 2.9.1 and Schedule 29 of the Food Standards Code); and, (b) the iron saturation is ~8.7% and ≤10.7% to comply with the mean iron content of the Application’s product and the proposed maximum specification, respectively.
[3]FSANZ’s Application Handbook section 3.6.2 A.3.1 (b) (iii) requires studies “must include a control group (i.e. an infant formula-fed group that is not exposed to the proposed compositional change), an exposure group (i.e. a formula-fed group that is exposed to the proposed compositional change), plus a breastfed reference group. If a breastfed reference group is not included, a rationale for its omission is required.” Section 4.2 of the current assessment addresses the comparison between bLf-IFP exposure group with the “breastfed reference group” and the “control group”.
[4]FSANZ’s Application Handbook section 3.6.2 A.3.1 (b) (i) requires studies measure “at least length and weight” and we widened the inclusion criteria to include any other growth and development outcome.
[5]Based on FSANZ’s Application Handbook requirements section 3.6.2 A.3.1 (b) (i).
[6]Quality determined based on an assessment using AMSTAR 2, ROBIS, or similar instrument.

The search retrieved 53 results. Screening of titles and abstracts led to two full text publications being retrieved (Lönnerdal & Hernell 1994; Björmsjö et al. 2021). Screening of full texts led to their exclusion (Lönnerdal & Hernell 1994; Björmsjö et al. 2021). With respect to the outcomes, growth and development, the Application provided five publications (Hernell & Lönnerdal 2002; King et al. 2007; Johnston et al. 2015; Li et al. 2019; Björmsjö et al. 2021) of which all except one (Hernell & Lönnerdal 2002) were captured in our search. Three studies provided by the Application did not include a human milk-fed sample of infants and their full text publications were, therefore, not retrieved (King et al. 2007; Johnston et al. 2015; Li et al. 2019). After screening the title and abstract of Hernell & Lönnerdal (2002), we retrieved this full text publication. Screening of the full text led to its exclusion.

A2.2 Effect on growth and development: comparison to infants consuming IFP without added bLf

A2.2.1 Methods and results

We identified and screened the literature using the strategy and study selection criteria described in section A2.1. The only modification was the replacement of the comparator group listed in Table A.1 with: consumption of an IFP of the same composition to that of the intervention group’s IFP but without bLf.

The search provided 53 results. Screening of titles and abstracts led to three full text publications being retrieved (Lönnerdal & Hernell 1994; King et al. 2007; Björmsjö et al. 2021). Screening of full texts led to their exclusion (Lönnerdal & Hernell 1994; King et al. 2007; Björmsjö et al. 2021).

With respect to the outcomes, growth and development, the Application provided five publications (Hernell & Lönnerdal 2002; King et al. 2007; Johnston et al. 2015; Li et al. 2019; Björmsjö et al. 2021) of which all except one (Hernell & Lönnerdal 2002) were captured in our search. Two studies provided by the Application did not use suitable IFP in the intervention or comparator group and their full text publications were, therefore, not retrieved (Johnston et al. 2015; Li et al. 2019). After screening the titles and abstracts of Li et al. (2019) and Johnston et al. (2015), we did not retrieve these full text publications as their intervention group’s IFP included both bLf as well as additional substances not contained in the comparator group’s IFP. The intervention group’s IFP tested by Li et al. (2019) contained bLf and bovine milk fat globule membrane. The intervention groups’ IFP used by Johnston et al. (2015) contained bLf as well as a prebiotic blend of polydextrose and galactooligosaccharides, and lowered levels of arachidonic acid. These additional

dissimilarities with the comparator groups' IFP precludes their findings from being used to attribute any potential effects to bLf alone and draw conclusions about the effect of bLf. After screening the title and abstract of Hernell & Lönnerdal (2002), we retrieved this full text publication. Screening of the full text led to its exclusion.

A2.3 Bioavailability of bovine versus human lactoferrin

A2.3.1 Methods and results

We reviewed the literature published since date of inception to June 2022. On 7th July 2022, we searched PubMed using ("infan*"[All Fields] AND ("human milk"[All Fields] OR "breast*"[All Fields] OR "human lactoferrin"[All Fields]) AND "bovine lactoferrin"[All Fields] AND ("biological availability"[All Fields] OR "bioavailab*"[All Fields] OR "bioequival*"[All Fields] OR "bioactiv*"[All Fields] OR "equival*"[All Fields] OR "absor*"[All Fields] OR "digest*"[All Fields] OR "metabol*"[All Fields] OR "excret*"[All Fields] OR "stor*"[All Fields] OR "deliver*"[All Fields] OR "assimil*"[All Fields] OR "utili*"[All Fields] OR ("uptake"[All Fields] OR "uptakes"[All Fields] OR "uptaking"[All Fields]) OR "intestin*"[All Fields] OR "structur*"[All Fields] OR "function*"[All Fields] OR "activ*"[All Fields] OR "saturat*"[All Fields] OR "replet*"[All Fields] OR "deplet*"[All Fields] OR "apo"[All Fields] OR "holo*"[All Fields] OR "metal*"[All Fields] OR ("nutrient s"[All Fields] OR "nutrients"[MeSH Terms] OR "nutrients"[All Fields] OR "nutrient"[All Fields]) OR "trace element"[All Fields] OR "mineral"[All Fields] OR "vitamin"[All Fields] OR "deficien*"[All Fields] OR "anaemi*"[All Fields] OR "anemi*"[All Fields] OR "iron*"[All Fields] OR "copper*"[All Fields] OR "zinc*"[All Fields] OR "manganese*"[All Fields])) and filtered results by studies in humans, published in English, and available in full text.

The search provided 35 results. We identified primary research by screening individual publications against inclusion criteria (Table A.1) and included moderate or high quality reviews of such primary research. The only modifications were: the replacement of the outcome criteria listed in Table A.1 with any outcome related to the bioavailability of lactoferrin; and, the replacement of the time criteria listed in Table A.1 with any duration.

Screening of titles and abstracts led to zero full text publication being retrieved. We screened additional full texts provided by the Application (sections 2.3.1.1.3, 2.3.1.2.2, 2.3.1.3.2, 3.2.1.2.1, 3.2.1.2.2, and 3.2.2.1) and excluded them as they did not meet the study selection criteria.

A2.4 The effect of bovine versus human lactoferrin on nutrient bioavailability

A2.4.1 Methods and results

We identified and screened the literature using the strategy and study selection criteria described in section A2.3.1. The only modifications were: the replacement of the outcome criteria listed in Table A.1 with any outcome related to nutrient bioavailability; and, the replacement of the time criteria listed in Table A.1 with any duration. For time efficiency, we did not check studies' non-compliance with any of the compositional requirements of Standard 2.9.1 other than iron, or the pH of IFP (which may affect bLf's ability to bind iron). As we did not exclude studies on this basis, included studies may have used non-compliant IFP.

The search provided 35 results.

Screening of titles and abstracts led to five full text publications being retrieved (Lönnerdal & Hernell 1994; Hernell & Lönnerdal 2002; Chierici et al. 1992; Björmsjö et al. 2021; Björmsjö et al. 2022) of which none met the inclusion criteria. Screening of full texts led to the exclusion of Lönnerdal & Hernell (1994), Hernell & Lönnerdal (2002) and Björmsjö et al. (2021) for the reasons stated above (sections 4.2.2 and A2.1.1). Björmsjö et al. (2021) and Björmsjö et al. (2022) report on the same study. Björmsjö et al. (2022) was excluded for these reasons as well as not assessing a relevant outcome. Chierici et al. (1992) was excluded because: the study's IFP had an iron content substantially lower than the Code's compositional requirements (82% lower than the Code's minimum iron level); the intervention and comparator groups were samples of only 14 and 10 infants, respectively, and is unlikely to be powered to detect differences in outcomes, if they existed; iron saturation of bLf (20%) is twice as high as the maximum level specified by the Application; and, the intervention groups' infants' age at which they began consuming bLf-IFP is not specified but implied as being from birth.

We screened additional full texts provided by the Application (sections 2.3.1.1.3, 2.3.1.2.2, 2.3.1.3.2, 3.2.1.2.1, 3.2.1.2.2, and 3.2.2.1) and excluded them as they did not meet the study selection criteria. Schulz-Lell et al. (1991) was excluded because: the study's bLf and non-bLf groups IFP' contained 81% and 86% lower iron content than the Code's minimum compositional requirements, respectively; the non-equivalence of two groups' IFP iron content; and the small sample size (n=7 or 9 for each group). The iron saturation of the bLf was not stated. Last, we note that the study by Schulz-Lell et al. (1991) was not randomised. Although this was not a reason to exclude any study, it increases the risk of bias.