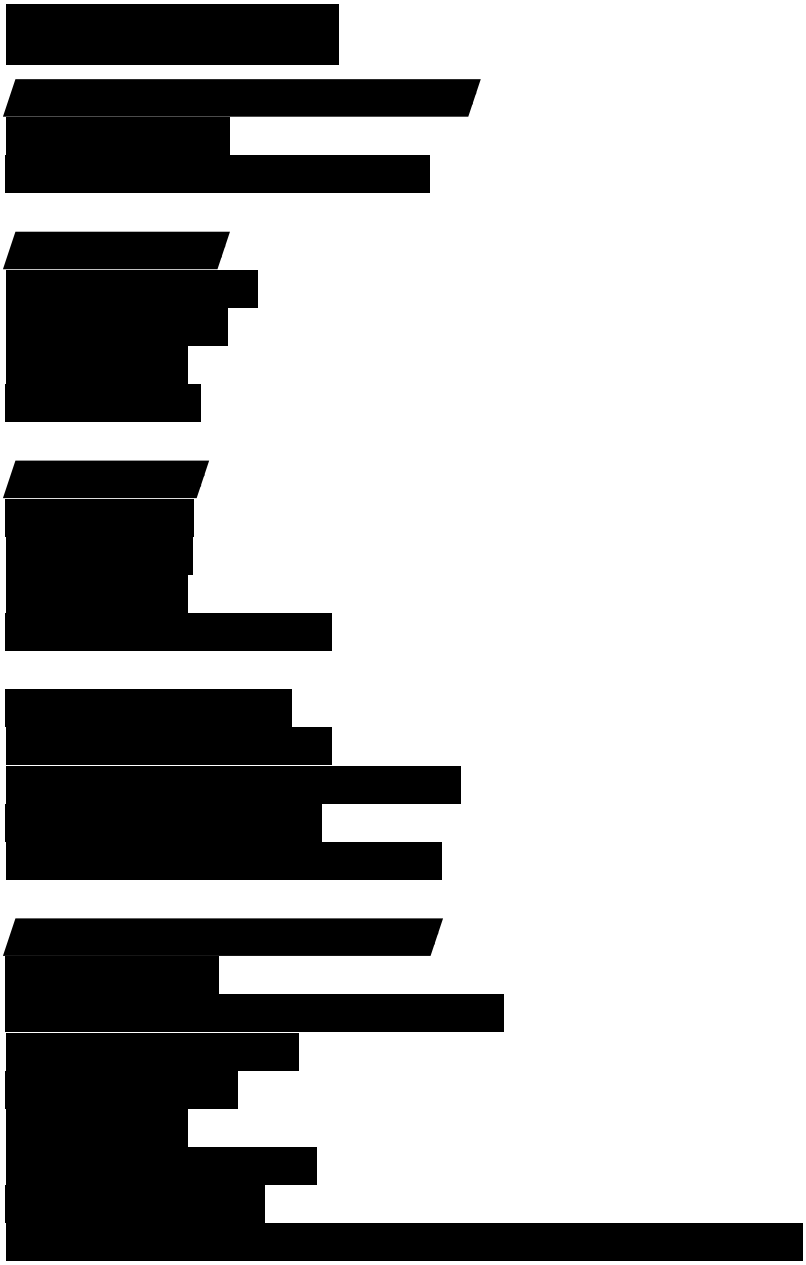
APPLICATION TO PERMIT THE OPTIONAL USE OF BOVINE LACTOFERRIN IN INFANT FORMULA PRODUCTS

Applicant:

[redacted]

Date:
7 April 2022

[redacted]



Statutory declaration

(Oaths and Declarations Act 1957)

I [redacted]

1. the information provided in this application fully sets out the matters required; and
2. the information is true to the best of my knowledge and belief; and
3. no information has been withheld which might prejudice this application to the best of my knowledge and belief.

And I make this solemn declaration conscientiously believing the same to be true and by virtue of the Oaths and Declarations Act 1957. Declared at Lyttelton this 7 April 2022.

Signature

[redacted]

Declared before me

[redacted]

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Abbreviations

AE	Adverse event
ANZ	Australia and New Zealand
Apo-bLf	Iron-depleted bovine lactoferrin
ARA	Arachidonic acid
BALF	Bronchoalveolar lavage fluid
BBB	Blood-brain barrier
bLf	Bovine lactoferrin
BW	Body weight
°C	Degrees Celsius
CAGR	Compound annual growth rate
CAS	Chemical Abstracts Service
CD	Cluster determinant
CCP	Critical control points
CFR	Code of Federal Regulations
cfu	Colony forming units
CI	Confidence interval
CMA	Cow's milk allergy
CMV	Cytomegalovirus
CNS	Central nervous system
CSF	Cerebrospinal fluid
d	Day(s)
Da	Dalton
DEX	Dexamethasone
DHA	Docosahexaenoic acid
EC	European Commission
E. coli	*Escherichia* coli
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
EOS	Early-onset sepsis
ETEC	Enterotoxigenic *Escherichia* coli
EU	Endotoxin units
FDA	Food and Drug Administration
Fe	Iron
FIA	Fractional iron absorption
FOF	Follow-on formula
FSANZ	Food Standards Australia New Zealand
FSC	Food Standards Code

g	Gram(s)
GI	Gastrointestinal
GOS	Galactooligosaccharides
GRAS	Generally regarded as safe
GRN	GRAS Number
h	Hour(s)
HACCP	Hazard analysis critical control point
Hb	Haemoglobin
HH	Hereditary hemochromatosis
HIF	Hypoxia inducible factor
hLf	Human lactoferrin
hLfR	Human lactoferrin receptor
Holo-bLf	Iron-saturated bovine lactoferrin
hPIV	Human parainfluenza virus
HPLC	High-performance liquid chromatography
HSV	Herpes simplex virus
ID	Iron deficiency
IDA	Iron deficiency anaemia
IEC	Intestinal epithelial cell
IF	Infant formula
IFN	Interferon
IFSDN	Infant formula for special dietary needs
Ig	Immunoglobulin
IL	Interleukin
IP	Intellectual Property
IUGR	Intrauterine growth restriction
kDa	Kilodalton
kg	Kilogram(s)
kJ	Kilojoule(s)
L	Litre(s)
LAL	*Limulus* Amebocyte Lysate
Lf	Lactoferrin
LfR	Lactoferrin receptor
LOS	Late-onset sepsis
LPS	Lipopolysaccharide
LBW	Low-birth-weight
m/m	Mass per mass
max	Maximum
MCV	Mean corpuscular volume

MFGM	Milk fat globule membrane
mg	Milligram(s)
min	Minimum
mL or ml	Millilitre(s)
MLN	Mesenteric lymph nodes
MPI	Ministry for Primary Industries
MPN	Most probable number
mRNA	Messenger ribonucleic acid
mo	Month(s)
M_w	Molecular weight
µg	Microgram(s)
µL or µl	Microlitre(s)
µm	Micrometre(s)
n or N	Number
N/A	Not applicable or Not available
NaCl	Sodium chloride
NEC	Necrotizing enterocolitis
ng	Nanogram(s)
NIFN	New Infant Formula Notification
NIP	Nutrient information panel
NIS	Nutrient information statement
NK cells	Natural killer cells
nm	Nanometre(s)
NOAEL	No-observed-adverse-effect-level
NZ	New Zealand
PDX	Polydextrose
PEG	Polyethylene-glycol
PES	Permeable polyethersulfone
PND	Post-natal day
RES	Reticulo-endothelial system
RFC	Radial flow column
RH	Relative humidity
RMP	Risk Management Programme
RNA	Ribonucleic acid
RO	Reverse osmosis
RP-HPLC	Reverse-phase high-performance liquid chromatography
RR	Relative risk
RSV	Respiratory syncytial virus
RV	Rhinovirus

SAE	Serious adverse event
SARS-CoV--2	Severe acute respiratory syndrome coronavirus 2
SD	Standard deviation
TGF	Transforming growth factor
Th	T helper
TIA	Total iron absorption
TNF	Tumour necrosis factor
UF	Ultrafiltration
US(A)	United States (of America)
USD	United States Dollars
USFDA	United States Food and Drug Administration
UV-Vis	Ultraviolet visible
VLBW	Very low birth weight
WHO	World Health Organization
wk	Week(s)
WPC	Whey protein concentrate
w/v	Weight per volume
w/w	Weight per weight
yr or yrs	Year(s)

Executive Summary

Synlait Milk Ltd. (Synlait) seeks permission under the Australia New Zealand Food Standards Code (FSC) for the optional addition of bovine lactoferrin (bLf), as a nutritive substance, to foods regulated within the FSC Part 2.9 Special purpose foods, specifically Standard 2.9.1 Infant formula products (infant formula [birth to 6 months], follow-on formula [6 to 12 months] and infant formula for special dietary needs [birth to 12 months]).

The purpose of the use of bLf in Infant formula products is based on the weight of evidence for a reduced risk of infection in formula-fed infants receiving bLf-fortified formula compared to standard formula not fortified with bLf. This is backed up by significant evidence supporting the safe use of bLf in infants. Breastfed infants benefit from lactoferrin naturally present at high levels in human milk. Infants who cannot be breastfed and rely on Infant formula products to support development and growth miss out on the benefits of lactoferrin unless bLf is added to Infant formula products. As well as providing a physiological benefit to formula-fed infants, Infant formula products containing added bLf will more closely reflect the lactoferrin composition in human milk. As the addition of bLf to Infant formula products is already approved in many countries, the ability to include bLf in Infant formula products for sale in Australia and New Zealand will facilitate trade with countries where bLf is already permitted due to exemptions for export of Infant formula products containing added bLf no longer being required. This will help level the playing field for ANZ manufacturers, providing a better competitive position.

Lactoferrin (Lf) is an iron-binding protein that is naturally present in the body. It is present in mammal milks, notably at high levels in human milk (around 1230-1420 mg/L in Australian mothers), and at significantly lower levels in bovine milk (~100mg/L), and consequently infant formula not fortified with bLf (~15mg/L). While bovine and human Lf (hLf) are not identical, differences in structure result in only small differences in cellular uptake and functionality, and bLf has been shown to provide benefits similar to those provided by hLf.

The proposed maximum permitted level of bLf in Infant formula products is 40mg/100kJ across all Infant formula products, which is equivalent to around 1100mg/L made-up infant formula and 1200mg/L follow-on-formula (using the midpoints of the energy ranges in Standard 2.9.1).

Proposed maximum permitted levels of bLf in foods defined within Standard 2.9.1 Infant formula products

Standard	**Target population**	**Specific category**	**Maximum permitted levels**
2.9.1 Infant formula products	Infants 0-12 months	Infant formula	40 mg/ 100kJ
		Follow-on formula	40 mg/ 100kJ
		Infant formula for special dietary use	40 mg/ 100kJ

Synlait bLf is available in powdered form, with specification parameters comprising physical appearance, purity, total bLf levels, moisture, among others, as well as limits for potential chemical and microbiological impurities, and contaminants. Synlait bLf is derived from skim milk using ion exchange technology, a process resulting in a bLf ingredient with high purity and proven bioactivity relevant for infant development.

Based on the totality of information, Synlait concludes there is compelling evidence that a substantial proportion of both intact bLf and its peptides resist gastric digestion and persists throughout the gastrointestinal tract. This resistance to digestion is important for bLf to be able to exert some of its benefits, in particular its bacteriostatic effect. Some bLf is also absorbed in the intestinal lumen via lactoferrin receptors, exerting a range of systemic effects. This duplicity of fates affords it to play a range of different metabolic roles and manifest its bioactivity via a range of different mechanisms. This underlies the clinical benefits associated with the inclusion of bLf in milk-based infant formula products. Based on the results from acute, sub-chronic and chronic animal toxicity studies, Synlait concludes that bLf is well tolerated with no significant adverse effects or toxicity at the concentrations tested. The no-observed-adverse-effect level (NOAEL), based on these toxicity studies, is determined to be 2,000 mg bLf/kg BW/day. The compound bLf is also non-genotoxic, as determined by the Ames mutagenicity test.

Further support for the safe use of bLf comes from studies in term infants, with normal growth seen in infants receiving bLf at levels up to 1000mg/L made-up formula, and no safety or tolerance issue being reported. Compelling evidence for the safety of bLf also comes from numerous studies in preterm and low-birth-weight infants, a particularly vulnerable population group, with no safety or tolerance issues relating to bLf administration being reported. Administered doses were equivalent to levels of 370-1960mg/L formula. There is also a history of safe consumption of bLf in countries that have had bLf permitted for use in Infant formula products for many years.

There is considerable evidence from *in vitro*, animal and human studies supporting a benefit of bLf in formula-fed infants, notably reduced risk of infection in formula-fed infants receiving bLf versus formula-fed infants not receiving bLf. Several mechanisms underlying this benefit of bLf have been identified, including proven antibacterial effect, antiviral effect, and immunomodulatory effect of bLf. bLf can directly bind to bacteria and viruses and inactivate them, and can bind to receptors in the intestine, blocking entry of pathogens, and can also be internalised and thereby exert immunomodulatory functions. The antibacterial effect of bLf is also partly due to bLf's high affinity to iron. By binding iron, iron is made unavailable to pathogens, which require iron as food. However, iron bound to bLf remains available to the infant, being absorbed together with bLf via lactoferrin receptors. This is supported by evidence from human studies showing that addition of bLf to formula supports normal iron absorption and homeostasis.

Evidence from studies in term formula-fed infants support a reduced risk of respiratory and gastrointestinal infections through bLf addition to infant formula, with both incidence and severity being reduced. Further supporting evidence on reduced risk of infection comes from animal studies. Evidence for a beneficial effect of bLf also comes from the highly vulnerable group of pre-term infants and low-birthweight infants. This population group has an especially high risk of infections, meaning that effective dietary interventions are of particular importance. The use of bLf has been found to reduce the risk of late-onset-sepsis in this vulnerable population group.

Dietary exposure was assessed, and exposure to bLf in formula-fed infants is expected to be somewhat lower than exposure of breastfed infants to hLf. Exposure levels are considered safe. Whilst bLf is an iron-binding protein and contains iron up to 15mg/100g bLf, the contribution of bLf at proposed levels to total

iron content in infant formula is negligible. Any iron contributed from added bLf will contribute to the total iron content of formula. Synlait does not anticipate that adding bLf to Infant formula product will have any adverse safety or nutritional outcomes. The addition of bLf to formula increases choice for parents of formula-fed babies, and bLf containing formula may replace formula not containing bLf. Synlait does not anticipate any nutritional concerns with this replacement as any Infant formula products sold in Australia and New Zealand must meet the requirements of Standard 2.9.1 Infant formula products. Based on published evidence, Synlait does not anticipate that parents who are breastfeeding will choose to switch to formula as a result of the addition of bLf to formula.

Overall, there is significant benefit in permitting bLf as a nutritive substance to Infant formula products, in particular physiological benefits for formula-fed infants. There is convincing evidence for the safe use of bLf in Infant formula products.

1. General Requirements (3.1.1.)

1.1. Applicant details

See Page 2 for applicant details.

1.2. Purpose of application

This application seeks permission under the Australia New Zealand Food Standards Code (FSC) for the optional addition of bovine lactoferrin (bLf), as a nutritive substance, to foods regulated within the FSC Part 2.9 Special purpose foods, specifically Standard 2.9.1 Infant formula products (infant formula [IF, birth to 6 months], follow-on formula [FOF, 6 to 12 months] and infant formula for special dietary needs [IFSDN, birth to 12 months]).

Permission to add nutritive substances to foods is regulated by Part 2.9 Special purpose foods, this application seeks to vary Standard 2.9.1 Infant formula products. Proposed options are laid out, as follows.

2.9.1-5 Use of substances as nutritive substances

The optional use of nutritive substances under 2.9.1- 5 refers to the table of Schedule 29 section S29-5. Permission to add bovine lactoferrin to infant formula products would be addressed by amending the table to S29-5 to include bovine lactoferrin with the inclusion of the following:

Column 1	*Column 2*	*Column 3*	*Column 4*
Substance	*Permitted form*	*Minimum amount per 100kJ*	*Maximum amount per 100kJ*
Lactoferrin	Bovine lactoferrin	-	40 mg

Conditions of use as outlined in Table 2-1. in Section 2.1.1, may be stipulated within each Standard, as referenced in Standard 2.9.1 Infant formula products.

The purpose of this application is consistent with guideline policies as set out by the Australia and New Zealand Ministerial Forum on Food Policy (previously the Australia and New Zealand Food Regulation Ministerial Council). This includes the 'Policy Guideline for the Addition to Food of Substances other than Vitamins and Minerals' for the addition of bLf to Special purpose foods. The addition of bLf is aligned with the 'High Order' Policy Principles of the protection of public health and safety, informed consumer choice and the prevention of misleading or deceptive conduct. The permission to add bLf to Infant formula products will promote consistency between domestic and international food standards and will help promote an efficient and internationally competitive food industry. The application is further aligned with the 'Specific Order Policy Principles – Any Other Purpose', where the purpose for adding bLf to Infant

formula products is the provision of a safe bioactive substance that supports wellbeing associated with reduced risk of infection in infants. Lactoferrin is naturally present in mammalian milks and as such has a safe history of consumption. The addition of bLf to human food also has a history of safe use, having typically been added in the form and in quantities consistent for delivering the benefits subject of this application. The addition of bLf to Infant formula products will not create a significant negative public health impact to the general population or sub-populations, nor will the presence of bLf mislead consumers as to the nutritional quality of the food.

For products subject to Standard 2.9.1 Infant formula products, the Australia and New Zealand Ministerial Forum on Food Policy (Food Regulation Standing Committee – Regulation of Infant Formula Products) Policy Guideline provides guidance on expectations in the setting of new regulation for Infant formula products, in addition to the 'High Order' Policy Principles as above. This application is aligned with the Specific Policy Principles, in that:

- the addition of bLf to Infant formula products does not negate the overarching recognition that breastfeeding is the normal and recommended way to feed an infant;
- the addition of bLf to Infant formula products is consistent with national nutrition policies and guidelines of ANZ that are relevant to infant feeding;
- the addition of bLf to Infant formula products is based on a safe history of use, outside of ANZ, and takes into account the vulnerability of the infant population, recognising the importance of infant formula products in the diets of formula-fed infants;
- when used as the sole source of nutrition, infant formula containing bLf supports the normal growth and development of healthy term infants similar to that of exclusively breastfed infants;
- Infant formula products containing bLf are safe, suitable and meet the nutritional requirements to support the growth, development and dietary management of the infants for whom they are intended;
- used as the sole source of nutrition, infant formula containing bLf supports the normal growth and development of pre-term infants;
- Infant formula products, including infant formula, follow-on formula and infant formula for special dietary needs, that contain bLf are safe, suitable for the intended use;
- the addition of bLf to Infant formula products does not impact the essential composition of Infant formula products prescribed in the FSC, formulas in accordance with Standard 2.9.1 that contain bLf satisfy the nutritional requirements of infants;
- the addition of bLf to Infant formula products results in a composition that is more closely aligned with that of breastmilk which contains high levels of lactoferrin;
- the addition of bLf to Infant formula products available for sale in ANZ requires pre-market assessment. Whilst bLf has been added to Infant formula products manufactured in ANZ for a number of years, those products have been manufactured for export markets, and therefore the addition of bLf at the proposed level does not have a history of use in ANZ;

– the addition of bLf to infant formula products has a substantiated beneficial role in reducing risk of infection compared to formula-fed infants consuming formula not fortified with bLf.

1.3. Justification for the application

Lactoferrin is a functional dairy protein, increasingly recognised for providing health benefits across different age groups, with evidence supporting a key role in early life development. Lactoferrin is naturally present in mammal milk, including in human milk. Lactoferrin has been found to play a key role in early life, in particular, reducing the risk of infection, as discussed in more detail in Section 3.2. Since bovine milk is significantly lower in lactoferrin compared to human milk, the levels in non-fortified formula are significantly lower compared to human milk. Adding lactoferrin to Infant formula products can help deliver the benefits outlined in Section 3.2. Since neither human lactoferrin nor recombinant human lactoferrin are available for addition to Infant formula products, the option of lactoferrin derived from bovine milk has been extensively studied and is widely used in international markets. Bovine lactoferrin (bLf) is similar in structure and function to human milk, as outlined in more detail in Sections 2.2.2 and 3.2.

Globally available, and popular, as dietary supplement, bLf is increasingly being used as a nutritive ingredient in foods, particularly Infant formula products and other dairy-based foods. In New Zealand, MPI has previously commented on the ambiguity of Clause 6 (1) in Standard 2.9.1 regarding the addition of ingredients derived from milk into infant formula (Ministry for Primary Industries, 2012), concluding that the addition of bLf to Infant formula products may breach the FSC unless specific permission is expressly given. This application intends to address that ambiguity and provide clarity on the addition of bLf to Infant formula products.

The optional addition of bLf to Infant formula products will extend the option of naturally beneficial ingredients that can be used and hence provide additional benefits and increase consumer choice.

1.3.1. Regulatory Impact

This application will provide regulatory clarification in Australia and New Zealand with regards to the permitted use of bLf in Infant formula products.

1.3.1.1. Cost and Benefits

The main benefits and purposes of adding bLf to Infant formula products are:

- To provide a beneficial physiological effect over and above standard products, including helping reduce risk of infection in formula-fed infants as outlined in Section 0;
- The inclusion of bLf in Infant formula products will more closely reflect the lactoferrin composition of human breast milk and will give parents and caretakers increased choice when needing to formula-feed;

- The ability to include bLf in Infant formula products will increase alignment for trade with countries where bLf is permitted. Globally bLf is increasingly being used as a nutritive ingredient in foods, particularly Infant formula products and dairy-based foods. The ability to include bLf in Infant formula products enables our own diverse ANZ intended infant population to receive these nutritional benefits that they seek from overseas foods, and also enables New Zealand and Australian manufacturers and exporters to innovate with speed and respond to international customer demand against competitor countries, by removing the requirement and extended timeline to exempt food for export (destinations other than Australia) from the requirements of the FSC, under S347 of the Food Act 2014;
- The permission to use bLf as a nutritive substance in Infant formula products will provide a further benefit to international trade due to consumers overseas often looking for the same ingredients present in imported products they buy in products on the market in the country of manufacture, giving them further confidence that the ingredients used are safe.

Both New Zealand and Australia export significant quantities of Special purpose foods, in particular Infant formula products, to a number of countries in South-East Asia and increasingly other markets such as the USA where the addition of bLf is permitted, either expressly or by broader terms that permit the addition of ingredients based on scientific evidence e.g., Codex Alimentarius standards and guidance documents. Approval of this application will facilitate international trade, with the ability to export locally compliant value-added products to various markets around the world. It will enable brand owners to globally align products, and local manufacturers to meet customer requests for the inclusion of lactoferrin.

The cost of the application has been considered in the context of overall potential to increase trade, both in finished consumer-ready goods and of bLf as an ingredient.

The incremental costs of adding bLf to Infant formula products is normally reflected in nominal price premiums of products that include bLf and potentially other optional ingredients. In Infant formula products, historically the use of optional ingredients and associated price premiums has resulted in market segmentation into standard and premium product ranges, that have provided consumers with product and price range options. Such segmentation remains, however, there is a convergence of the segments as optional ingredients such as bLf become more mainstream in offshore markets where addition is permitted.

At a nominal value of USD500 to 700/kg bLf, the addition of bLf in Infant formula products at the proposed maximum level of 40mg/100kJ (~110 mg/ 100mL) of formula would add approximately 5.5-7.7 cents (US) per 100mL of made-up formula, or 40-60 cents (US) per 100g of powder.

FSANZ is reviewing standards that are pertinent to this application, P1028 - Infant Formula, the three Consultation papers covering safety and technology, nutrient composition and the regulatory framework and definitions are now closed. The 1st Call for Submissions is scheduled for an 8-week comment period in the first quarter of 2022, with an overall target for gazettal March 2023. P1024 – Revision of the Regulation of Nutritive Substances & Novel Foods, work has been suspended on the proposal until modernisation amendments are made to the FSANZ Act 1991.

This application will not result in any additional compliance or regulatory costs to Government but may provide benefits as fewer exemptions will need to be processed.

1.3.1.2. Impact on International Trade

The permitted addition of bLf to Infant formula products (regulated under Standard 2.9.1) will better align products manufactured in New Zealand and Australia with existing standards in other countries, thus facilitating international trade. For New Zealand this will remove the requirement to exempt food for export to destinations other than Australia (currently in place for export to the USA, China and Hong Kong) from the requirements of the FSC, under S347 of the Food Act 2014. Future potential exports to the European Union would also not require S347 exemptions to permit export. The permitted addition of bLf to Infant formula products will further align trade opportunities with a range of countries that already permit the addition of bLf to a range of foods.

In addition to the alignment of trade, being able to add bLf into products sold in Australia and New Zealand also beneficially affects consumer perception of this nutritive substance overseas. Overseas consumers look at products in the market of origin of products they consider purchasing, and they want to see the same ingredients being freely sold, used and consumed in the country of origin to give them confidence in the products they purchase. Therefore, permission of bLf in Infant formula products in Australia and New Zealand will support international trade via increased consumer acceptance and uptake.

1.4. Assessment procedure

Based on the criteria set out in the FSANZ "Application Handbook" (1 July 2019) this Application will fall within the scope of a General Procedure. The application is for the variation of food regulatory measures that include the addition of a new nutritive substance to foods for vulnerable populations (infants) and the requirement for pre-market approval of the substance.

1.5. Confidential commercial information

The following information is commercially sensitive, confidential information not to be shared with public:

- **Appendix 1 (all pages)**: Appendix 1 contains confidential commercial information relating to the manufacture of bLf, specifically:
    - A detailed manufacturing process flow, which is a trade secret and therefore Synlait IP; a non-confidential simplified process flow is presented in Section 2.2.4.1, Figure 2-6.
    - Specifications of materials and processing aids used in the manufacture of bLf, which are either Synlait trade secret, or are IP of our suppliers and provided to Synlait in confidence; a non-confidential summary of materials and processing aids used can be found in Section 2.2.4.2, Table 2-7 .

- **Appendix 3 (all pages):** contains confidential commercial information relating to test method and results, specifically:
    - A detailed description of the Synlait test method for lactoferrin. The test method is Synlait's IP/trade secret. A summary of the test method is provided in Section 2.2.7.
    - Results on particle size distribution; this information is commercially sensitive as it can provide information to competitors that they may use to their advantage. A brief discussion of results is included in Section 2.2.4.1.

1.6. Other confidential information

No other confidential information provided.

1.7. Exclusive capturable commercial benefit

Synlait expects the application to confer an exclusive capturable commercial benefit (ECCB) to Synlait once amendments to the Food Standards Code are made, and provided Exclusivity is granted to Synlait. Therefore, Synlait intends to pay the fee to cover the assessment of the application. Justification for obtaining exclusivity and information to support an ECCB to Synlait are outlined in Table 1-1.

Table 1-1 Justification for obtaining Exclusivity and information to support an Exclusive capturable commercial benefit to Synlait

Why are you making this application? What are you hoping to get out its approval?	The purpose of this application, as outlined above, is to get permission to add bLf to Infant formula products in order to deliver health benefits. Being able to add bLf to products will create more demand for Synlait bovine lactoferrin; will provide Synlait's customers with an option to align their international product portfolios; will enable product innovation and give Synlait and their customers the opportunity to provide differentiated product offerings, and will provide more consumer choice. Permission of bLf addition to Infant formula products will also enable easier trade for Synlait and Synlait's customers due to alignment with international regulations and exemptions for export no longer being needed for these products. Synlait has made significant investment in the development of a high-quality bovine lactoferrin ingredient suitable for infant application, and in state-of-the art manufacturing facilities. Synlait also committed significant resource in drafting this application and is paying the applicable fees in full. Synlait therefore requests for Exclusivity to be granted, enabling Synlait to capture an Exclusive capturable commercial benefit.

How will you benefit from the approval of your application?	From a commercial point of view, Synlait aims to work with key customers to commercialise bLf-containing Infant formula products in Australia and New Zealand, which will provide a significant commercial benefit to the business. Synlait is aware of several customers having interest in adding bLf to their Infant formula products. Aside from the direct financial value of being able to use bLf in Infant formula products in Australia and New Zealand, Synlait expects that the permission of Synlait's bLf will also indirectly positively impact product sales in other markets, both for Synlait and Synlait's key customers.
Who besides you, will benefit from the approval of your application? How and why will they benefit?	Other key beneficiaries will be Synlait's key customers. Obtaining Exclusivity will enable Synlait's customers to be the first in the market to provide a differentiated product containing bLf and to be able to align their international product portfolio.
If your application is approved, whose permission will be required before anyone can derive a benefit from that approval?	Once the application is approved, Synlait does not need permission from anyone else to derive a benefit from the approval. The manufacturing process is Synlait IP, and Synlait has all regulatory approvals in place to manufacture lactoferrin for use in Infant formula products. A Freedom-to-Operate search completed in 2022 by external IP lawyers has resulted in no patents being identified that would hinder Synlait or Synlait's customers from using bLf in their Infant formula products destined for the Australian and New Zealand markets.
Who holds the intellectual property in the subject matter of your application?	The use of bLf in Infant formula (and other) products is now permitted in many regulatory jurisdictions and there are no IP constraints for its use in these processes. Early patents for extraction and use have long since expired. The IP for the manufacture of Synlait bLf is the property of Synlait. As mentioned above, a Freedom-to-Operate search completed in 2022 by external IP lawyers has resulted in no patents being identified that would hinder Synlait or Synlait's customers from using bLf in their Infant formula products destined for the Australian and New Zealand markets.

1.8. International and other national standards

1.8.1. International standards

The addition of bLf to Infant formula products is consistent with the intent and recommendations of relevant internationally recognised codes of practice and guidelines, and in particular with those by the Codex Alimentarius Commission (Codex). Specifically with regard to the use of bLf in Infant formula products the safe history of use of bLf, together with extensive safety and clinical data, addresses the recommendations for data requirements for changes to infant formula as recommended by the National Academy of Medicine (NAM), formerly called the US Institute of Medicine (IOM), Food and Nutrition Board guidelines that clarify the types and extent of safety testing necessary for new formula ingredients, particularly unconventional substances derived from novel sources or technologies (Institute of Medicine, 2004).

The Codex Alimentarius Commission (Codex)[1] provides standards, codes of practice and guidelines relevant to Infant formula products, and for which the use of bLf is consistent with their intent.

The Codex Standards for Special purpose foods for infants (Codex Standard for Infant Formula and Formulas for Special Medical Purposes Intended for Infants[2] (from birth through to 12 months)) allows for the addition of other ingredients which provide "substances ordinarily found in human milk and to ensure that the formulation is suitable as the sole source of nutrition for the infant or to provide other benefits that are similar to outcomes of populations of breastfed babies. The suitability for the particular nutritional uses of infants and the safety of these substances shall be scientifically demonstrated. The formula shall contain sufficient amounts of these substances to achieve the intended effect, taking into account levels in human milk" (Codex Alimentarius, 2020).

The Codex Standard for Follow-up Formula[3] (for infants from 6 months and young children through 3 years) is less definitive for optional ingredients, however, the addition of bLf would be consistent with the requirements of the standard in that other nutrients may be added when required to ensure that the product is suitable to form part of a mixed feeding scheme intended for use from the 6th month on. That the usefulness of those ingredients is scientifically proven, and that when added, the food will contain significant amounts of the nutrients, based on the requirements of infants from the 6th month on and young children (Codex Alimentarius, 2017).

The proposed addition of bLf for Infant formula products under Standard 2.9.1 is aligned with the intent of the Codex infant and follow-up formula standards. Furthermore, the production and specifications set for bLf are consistent with the Codex recommendations for raw materials and ingredients for use in infant and

[1] http://www.fao.org/fao-who-codexalimentarius/en/
[2] https://www.fao.org/fao-who-codexalimentarius/sh-proxy/es/?lnk=1&url=https%253A%252F%252Fworkspace.fao.org%252Fsites%252Fcodex%252FStandards%252FCXS%2B72-1981%252FCXS_072e.pdf
[3] (https://www.fao.org/fao-who-codexalimentarius/sh-proxy/zh/?lnk=1&url=https%253A%252F%252Fworkspace.fao.org%252Fsites%252Fcodex%252FStandards%252FCXS%2B156-1987%252FCXS_156e.pdf

follow-up formula and formulas for special medical purposes for infants (Codex Code of Hygienic Practice for Powdered Formulae for Infants and Young Children[4] (Codex Alimentarius, 2008).

For Infant formula products, the addition of bLf and labelling of infant formula products fits consistently with the intent of infant formula marketing codes of practice; Marketing in Australia of Infant Formulas: Manufacturers and Importers Agreement 1992 (The MAIF Agreement, 1992); WHO International Code of Marketing of Breast-milk Substitutes (World Health Organization, 1981); The Infant Nutrition Council Code of Practice for the Marketing of Infant Formula in New Zealand (Infant Nutrition Council, 2007).

1.8.2. Other standards

No other relevant standards were identified.

1.9. Statutory declaration

See Page 3 for statutory declaration.

1.10. Checklist

Requirements	Comment and relevant sections covered	Page No
General requirements (3.1.1)	**Section 1**	**13**
A Form of application		
Application in English	Yes	
Table of content, table of tables, table of figures	Yes	
Executive Summary (separated from main application electronically)	Yes	13 (and separate document)
Relevant sections of Part 3 clearly identified	Yes	
Pages sequentially numbered	Yes	
Electronic copy (searchable)	Yes	
All references provided	Yes	
B Applicant details	Section 1.1	2, 16
C Purpose of the application	Section 1.2	16

[4] https://www.fao.org/fao-who-codexalimentarius/sh-proxy/en/?lnk=1&url=https%253A%252F%252Fworkspace.fao.org%252Fsites%252Fcodex%252FStandards%252FCXC%2B66-2008%252FCXP_066e.pdf

D Justification for the application	Section 1.3	18
Regulatory impact information	Section 1.3.1	18
Impact on international trade	Section 1.3.1.2	20
E Information to support the application	Sections 2 and 3	28 and 88
Data requirements	Section 2.1.2	29
F Assessment procedure	Section 1.4	20
G Confidential commercial information	Section 1.5	20
CCI material separated from other application material	Yes	Appendices
Formal request including reasons	Yes	20
Non-confidential summary provided	Yes	
H Other confidential information	Section 1.6	21
I Exclusive capturable commercial benefit	Section 1.7	21
Justification provided	Yes	
J International and other national standards	Section 1.8	23
International standards	Section 1.8.1	23
Other national standards	Section 1.8.2	24
K Statutory declaration	Yes	2
L Checklist provided	Section 1.10	24
Substances used for a nutritive purpose (3.3.3)	**Section 2**	**28**
A Information on the use of the nutritive substance	Section 2.1	28
A.1 Purpose of the use of the substance	Section 2.1.1	28
A.2 General data requirements for supporting evidence	Sections 2.3 & 3.1	58, 88
B Technical information on the use of the nutritive substance	Section 2.2	34
B.1. Identification	Section 2.2.1	36
B.2 Chemical and physical properties	Section 2.2.2	36
B.3 Impurity profile	Section 2.2.3	37
B.4 Manufacturing process	Section 2.2.4 and Appendix 1	41

B.5 Specification for identity and purity	Section 2.2.5	45
B.6 Analytical method for detection	Section 2.2.7	55
B.7 Proposed food label	Section 2.2.8	55
C Information related to the safety of bovine lactoferrin	Section 2.3	58
C.1. Toxicokinetics and metabolism, degradation products and major metabolites	Sections 2.3.1, 2.3.2, 2.3.3, 2.3.4, 2.3.5	58, 63, 71, 75, 76
C.2 Animal or human studies	Sections 2.3 & 3.2.2	58, 97
C.3 International safety assessments	Section 2.3.7	78
D Information on dietary intake of the nutritive substance	Section 2.4	80
D.1. List of food groups or foods proposed to contain the nutritive substance	Sections 2.4.1	80
D.2 Proposed maximum levels in food groups or foods	Sections 2.4.2	80
D.3 Likely level of consumption foods containing nutritive substance	Section 2.4.3	81
D.4 Percentage of food group to use the nutritive substance	Section 2.4.4	82
D.5 Use in other countries (if available)	Section 2.4.5	82
D.6 Where consumption has changed, information on likely consumption	Section 2.4.6	83
E Information related to the nutritional impact of a vitamin or mineral	Not relevant to application	
F Information related to the nutritional impact of a nutritive substance other than vitamins and minerals	Section 2.5	84
F.1 Nutritional purpose (other than vitamins and minerals)	Sections 2.5.1 and 3.1.1	84 and 88
G Information related to potential impact on consumer understanding and behaviour	Section 2.6	84
G.1 Consumer awareness and understanding	Section 2.6.1	84
G.2 Actual or potential behaviour of consumers	Section 2.6.2	86

2. Substances for a nutritive purpose (3.3.3.)

2.1. Information on the use of the nutritive substance

2.1.1. Information on the purpose of the use of bLf

The purpose of the use of bLf in Infant formula products is based on the weight of evidence for the reduced risk of infection in formula-fed infants receiving bLf-fortified formula compared to standard formula not fortified with bLf. Breastfed infants benefit from lactoferrin (Lf) naturally present in human milk, however, infants who cannot be breastfed and rely on infant formula products to support development and growth may miss out on the benefits of Lf unless bLf is added to Infant formula products.

Significant levels of Lf are present in human milk (see Section 3.2.1), which suggests Lf being an important component in infant nutrition. Lactoferrin provided by human milk is known to exert immunoregulatory, antibacterial, and antiviral activity, and is involved in iron homeostasis (Demmelmair *et al.*, 2017; Lönnerdal, 2016). Bovine and human Lf (hLf) are not identical, but show a 69% amino acid sequence identity, which is associated with some differences in tertiary structure (see Section 2.2). However, this results in only minor differences in cellular uptake and bLf and hLf have similar functions (Demmelmair *et al.*, 2017). bLf has been shown to provide similar benefits to hLf, as discussed in detail in Section 3.2.3.

Human lactoferrin is unavailable for addition to Infant formula products, and while manufacture of hLf through recombinant technology using genetically modified microorganisms is possible, this faces many regulatory hurdles for approval and is not the subject of this application. Bovine lactoferrin recovered from bovine milk is readily available and is safely used in Infant formula products overseas.

Bovine milk is naturally low in bLf in comparison to hLf levels in human milk. Consequently, standard Infant formula products on the market that have no bLf added contain significantly lower levels of Lf compared to human milk, meaning that infants that cannot be breastfed miss out on the beneficial effects of Lf. The purpose of adding bLf to Infant formula products is to allow parents who formula-feed their baby to choose a product that provides benefits similar to those provided by hLf.

This application proposes the maximum permitted levels of bLf in the foods as specified in Section 2.4.2, and as also presented in Table 2-1 in this section. Further details on the purpose of adding bLf to Infant formula products, including infant formula (birth to 6 months), follow-on-formula (6 to 12 months) and infant formula for special dietary use (birth to 12 months), including comparisons with levels found in human breastmilk and unfortified formula, are presented in Sections 3.1.1 and 3.2.1.

Table 2-1 Proposed maximum permitted levels of bLf in foods defined within Standard 2.9.1 Infant formula products			
Standard	**Target population**	**Specific category**	**Maximum permitted levels**
2.9.1 Infant formula products	Infants 0-12 months	Infant formula	40 mg/ 100kJ
		Follow-on formula	40 mg/ 100kJ
		Infant formula for special dietary use	40 mg/ 100kJ

2.1.2. General data requirements for supporting evidence

Lactoferrin, including bLf, provides a range of biological activities that deliver key benefits for infants. Significant support for the benefits and safety of bLf in infants comes from animal studies and human intervention studies. Literature searches on PubMed were carried out to identify animal and human intervention studies relevant to the target population (infants) and supporting the benefit and safety of bLf.

The following literature searches to identify relevant studies were carried out on PubMed.

Human intervention studies

- Search terms: lactoferrin[Title/Abstract] OR lactoferrin[MeSH]
- Filters: Clinical Study, Clinical Trial, Controlled Clinical Trial, Multicenter Study, Randomized Controlled Trial, Child: birth-18 years
- Inclusion criteria: intervention study; in humans; bLf given as in a food or as a supplement; assessing a physiological effect relevant to this application (risk of infection, mineral homeostasis, safety parameters [anthropometric, tolerance]); generally healthy term infants (≤12 months) but including those with micronutrient deficiencies; preterm and low-birth-weight infants.
- Exclusion criteria: non-bovine lactoferrin used as intervention; bLf used for treatment of health issues (e.g. cancer, existing infections); bLf not given as part of food or supplement (e.g. applied topically, as mouthwash); non-intervention studies (e.g. observational); non-human studies; reviews.
- Further exclusion criterion for studies supporting benefits (but included in safety assessment): mixed interventions (i.e. bLf given with other active components, where effect of bLf cannot be discerned from effect of other components).

In addition to the search on PubMed, references in relevant papers and reviews were searched for any further studies not identified through the PubMed search.

Studies investigating benefits of bLf are discussed in Section 3.2.3; for evidence on safety of bLf consumption in infants see Section 3.2.2, while mineral homeostasis is discussed in Section 3.2.1.2.

The search carried out in October 2021 resulted in 89 citations (Figure 2-1); following review of headings and application of inclusion and exclusion criteria, a total of 49 studies were further considered. Following review of abstracts, full papers for 8 studies in term infants and 9 studies in preterm or low-birthweight infants were obtained for review. An additional 4 studies in term infants were identified on review of reference lists of the studies identified and of other publications. Two studies in infants were published after the search was carried out and these were also included for full paper review. See Figure 2-1 for an overview of the literature search.

A summary of excluded studies, with reason for exclusion, is included in Appendix 2 (A2:2).

Several other human studies not specifically relating to efficacy or safety did not form part of the systematic search are included in various parts of the application.

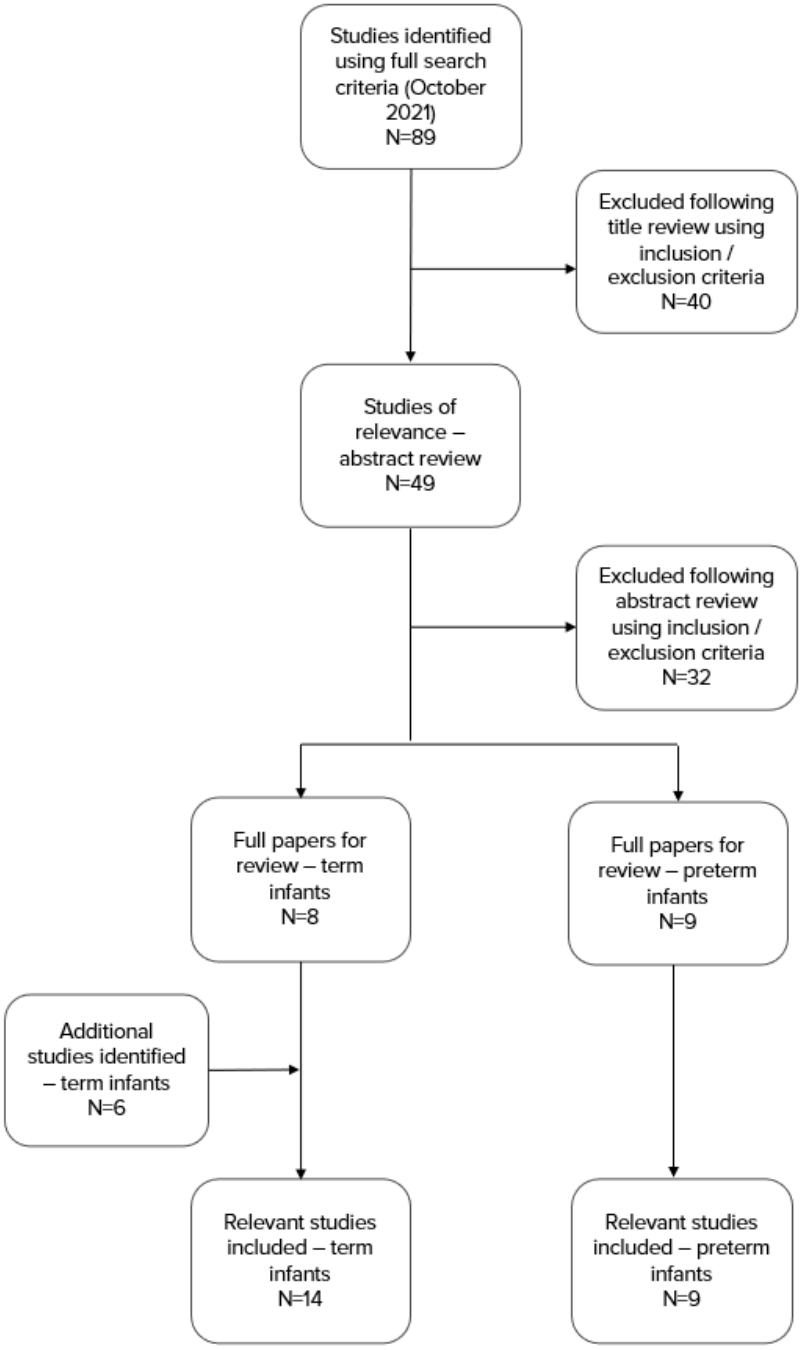

Figure 2-1: Literature search of human intervention trials in infants used to support benefits and safety of bovine lactoferrin.

Animal studies

- Search terms: (lactoferrin[Title] OR lactoferrin[MeSH]) AND bovine[Title/Abstract] AND (rat[Title/Abstract] OR rats[Title/Abstract] OR mouse[Title/Abstract] OR mice[Title/Abstract] OR murine[Title/Abstract] OR pig[Title/Abstract] OR pigs[Title/Abstract] OR piglet[Title/Abstract] OR primate[Title/Abstract] OR primates[Title/Abstract])
- Filters: Other animals
- Inclusion criteria: oral bLf application, assessing risk of infections relevant to population group (respiratory and gastrointestinal infections) or supporting mechanistic data relevant to benefit or safety-related outcomes (incl. toxicity, absorption, digestion, metabolism, excretion), in vivo study.
- Exclusion criteria: non-bovine lactoferrin used as intervention; mixed intervention, treatment of existing infection, unrelated health outcomes, studies investigating infections not relevant for target population (e.g. parasites, infections not present in ANZ), in vitro study, reviews.

The search carried out in February 2022 resulted in 358 citations (Figure 2-2); following review of headings and application of inclusion and exclusion criteria, a total of 64 studies were further considered. Following review of abstracts, full papers for 22 studies supporting benefits of bLf were reviewed, of which 19 were included.

Animal studies supporting safety or absorption, digestion, metabolism and excretion (ADME), were largely identified from relevant scientific reports and other regulatory applications. Only 6 included studies came from the PubMed search, while 24 studies were identified from the existing database of authors. Overall, 30 animal studies were included in the safety and ADME sections.

A summary of excluded studies animal studies, with reason for exclusion, is provided in Appendix 2 (A2:8).

In addition to human and animal studies, evidence from *in vitro* studies is provided to support the mechanisms underpinning the proposed health benefits.

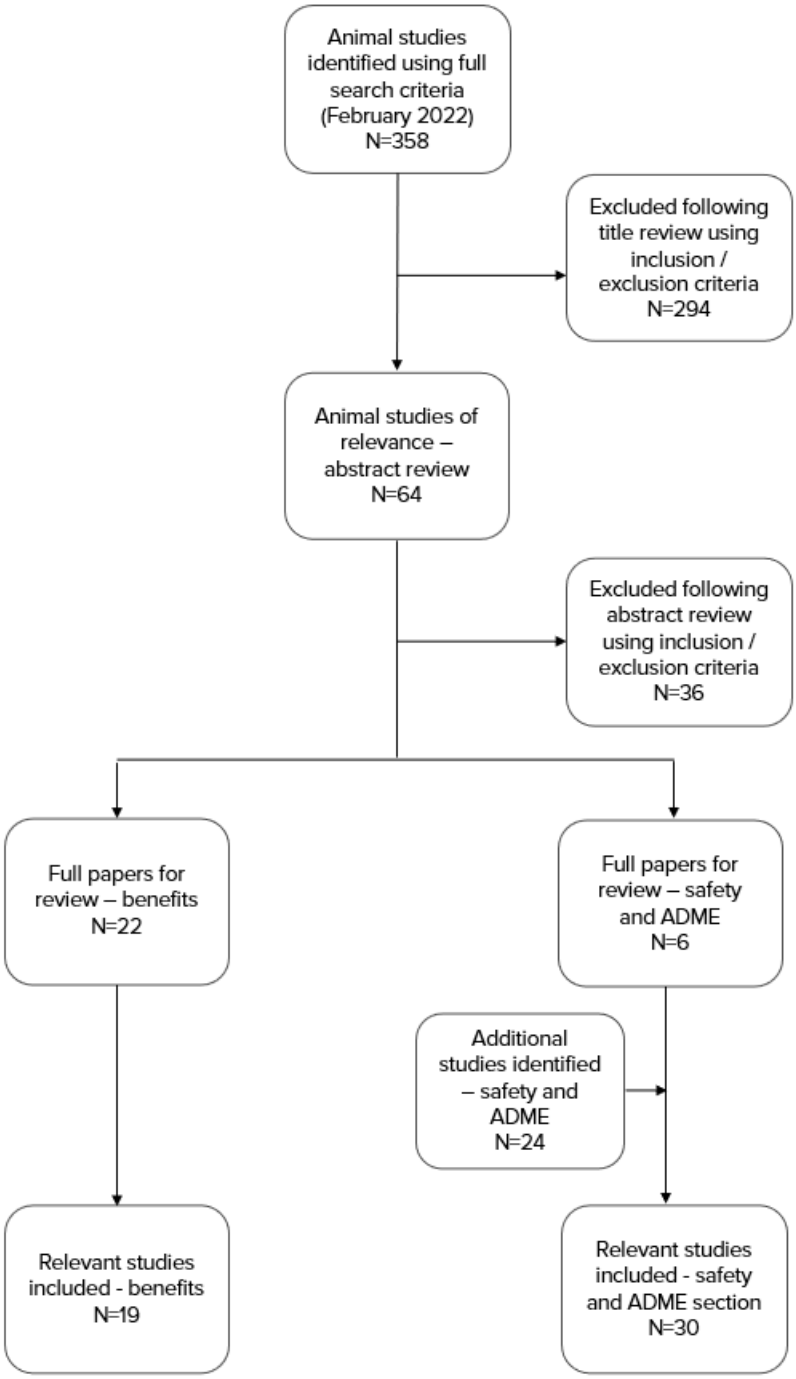

Figure 2-2: Literature search of animal studies used to support benefits and safety of bovine lactoferrin.
ADME = absorption, digestion, metabolism, excretion

2.2. Technical information on the use of bovine lactoferrin

Lactoferrin (Lf) is a non-haeme iron-binding protein that is naturally present in the body and is found in mucosal secretions such as tears, saliva, and nasal and bronchial secretions. It is also present in mammal milks, notably at high levels in human milk, and at lower levels in bovine milk (Demmelmair *et al.*, 2017). The presence of Lf, described as a red protein, was first reported in bovine milk in 1939, however, it was not until 1960 that the isolation, and identification, was successful from both bovine milk (Groves, 1960), and human milk (Johanson, 1960). Lactoferrin 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 (Mead & Tweedie, 1990). Lactoferrins are glycoproteins which are expressed in most mammalian biological fluids and are a major component of the mammalian immune defence system (Baker & Baker, 2012; Legrand *et al.*, 2008). Typically, Lfs have a molecular weight of about 80 kDa with 670-690 amino acid residues, with an interspecies sequence identity of about 70% (Baker *et al.*, 2002), the major differences being in the N-terminal amino acid sequences (Baker & Baker, 2012).

Lactoferrin is made up of two main lobes, with the N-terminal and C-terminal lobes of Lf (Figure 2-3) being unevenly glycosylated, the C-lobe typically containing more N-linked glycosylation sites (Albar *et al.*, 2014). The glycosylation state of Lf can modify the structural conformation of the protein, its susceptibility to proteolysis and consequently its biological activity (Le Parc *et al.*, 2014; van Veen *et al.*, 2004). The glycoprofile of Lf shows a degree of inter-species homology (Table 2-2), however known inter-species variation occurs (Le Parc *et al.*, 2014). While bovine and human Lf are not identical, showing 69% amino acid sequence identity which is associated with some differences in tertiary structure, this results in only minor differences in cellular uptake, and bovine and human Lf have similar functions (Demmelmair *et al.*, 2017).

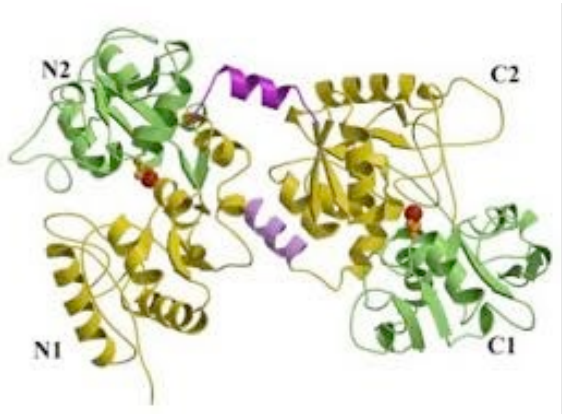

Figure 2-3 Schematic structure of bovine lactoferrin (from Baker et al. (2002))

The structure of Lf is similar to that of transferrin and ovotransferrin and is characterised by the presence of two homologous lobes (N and C), each capable of binding a ferric iron molecule together with a

synergistically bound carbonate ion (Baker & Baker, 2012; Lien *et al.*, 2004). The conformational state of lactoferrin is highly dependent on its metal ion status. In the metal-bound state (holo-lactoferrin) it has a closed highly stable and relatively rigid form, in contrast to the metal-free state (apo-lactoferrin) where the lobes are open (Anderson *et al.*, 1990; Moore *et al.*, 1997). Apo-lactoferrin typically has less than 5% iron saturation[5], in contrast to the iron saturated form (holo-lactoferrin) with an iron saturation approximating 100% (Bokkhim *et al.*, 2013). The degree of iron saturation also affects lactoferrin's bacteriostatic ability (Bullen *et al.*, 1972) (see Section 3.2.3.1.1 for further detail).

Peptides from lactoferrin (lactoferricin, lactoferrampin) are also known to exhibit strong antibacterial activity both *in vitro* and *in vivo* (Vogel, 2012).

Human lactoferrin (hLf) and bLf exhibit a high degree of similarity both structurally and functionally, whilst differing in some properties as summarised in Table 2-2 (adapted from Latorre *et al.*, 2012).

Table 2-2. Similarities and differences between human and bovine lactoferrin (adapted from Latorre et al. (2012))

Lactoferrin properties	**Human versus bovine Lf**
Nucleic acid sequence homology	77%*
Amino acid sequence homology	69%*
Secondary structure	100%*
Disulfide bonding	100%*
Lobe orientation	Different in relative orientation
Glycosylation sites	hLf has 3 sites compared to 5 for bLf
N-acetyllactosamine glycans	Different – glycans present are species specific
Thermoresistance	hLf is more thermo resistant
Proteolysis resistance	hLf is more resistant to proteolysis than bLf possibly due to conformation
DC-SIGN (dentritic-cell-specific intercellular adhesion molecule-3-grabbing non-integrin) binding	bLf shows higher capacity of binding than hLf, resulting in reduced transmission of human immunodeficiency virus 1
Nuclear factor (NF)-κB activation	Difference in glycans results in different levels of activation
Lipopolysaccharide (LPS) binding	100%*
Porin binding	100%*
*Percent similarity	

[5] One molecule of lactoferrin can bind two ferric ions. Iron saturation is calculated using the molecular weights of ferric iron (56g/mol) and lactoferrin (80,000g/mol), whereby the maximum amount of ferric iron bound to lactoferrin (100% saturation) is 112g/80,000g, which equals 140mg/100g. The following formula can be used to calculate iron saturation: iron saturation = iron content per 100g (mg) x 100 / 140. E.g. 15mg iron per 100g bLf equals 10.7% (15x100/140 =10.7).

2.2.1. Information to enable identification of bovine lactoferrin

The Chemical Abstracts Service (CAS) Registry Number for bovine lactoferrin is CAS Reg. No.146897-68-9. Bovine lactoferrin is a 689 amino acid glycoprotein, with 5 potential glycosylation sites (Latorre *et al.*, 2012), the mature bLf protein associated with a 19 amino acid signal peptide (Lönnerdal, 2003). It contains N-glycosidically-linked glycans possessing N-acetylneuraminic acid, galactose, mannose, fucose, N-acetylglucosamine, and N-acetylgalactosamine (Coddeville *et al.*, 1992). van Leeuwen *et al.* (2012) identified and quantified 42 different N-glycan structures in bLf. A schematic of the 3-dimensional structure of bLf is shown in Figure 2-3 in the previous section.

2.2.2. Information on the chemical and physical properties of bLf

The generally accepted physical and chemical properties of bLf are outlined in Table 2-3. Currently there is no monograph for bovine lactoferrin in the Food Chemicals Codex (FCC).

Table 2-3 Physical and Chemical Properties of Bovine Lactoferrin

Property		**Value**	**Reference**
Molecular Mass (Da)			
	Sedimentation co-efficient (aqueous)	77,100 ± 1,500	(Castellino *et al.*, 1970)
	SDS-PAGE	76,000 ± 2,400	(Castellino *et al.*, 1970)
	Iron Titration	78,500	(Aisen & Leibman, 1972)
Isoelectric Point (pH)			
	Chromatofocusing	8.2-8.9	(Shimazaki *et al.*, 1993)
	Isoelectric focusing	9.5-10.0	(Yoshida & Xiuyun, 1991)
Absorption Spectra			
	Apo-form at 280 nm	12.7	(Aisen & Leibman, 1972)
	Holo-form at 470 nm	0.400	(Aisen & Leibman, 1972)
Protease sensitivity		Relatively low (hLf < bLf)	(Brines & Brock, 1983)
Iron-binding			
	Equilibrium dialysis (K1 x 10-4)	3.73	(Aisen & Leibman, 1972)
Thermal Denaturation			
	Apo-Lf denaturation (Tmax: °C)	71 ± 0.3 and 90 ± 0.3	(Paulsson *et al.*, 1993)
	Apo-Lf enthalpy (ΔHcal: J/g)	12 ± 0.4 and 2 ± 0.5	(Paulsson *et al.*, 1993)
	Holo-Lf denaturation (Tmax: °C)	65 ± 0.3 and 93 ± 0.3	(Paulsson *et al.*, 1993)
	Holo-Lf enthalpy (ΔHcal: J/g)	2 ± 1 and 37 ± 1	(Paulsson *et al.*, 1993)

The heat stability of bLf is a function of iron binding status and pH of the environment. Holo-lactoferrin is more resistant to heat induced changes than apo-lactoferrin, with the ability of both forms to bind a range of bacterial species not affected by pasteurisation (72°C for 15 seconds) conditions (Paulsson *et al.*, 1993).

Bokkhim *et al.* (2014) established that mono- and di-saturated bLf display similar thermal stability and tertiary structure, and that the increased thermal stability can be attributed to the binding of the first iron ion of bLf. Apo-lactoferrin is heat stable at pH 4, resisting heating at 90°C for 5 minutes without any significant loss of iron-binding capacity, antigenic activity, or antibacterial activity (Abe *et al.*, 1991). Sanchez *et al.* (1994) showed first order reaction kinetics for denaturation of bLf between 72°C and 85°C and concluded that the standard pasteurisation regimes used in the dairy industry had practically no effect on lactoferrin structure (Steijns & van Hooijdonk, 2000). More recently, the rapid heating conditions typical of pasteurisation were shown to minimise conformation changes even in slightly alkaline conditions (pH 7.5) (Schwarcz *et al.*, 2008). This work addresses concerns regarding the potential for heat-induced conformational changes (Stanciuc *et al.*, 2013), and subsequent loss of bioactivity, that may occur during pasteurisation and spray-drying of lactoferrin. Spray-dried bLf is known to retain its bioactivity (Wang *et al.*, 2017).

For the purposes of bovine lactoferrin use as a novel food ingredient, the European Commission has defined bovine lactoferrin as:

‘Bovine lactoferrin (bLf) is a protein that occurs naturally in cow's milk. It is an iron-binding glycoprotein of approximately 77 kDa and consists of a single polypeptide chain of 689 amino acids. Bovine lactoferrin can be isolated from skimmed milk or cheese whey via ion exchange and subsequent ultra-filtration steps, and is dried by freeze drying or spraying. It is a virtually odourless, light pinkish powder’ (European Commission, 2017).

2.2.3. Information on the impurity profile

The specifications for bLf address the potential presence of impurities such as foreign matter, heavy metals, and contaminants (Section 2.2.5). Other potential impurities include protein fractions and endotoxins, as discussed below.

2.2.3.1.Protein impurities

Information on protein impurities identified in Synlait bLf in an analysis completed by Callaghan Innovation, a New Zealand Government Research Institute that includes accredited test analytical facilities (www.callaghaninnovation.govt.nz/), is shown in Table 2-4. The protein impurities, identified in HPLC profiles, are either intact or truncated protein impurities. This data was processed from a total of 102 data sets from 17 batches. The exact type of protein impurities was not identified by Callaghan Innovation.

Table 2-4 Protein impurity profile of Synlait bLf		
Protein Impurity	**Retention time (Min)**	**Peak (%)**
Impurity 1	6.47±0.02	0.43±0.09
Impurity 2	6.78±0.19	0.46±0.25
Impurity 3	7.78±0.08	0.85±0.27
Impurity 4	7.87±0.04	1.67±1.41
Impurity 5	7.92±0.06	0.81±0.49
Lactoferrin	**8.39±0.08**	**96.09+±0.66**
Impurity 7	8.98±0.09	0.24±0.14
Impurity 8	9.11±0.07	0.66±0.37
Impurity 9	9.23±0.04	0.60±0.33
Impurity 10	10.56±0.37	1.09±0.25

More recently, the impurity profile of Synlait bLf was independently investigated by researchers at the University of California, Davis (Lönnerdal *et al.*, 2020). The researchers used proteomics analysis, which allows identification of specific proteins. The method is unable to provide quantitative levels of impurity proteins, but instead can show relative quantities compared to other samples (Figure 2-4). The study by Lönnerdal *et al.* (2020) confirmed the high degree of relative purity of Synlait lactoferrin.

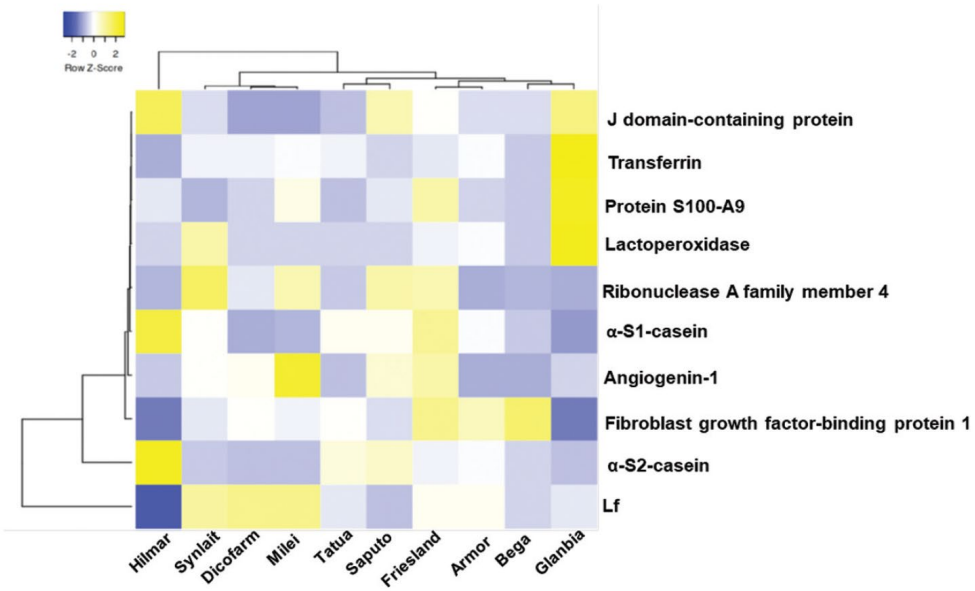

Figure 2-4 Protein impurity profiles of commercial lactoferrin samples as measured by proteomics analysis (From Lönnerdal et al. (2020))

2.2.3.2. Endotoxins

Endotoxin levels in lactoferrin products are a quality parameter controlled and monitored when manufacturing lactoferrin. Endotoxin levels in lactoferrin can vary depending on type of feedstock used (milk or whey) and manufacturing process; for example, salt recycling leads to higher endotoxin levels compared to using salt for only one cycle. Endotoxin measurement is completed by an independent test facility, Callaghan Innovation, a New Zealand Government Research Institute that includes accredited test analytical facilities (www.callaghaninnovation.govt.nz/). Endotoxin levels are measured using the internationally recognised and approved *Limulus* Amebocyte Lysate (LAL) method. The endotoxin levels presented in Table 2-5 are consistently <0.1 endotoxin units (EU)/mg bLf, which means the contribution from lactoferrin to finished products at such low endotoxin levels is negligible.

Typical results for endotoxin levels for products using our standard manufacturing process are presented in Table 2-5 for Batches 1810005089 to 1810006185 (manufactured between November 2018 to December 2018, batches manufactured following a plant upgrade), where salt recycling was not done. The data shows endotoxins effectively being absent. Synlait carried out a salt recycling trial early 2019 to understand how salt cycling may impact endotoxin levels, and observed endotoxin levels up to levels of 68.3 EU/mg bLf. On this basis, Synlait decided to not move ahead with salt recycling at the time.

Table 2-5. Endotoxin Levels in Synlait bLf

Lot number	**Endotoxin (EU/mg bLf)[1]**
1810005089	<0.059
1810005342	<0.048
1810005465	<0.049
1810005792	0.006
1810005578	0.007
1810005581	<0.004
1810006185	0.171
1910003113	0.0068

[1] Endotoxin measured using the LAL method (Endosafe LAL Test Kit, SC Bio Limited, NZ).

While endotoxin levels in some pharmaceutical preparations are tightly regulated (where they bypass the natural barriers of the body, e.g., parenteral nutrition, injections), there are no specifications set for endotoxin levels in foods. In a patented process to produce “endotoxin free” bovine lactoferrin for pharmaceutical-type applications, Thomson *et al.* (2013) described “endotoxin free” bLf as “lactoferrin compositions comprising less than about 20 EU/mg of protein, more preferably less than about 10 EU/mg, and even more preferably less than about 1 EU/mg”. Furthermore they identified that bLf derived from sweet whey typically contained endotoxin levels of at least about 250 EU/mg (with reports of up to 1250

EU/mg), compared to bLf derived from milk typically being at least about 20 EU/mg (Thomson *et al.*, 2013). Ando *et al.* (2010) reported the endotoxin level of commercially available human lactoferrin as ranging between 15-26 EU/mg of protein. Therefore, even when salt recycling was used in trials, endotoxin levels were still well within the boundaries of typical endotoxin levels found in bLf ingredients.

For general food use, no specifications are set for endotoxin levels in the finished product. Pharmaceutical grade bLf may have a maximum level of approximately 1 EU/mg bLF, in some instances.

Although it has been postulated that endotoxin levels in bLf may compromise its potential bioactivity in infant formula (Lönnerdal, 2014), more recently Wakabayashi *et al.* (2018) provided a pragmatic argument to counter that hypothesis, based on the relative abundance (molecular ratio) of bLf that is not associated with endotoxin.

Based on the average molecular weights of endotoxin and bLf (10,000 and 80,000 g/mol, respectively) Wakabayashi *et al.* (2018) calculated that in the most abundant case of endotoxin reported in bLf (72,000 ng LPS/g bLf) (Na *et al.*, 2004), the LPS/Lf molecule ratio is calculated as 1/1724, which means only one endotoxin molecule is contaminated in 1724 Lf molecules and 99.9% of Lf molecules are endotoxin free (Figure 2-5). Endotoxin levels in bLf and estimated molar ratio of endotoxin to bLf are outlined in Table 2-6.

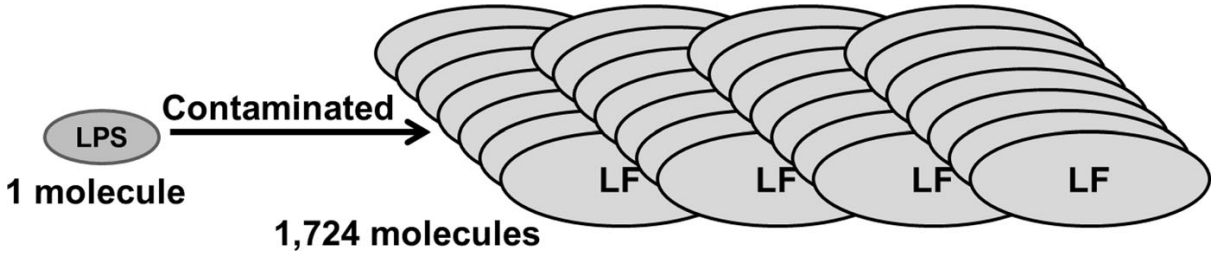

Figure 2-5 Schematic diagram of the endotoxin (LPS):Lf molecular ratio in an endotoxin-abundant bLf sample (from Wakabayashi et al. (2018))

Table 2-6 Endotoxin levels in bLf and the estimated molar ratio of endotoxin to bLf

	LPS level	**Molar ratio of LPS: Lf**
Synlait bLf, typical	<1 EU/mg (≈<200 ng/g, calculated) *	1: 6.3 x 10^5
Trial bLf using salt-recycling, observed highest level	68 EU/mg (≈13,600 ng/g, calculated) *	1: 1.7 x 10^4
Lf, worst case scenario estimate by Wakabayashi *et al.* (2018)	72,500 ng/g	1: 1.7 x 10^3
Lf, best case scenario estimate by Wakabayashi *et al.* (2018)	5 ng/g	1: 2.5 x 10^7

**: Values used in the calculations are 1 EU = 0.2 ng LPS; M_w (Lf) = 80000 g/mol; M_w (LPS) = 10000 g/mol. LPS = lipopolysaccharide*

Ultimately, any potential contribution of endotoxin from the addition of bLf, must be kept in perspective against the background levels of endotoxin inherently present in infant formula as identified by Townsend *et al.* (2007) (40 to 5.5 x 10^4 EU/g or approximately 4 to 550 ng/g of formula powder). Previously Morinaga researchers have shown that even in the presence of excess endotoxin (LPS:bLf w/w ratio 1:100) the antimicrobial activity of bLf against *E. coli* was not changed (Wakabayashi *et al.*, 2018).

In summary, endotoxin levels of commercial bLf can be considered a quality control factor in the manufacture of bLf. There is no evidence to suggest any adverse effects of endotoxins on the performance or safety of bLf, particularly in the context of endotoxin levels inherently present in food products.

2.2.4. Manufacturing process

2.2.4.1.General description of the manufacturing process for bLf

The detailed Synlait manufacturing process for bLf is outlined in Appendix 1 (A1:2) [CONFIDENTIAL], and a simplified process flow is outlined in Figure 2-6. In brief, raw milk is separated, the skim milk stream providing the feedstock used for the chromatographic separation of the bLf. Skim milk is cooled to below 8°C to prevent microbial growth and then clarified and filtered (1 µm filter) to remove any particulate matter, fine insoluble material, reduce the microbial load, and remove fat and fat-soluble compounds.

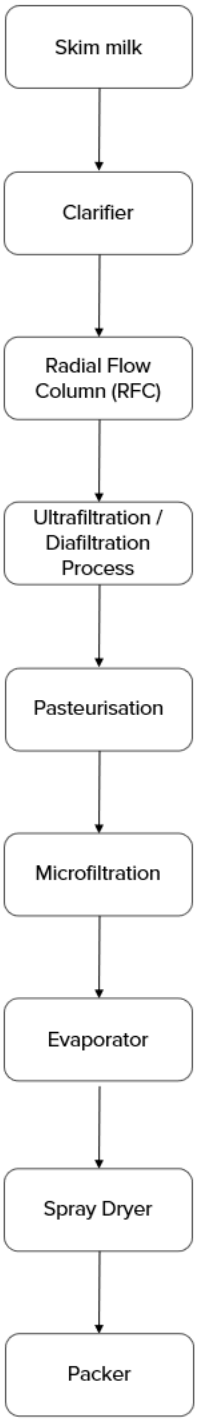

Figure 2-6 Simplified process flow of bLf manufacture

The skim milk filtrate is then passed over a radial flow ion exchange column containing Sepharose Big Beads (GE Healthcare). The filtrate bound to the column is thoroughly rinsed with demineralised water, and then washed with a dilute sodium chloride (NaCl) solution to remove potential contaminants that are weakly bound to the column. The bound bLf is then eluted with a more concentrated NaCl solution (approximately 10% w/v) and desalted using ultrafiltration. The concentrated NaCl removed via ultrafiltration is then recovered using a salt recovery ultrafiltration as diluted NaCl that is used subsequently for removal of protein contaminants in the elution process, after which it is disposed of.

The pH of the bLf ultrafiltrate solution is adjusted to, and maintained at, below pH 6.5; the solution is then pasteurised at 73.5°C for 18 seconds. These conditions exceed the pasteurisation requirements for both NZ as defined in DCP3 (New Zealand Food Safety Authority, 2010) and the heat treatment conditions described for "Grade A' Pasteurized Milk (USFDA Department of Health and Human Services, 2015). The pasteurisation ensures the final bLf product is produced in compliance with mandatory heat treatments for all milk and milk products in final packaged form intended for direct human consumption.

The pasteurised bLf solution is further concentrated with a microfiltration unit (1.2µm) prior to evaporation and spray drying. Spray drying provides a controlled powder particle size, and a less hygroscopic finished material compared to freeze-dried bLf. Spray-drying is the drying technology used in the manufacture of Synlait bLf. Thanks to bLf's relatively high heat stability once isolated, any heat treatment of the bLf isolate has a negligible impact on bLf's integrity.

The typical particle size distribution of bLf from this process is provided in Appendix 3 (A3:19) [CONFIDENTIAL]. Typically, more than 95% will pass through US 120 mesh. The dried bLf powder is hygienically packed into food grade packaging and sealed to protect it from light, air, and moisture.

Each batch of bLf powder is tested to ensure it meets the specifications (Table 2-8). The bLf content in the finished product is determined using a high-performance liquid chromatography (HPLC) method, details of which are presented in Section 2.2.7 *Analytical Method for Detection*.

Critical (quality) control points (CCP) are monitored routinely as a part of the third party audited Risk Management Programme (RMP) Hazard Analysis Critical Control Point (HACCP) process.

2.2.4.2. Materials and Processing Aids

Details of the raw materials and processing aids used in the manufacture of bLf are presented in Table 2-7. The primary raw material used in the production of bLf is raw milk. All milk is sourced from registered and accredited suppliers of fresh cow's milk, collected from the farms in the wider Canterbury region of New Zealand, by Synlait Milk Ltd and processed under an approved "Risk Management Programme" (RMP) certified and approved by the New Zealand (NZ) Government agency, the Ministry for Primary Industries (MPI). The RMP is a program designed to identify, control, manage, eliminate, or minimise hazards and other risks during the processing of animal materials and products. Raw milk supply in NZ is governed by the Animal Products Act (1999), and the Animal Products (Raw Milk Product Specifications) Notice 2009. The Synlait Raw Milk (Monitoring) Specification is presented in Appendix 1 (A1:23) [CONFIDENTIAL] and the

Specification for Skim Milk (Monitoring) is also presented in Appendix 1 (A1: 32) [CONFIDENTIAL] and is fully compliant with the requirements of the Animal Products (Raw Milk Product Specifications) Notice 2009.

Synlait Milk Ltd is a registered Dairy Processor approved by MPI with the designated Unique Location Identifier – manufacturing 540. It is approved for the manufacture of a range of dairy products. The Synlait facility is also a USFDA registered facility (USDFA Registration No. 15930127872).

The ion exchange column material, Sepharose Big Beads, is approved for use as a food contact substance (FSANZ Application A1120 (approved 20 May 2016)), approved for food use as an ion exchange resin, and also for repeated use in extracting individual proteins or substances present in low concentrations from aqueous food materials such as milk, whey, fruit juice, beer and wine. The approved process conditions include pH 3-14 and temperatures of 5-60°C, which covers the operating range for the extraction of bLf from skim milk. An example of the manufacturer's Certificate of Analysis, including specification limits, and safety data sheet for Sepharose Big Beads are presented in Appendix 1 (A1: 3) [CONFIDENTIAL]. Following separation of the bLf by ion exchange, the bLf-containing solution is concentrated by ultrafiltration (UF). Details of the semi-permeable polyethersulfone (PES) UF membrane (Synder MK PES 30,000 Da Sanitary UF Membrane) are provided in Appendix 1 (A1: 5) [CONFIDENTIAL]. Membranes of this class have molecular cut-offs of 30,000 Da. Ultrafiltration membranes with molecular cut-offs of 5,000 Da (Tami Tubular Ceramic Membranes Isoflux) are also used for the recovery of the salt solution. Technical details of those membranes are also provided in Appendix 1 (A1: 7) [CONFIDENTIAL]. All materials and membranes used for separation, concentration and packaging of the Synlait bLf are safe and suitable and used in accordance with the regulations for food contact materials.

The processing aids used in the production of bLf are all designated food grade (Table 2-7). The potable water undergoes reverse osmosis treatment prior to use in this process. Specifications for the salt (sodium chloride) are presented in Appendix 1 (A1:37 and A1: 39) [CONFIDENTIAL]. Synlait draws its water from local subterranean aquifers, treats with chlorine to a residual of <5ppm, and filters prior to use in milk or product contact processing requirements. The testing schedule to ensure that the water quality meets the NZ Drinking Water Standards (Ministry of Health, 2008) is outlined in the water quality specification (Appendix 1, A1: 9) [CONFIDENTIAL].

Table 2-7. Raw materials and processing aids used in the production of Synlait milk-derived bLf					
Material	**CAS Number**	**Purity (%)**	**Function**	**Source**	**Regulatory Approvals**
Raw Materials					
Raw milk	N/A	100%	Raw material	Synlait Raw Milk Supply	Conforms to NZ Animal Products Act, and Animal Products (Raw Milk Product Specifications) Notice 2009
Skim milk (unpasteurised)	N/A	100%	Raw material	In process milk	N/A
Processing Aids					
Demineralised water	N/A	100%	Diluent for salt solution	On site RO water	Conforms to NZ Drinking Water Standards
Sodium Chloride	7647-14-5	min. 99.6%	Salt squeeze for ion exchange resin	Dominion Salt, NZ	Meets FSC requirements
Sepharose Big Beads	N/A	N/A	Ion exchange resin	GE Healthcare	FSANZ Application A1120 (approved 20 May 2016)
Ultrafiltration Membranes	N/A	N/A	Protein concentration, demineralization & salt recovery	Synder	USDA 3-A Standards and 21 CFR requirements
Microfiltration Membrane	N/A	N/A	Microbial load, bLf concentration and particulate reduction	Tami	USDA 3-A Standards

2.2.5. Specification for identity and purity

Specifications are presented below for Synlait manufactured spray-dried bLf (Table 2-8). The specification parameters comprise physical appearance, purity, total bLf levels, moisture, among others, as well as limits for potential chemical and microbiological impurities, and contaminants. Analytical results from five batches of powdered bLf manufactured January and June 2021 (Table 2-9) suggest that Synlait's spray-dried bLf consistently conforms with the food-grade specifications set for this product.

A comparison of the Synlait specification with those set for the European Union (European Commission, 2017) (Appendix A4:2), and for China (GB 1903.17 – 2016) (Appendix A4: 7) is provided in Table 2-10.

Table 2-8. Manufacturing Specifications for Synlait spray-dried bLf powder

Parameter	Specification	Method
General composition		
Protein (Nx6.38)	≥95 g/100g	ISO 8968-1/IDF 20-1:2014
Lactoferrin (Purity)	≥95 % of protein	RP-HPLC Method
Ash	≤1.3 g/100g	BS 1743:1968 (modified)
Moisture	≤4.5 g/100g	GB 5009.3-2016 (modified)
Iron	≤15 mg/100g	AsureQuality Method (ICP-OES)
Fat	≤1 g/100g	AsureQuality Method
Physical attributes		
Scorched Particles	A (/25g)	AsureQuality Method
Foreign Matter	Absent (/25g)	AsureQuality Method
pH (10% solution)	5.2 – 7.2	AS2300.1.6-2010
Solubility Transmittance	Transparent	Synlait Method TCH-05-0010
Transmittance of Lactoferrin	80-100%	Synlait Method TCH-05-0010
Contaminants		
Heavy metals	<10.0 mg/kg	AsureQuality Method (ICP-MS)
Melamine	Not detected	USFDA LIB 4421 Vol 24 Oct 2008 (modified)
Arsenic	<0.020 mg/kg	AsureQuality Method (ICP-MS)
Aluminium	<4.8 mg/kg	AsureQuality Method (ICP-MS)
Cadmium	<0.10 mg/kg	AsureQuality Method (ICP-MS)
Mercury	<0.10 mg/kg	AsureQuality Method (ICP-MS)
Lead	<0.020 mg/kg	AsureQuality Method (ICP-MS)
Aflatoxin M1	<0.05 µg/kg	AsureQuality Method (UPLC)
Nitrate	≤50.0 mg/kg	ISO 14673-3/ IDF 189-3: 2004 (modified)
Nitrite	≤2.0 mg/kg	ISO 14673-3/ IDF 189-3: 2004 (modified)
Microbiological Tests		
Aerobic Plate Count	<1000 cfu/g	ISO 4833-1:2013 (modified)
Yeasts and Moulds	<10 cfu/g	ISO 6611/IDF 94:2004 (modified)
Escherichia coli	Not detected (/g)	Synlait In-House Method
Salmonella	Not detected (/250g)	ISO 6579-1:2017 (modified)
Coagulase-positive Staphylococci Detection	Not detected (/25g)	ISO 6888-3:2003 (modified)
Coliforms Detection	Not detected (/g)	Synlait In-House Method
Cronobacter species	Not detected (/300g)	ISO 22964:2017 (modified)
Enterobacteriaceae Detection 37°C	Not detected (/g)	ISO 21528-1:2017 (modified)
Listeria	Not detected (/125g)	ISO 11290-1:2017 (modified) (ALOA 37°C, Palcam 35°C)

Table 2-8. Manufacturing Specifications for Synlait spray-dried bLf powder		
Parameter	**Specification**	**Method**
Bacillus cereus Count (presumptive) 30°C	<20 cfu/g	ISO 7932:2004 (modified)
Sensory attributes		
Appearance	Pink to reddish brown colored, free-flowing powder	Dairy Industry Standard Method

Table 2-9. Batch Data of Synlait spray-dried bLf powder

Specification Parameter	**Limit**	**LOT No. s**				
		LFN2010010379	LFN2110001431	LFN2110002430	LFN2110003132	LFN2110004046
General composition						
Protein (Nx6.38)	≥95 g/100g	96.2	97.0	97.3	97.9	98.1
Lactoferrin (Purity)	≥95 % of protein	96.9	97.2	97.2	97.6	97.0
Ash	≤1.3 g/100g	0.1	0.2	0.1	0.1	0.2
Moisture	≤4.5 g/100g	4.0	3.6	4.0	3.5	3.7
Iron	≤15 mg/100g	11	12	13	13	12
Fat	≤1 g/100g	<0.1	0.1	<0.1	<0.1	<0.1
Physical attributes						
Scorched Particles	A (/25g)	A	A	A	A	A
Foreign Matter	Absent (/25g)	Absent	Absent	Absent	Absent	Absent
pH (10% solution)	5.2 – 7.2	5.7	5.7	5.7	5.8	5.8
Solubility Transmittance	Transparent	Transparent	Transparent	Transparent	Transparent	Transparent
Transmittance of Lactoferrin	80-100%	96.1	95.8	96.1	96.4	96.3
Contaminants						
Heavy metals	<10.0 mg/kg	<3.0	<3.0	<3.0	<3.0	<3.0
Melamine	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected
Arsenic	<0.020 mg/kg	<0.020	<0.020	<0.020	<0.020	<0.020
Aluminium	<4.8 mg/kg	<1.0	<1.0	<1.0	<1.0	<1.0
Cadmium	<0.10 mg/kg	<0.0020	<0.0020	<0.0020	<0.0020	<0.0020
Mercury	<0.10 mg/kg	<0.010	<0.010	<0.010	<0.010	<0.010
Lead	<0.020 mg/kg	<0.010	<0.010	<0.010	<0.010	<0.010
Aflatoxin M1	<0.05 µg/kg	<0.01	<0.01	<0.01	<0.01	<0.01
Nitrate	≤50.0 mg/kg	24	30	21	27	29
Nitrite	≤2.0 mg/kg	≤0.1	0.2	0.1	0.1	<0.1

Table 2-9. Batch Data of Synlait spray-dried bLf powder

Specification Parameter	**Limit**	**LOT No. s**				
		LFN2010010379	LFN2110001431	LFN2110002430	LFN2110003132	LFN2110004046
Microbiological Tests						
Aerobic Plate Count	<1000 cfu/g	<10	<10	<10	<10	<10
Yeasts and Moulds	<10 cfu/g	<1	<1	<1	<1	<1
Escherichia coli	Not detected (/g)	Not detected	Not detected	Not detected	Not detected	Not detected
Salmonella	Not detected (/250g)	Not detected	Not detected	Not detected	Not detected	Not detected
Coagulase-positive Staphylococci Detection	Not detected (/25g)	Not detected	Not detected	Not detected	Not detected	Not detected
Coliforms Detection	Not detected (/g)	Not detected	Not detected	Not detected	Not detected	Not detected
Cronobacter species	Not detected (/300g)	Not detected	Not detected	Not detected	Not detected	Not detected
Enterobacteriaceae Detection 37°C	Not detected (/g)	Not detected	Not detected	Not detected	Not detected	Not detected
Listeria	Not detected (/125g)	Not detected	Not detected	Not detected	Not detected	Not detected
Bacillus cereus Count (presumptive) 30°C	<20 cfu/g	<10	<10	<10	<10	<10
Sensory attributes						
Appearance	Pink to reddish brown coloured, free-flowing powder	Typical	Typical	Typical	Typical	Typical

Table 2-10. Comparison of Regulatory and Synlait Specifications for bLf (powder form)

Parameter	**European Union** (European Commission 2017, Appendix A4:2)	**Peoples Republic of China** (GB 1903.17 – 2016, Appendix A4:7)	**Synlait Milk Ltd.**
Physical and Chemical Parameters			
Description	Virtually odourless, light pinkish powder	Pale pink to reddish brown powder	Pink to reddish brown coloured, free-flowing powder
Protein of which bovine lactoferrin of which other proteins	> 93.0% > 95.0% < 5.0%	≥ 93.0% ≥ 95.0%	≥ 95.0 g/100g ≥ 95.0%
Moisture (loss on drying)	<4.5%	≤4.5%	≤ 4.5 g/100g
Ash	< 1.5%	≤2.0%	≤ 1.3 g/100g
Arsenic	<2.0 mg/kg	≤ 1 mg/kg	≤ 0.02 mg/kg
Lead	-	≤ 1 mg/kg	<0.020 mg/kg
Iron	< 350 mg/kg	≤ 35mg/100g	≤ 15 mg/100g
pH (2% solution, 20°C)	5.2 to 7.2	5.2 to 7.2	5.2 to 7.2
Solubility (2% solution, 20°C)	Complete	Complete, transparent, no visible impurities	Transparent
Microbiological			
Total / Standard Plate Count	-	≤ 1000 cfu/g	< 1000 cfu/g
Yeasts & Moulds	-	≤ 10 cfu/g	< 10 cfu/g
Coliforms	-	< 3.0 MPN/g	Not detected /g
Salmonella	-	Not detected /25 g	Not detected /250 g
Staphylococcus aureus (coagulase positive)	-	Not detected /25 g	Not detected /25g

As recognised by the European Commission (European Commission, 2012a, 2012b, 2017) commercially available bLf is substantially equivalent when made to similar specifications. This is, for example, further highlighted in Table 2-11 comparing Synlait bLf to that of Morinaga Milk Industry Co. Ltd, and FrieslandCampina.

Of particular note is the similarity of composition between the different sources of bLf which is especially important as much of the early research across *in vitro, in vivo* animal models and human clinical studies were undertaken using bLf sourced from either Morinaga Milk or FrieslandCampina. Similarity across the sources infers that the research completed is transferrable across bLf in general, a fact recognised by the European Union in setting a general specification for bLf. Equivalence is discussed in more detail in Section 3.2.3.2.

Table 2-11. Batch Data of Synlait, Morinaga and FrieslandCampina bLf

Specification Parameter	**Synlait bLf Batches**					**Morinaga bLf Batch Data** (GRN 465, 2014)						**FrieslandCampina Batch Data** (EFSA Panel on Dietetic Products Nutrition and Allergies, 2012)					
	LFN201 0010379	LFN2110 001431	LFN2110 002430	LFN2110 003132	LFN2110 004046	131110	151110	171110	101110	161210	211210	103713 35	103880 40	103903 71	103915 87	103937 23	104096 99
General composition																	
Total Protein (g/100g)	96.2	97.0	97.3	97.9	98.1							97.4	97.7	97.7	97.8	96.6	97.3
Total Protein (%dry weight)						98.8	99.4	99.3	99.2	98.8	98.7						
Lactoferrin purity (% protein)	96.9	97.2	97.2	97.6	97.0	97.3	96.8	96.8	97.0	97.7	97.2	>95	>95	>95	>95	>95	>95
Ash (g/100g)	0.1	0.2	0.1	0.1	0.2							0.20	0.21	0.32	0.25	0.30	0.12
Ash (% dry weight)						0.13	0.17	0.11	0.07	0.09	0.05						
Moisture (% m/m)	4.0	3.6	4.0	3.5	3.7	0.41	0.05	0.35	0.54	0.70	0.43	3.04	3.39	3.25	3.29	3.53	2.95
Iron Content (mg/100g)	11	12	13	13	12	21.1	21.7	19.7	8.20	9.59	9.51						
Physical attributes																	
Foreign Matter (in 25g)	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent						
pH	5.7	5.7	5.7	5.8	5.8	5.53	5.58	5.75	5.50	5.59	5.20	5.8	5.8	5.8	5.8	5.7	5.8
Solubility (2% solution, 600 nm)	TP	TP	TP	TP	TP	100	100	100	100	100	100						
Transmittance (2% solution, 600 nm) (%)	96.1	95.8	96.1	96.4	96.3	91.9	92.0	90.6	81.4	95.2	85.7	91	88	91	90	90	93
Contaminants																	
Lead (Pb) (mg/kg)	<0.010	<0.010	<0.010	<0.010	<0.010	ND	ND	ND	ND	ND	ND						
Cadmium (Cd) (mg/kg)	<0.002	<0.002	<0.002	<0.002	<0.002	ND	ND	ND	ND	ND	ND						
Mercury (Hg) (mg/kg)	<0.010	<0.010	<0.010	<0.010	<0.010	ND	ND	ND	ND	ND	ND						
Arsenic (As) (mg/kg)	<0.020	<0.020	<0.020	<0.020	<0.020	ND	ND	ND	ND	ND	ND						
Aflatoxin M1	<0.01	<0.01	<0.01	<0.01	<0.01	ND	NT	NT	ND	NT	NT						
Microbiological Tests																	
Aerobic Plate Count (cfu/g)	<10	<10	<10	<10	<10	0	0	0	0	0	0	<1000	<1000	<1000	<1000	<1000	<1000
Coliforms (in 1g)	ND	ND	ND	ND	ND	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.						
Coagulase positive Staphylococcus aureus (in 1g)	ND	ND	ND	ND	ND	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
Yeasts and Moulds (cfu/g)	<1	<1	<1	<1	<1	0	0	0	0	0	0	<10	<10	<10	<10	<10	<10

Table 2-11. Batch Data of Synlait, Morinaga and FrieslandCampina bLf

Specification Parameter	**Synlait bLf Batches**					**Morinaga bLf Batch Data** (GRN 465, 2014)						**FrieslandCampina Batch Data** (EFSA Panel on Dietetic Products Nutrition and Allergies, 2012)					
	LFN201 0010379	LFN2110 001431	LFN2110 002430	LFN2110 003132	LFN2110 004046	131110	151110	171110	101110	161210	211210	103713 35	103880 40	103903 71	103915 87	103937 23	104096 99
Salmonella (in 25g)	Absent	Absent	Absent	Absent	Absent	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
Enterobacteriaceae (cfu/g)	ND	ND	ND	ND	ND							<10	<10	<10	<10	<10	<10
Cronobacter species / Cronobacter sakazakii (in 300g)	ND	ND	ND	ND	ND	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.						
Sensory properties																	
Appearance	Typical	Typical	Typical	Typical	Typical	Conform	Conform	Conform	Conform	Conform	Conform						
Minerals																	
Sodium (mg/100g)	55	63	62	52	45	38.0	39.9	42.0	33.5	47.1	48.2						
Potassium (mg/100g)	<0.91	<0.91	<0.91	<0.91	<0.91	1.00	5.36	5.30	1.09	1.11	2.70						
Magnesium (mg/100g)	<0.14	<0.14	<0.14	<0.14	<0.14	0.49	0.51	0.55	0.56	0.55	0.53						
Phosphorus (mg/100g)	2.6	2.0	1.1	1.6	1.2	3.25	3.87	3.99	2.95	3.14	3.57						
Calcium (mg/100g)	<0.445	<0.445	<0.445	<0.445	<0.45	8.14	8.87	9.15	7.81	7.93	7.69						
Chloride (%m/m)	0.751	0.755	0.753	0.729	0.755	0.766	0.816	0.758	0.795	0.764	0.889						
Copper (µg/100g)	12	<11	<11	<11	<11	280	90	310	ND	ND	ND						
Zinc (mg/100g)	0.63	0.57	0.35	0.32	0.17	0.15	0.64	0.32	0.32	0.29	0.25						
Manganese (mg/100g)	<0.007	<0.007	<0.007	<0.007	<0.007	ND	ND	ND	0.01	0.01	0.01						

*%m/m = % mass/mass, Neg. = Negative, ND= Not Detected; NT = Not Tested; TP = Transparent; Blank cells indicate no data available

2.2.6. Bovine lactoferrin stability

The stability of Synlait's bLf spray-dried powder was evaluated using an in-house accelerated storage protocol, as detailed below. Samples were packed as for commercial purpose, into multilayer oxygen and water vapor resistant 5kg packs and stored in temperature-controlled incubators at 40°±1°C for 36 weeks. The accelerated storage protocol is based on prediction of the kinetics of degradation described by the Arrhenius equation. This is one of the most common models used for shelf-life prediction (Calligaris *et al.*, 2019; Mizrahi, 2004; Taoukis *et al.*, 1997).

A historically useful generalisation based on the Arrhenius equation is that for many common reactions at room temperature, the reaction rate doubles for every 10°C increase in temperature (Aisami *et al.*, 2017). As such, the rate of degradation from a 20°C ambient study, will be increased fourfold if the study is conducted at 40°C. According to the accelerated storage protocol 1 week at 40°C is approximately equivalent to 1 month at ambient temperature. Hence, 36 weeks at 40°C represents an equivalent ambient storage period of 36 months (3 years). Analysis results of the samples at t=0, t=30 and t=36 weeks are presented in Table 2-12 and Table 2-13.

Results of the accelerated shelf-life test (Lot 1410001046; Date of Manufacture 01 May 2014) (Table 2-12 and Table 2-13) show no significant changes in any of the physical, chemical, physicochemical or microbiological parameters over the 36 weeks at 40°C. Based on the accelerated storage protocol, bLf powder manufactured by Synlait, and commercially packed, is stable and has a shelf life of at least 3 years (36 months) at ambient temperatures in unopened packs. Ambient (<25°C, <65% RH) shelf-life testing, in the 5kg commercial packs, has been evaluated at 12 months and has been on-going in order to validate the accelerated storage data in real time. At 12 months there were no notable changes to the product or its' microbiological status.

Table 2-12 Accelerated stability testing of Synlait bLf spray-dried powder- Physicochemical

Test Parameter	Units	Specification	Week 0	Week 30 (@ 40°C)	Week 36 (@40°C)
Lactoferrin	% protein	≥95*	94.6*	95.7	95.5
Protein	%m/m	≥95*	97.5	96.2	96.7
Ash	%m/m	≤1.3	0.2	0.2	0.3
Moisture	%m/m	≤4.5	3.8	4.3	3.9
Iron	mg/100g	≤15	11	11	11
Fat	%m/m	<1.0	Not tested	<0.1	0.1
Sediment (/25g)		A	A		
Foreign Matter (/25g)		Absent	Absent	Absent	Absent
pH (2% solution)		5.2-7.2	6.5	6.36	6.29
Solubility Transmittance	%	Transparent 80-100	Transparent 91.8	Transparent 94.0	Transparent 93.3
Appearance		Typical	Typical	Typical	
Nitrates	mg/kg	<150	115	83	76
Nitrites	mg/kg	<2	0.3	0.2	0.1
Heavy Metals	mg/kg	<3	<3	<3	<3
Arsenic	mg/kg	<0.02	Not detected	<0.02	<0.02
Aluminum	mg/kg	<4.8	<1	<1.0	<1.0
Cadmium	mg/kg	<0.1	<0.002	<0.002	<0.002
Mercury	mg/kg	<0.1	<0.01	<0.01	<0.01
Lead	mg/kg	<0.02	<0.01	<0.01	<0.01
Aflatoxin M1	µg/kg	<0.05	<0.02	<0.025	<0.025

**Note that at the time of manufacture of the powder used for shelf-life testing a different specification was in place requiring lower lactoferrin purity and protein content*

Table 2-13 Accelerated stability testing of Synlait spray-dried bLf powder - Microbiological

Test parameters	Unit	Specification	Week 0	Week 30 (@ 40°C)	Week 36 (@ 40°C)
Aerobic Plate Count	cfu/g	<1000	<10	<10	<10
Thermophilic Aerobic Spores	cfu/g		<10	<1	<10
Mesophilic Aerobic Spores	cfu/g		<1	<1	<1
Yeasts and Molds	cfu/g	<10	<1	<10	<10
Coliforms	/g	Not detected	Not detected	Not detected	Not detected
Escherichia coli	/g	Not detected	Not detected	Not detected	Not detected
Bacillus cereus	cfu/g	<10	<10	<10	<10
Enterobacteriaceae	/g	Not detected	Not detected	Not detected	<1
Coag. Positive Staph. aureus	/g	Not detected	Not detected	Not tested	Not detected
Chronobacter sakazakii	/300g	Not detected	Not detected	Not tested	Not detected
Listeria	/125g	Not detected	Not detected	Not tested	Not detected
Salmonella	/250g	Not detected	Not detected	Not tested	Not tested

2.2.7. Analytical method for detection

A method to determine the bLf content and purity of lactoferrin was developed by Callaghan Innovation, New Zealand. Using a modified method, HPLC analysis of bLf is carried out on a HPLC system equipped with a temperature-controlled column oven and UV-Vis detector recording at 280nm. Samples are dissolved in Milli-Q-grade water and diluted in HPLC solvent A and injected onto a selected reversed-phase (RP)-HPLC column. Protein peaks present in the chromatogram recorded at 280nm are integrated, and area of the Lf peak is used for determination of lactoferrin content, and the %bLf area is used to determine the bLf purity, respectively. The bLf content of products is expressed as weight percent (g/100g), and purity is expressed as %bLf. Identification of peaks is based on their retention times and absorption spectra at 280nm compared against a commercial lactoferrin standard by *Wako Laboratory Chemicals*, a division of *Wako Chemicals USA, Inc*. The repeatability and reliability stated in the reference method are within ±2% for bLf content and ±2% for purity testing. The repeatability and intermediate precision calculated from the last in-house reference validation for bLF content were 2% and 3.5% respectively, and for purity 0.5% and 1.1% respectively. A detailed description of the reference analytical method can be found in Appendix A3:2 [CONFIDENTIAL]. Synlait method – 01890 Determination of Lactoferrin by RP-HPLC is a modified version of the reference method.

2.2.8. Information on the proposed food label

For the ingredients listing, the proposed labelling is “lactoferrin" in accordance with the definition of the European Commission outlined in Regulation (EU) 2017/2470 (European Commission, 2017). Statement of the level of bLf in the nutrition information statement (NIS) should be listed as “Lactoferrin”.

As per FSC requirements (Standard 2.9.2-21(iii)) the quantity of bLf must be expressed in weight /100mL (inclusive of any naturally occurring amount).

Given that there is emerging interest in, and the development of lactoferrin sources from other species, together with the possibility of future production of recombinant human lactoferrin, ensuring the use of bLf is appropriately labelled will mitigate potential future ambiguity.

As bLf is a cows’ milk protein, if added to products that otherwise do not contain dairy ingredients, there will be a requirement to include “milk” in the statement of ingredients as per Standard 1.2.3 – Information requirements – warning statements, advisory statements and declarations.

2.2.8.1. Information related to labelling requirements under 2.9

The option of bLf as a nutritive substance for infant formula products (Standard 2.9.1) requires labelling in accordance with Standard 1.2.4 Information requirements – statement of ingredients. The proposed labelling is “lactoferrin” as commonly known either as an ingredient of a compound ingredient or individually as an ingredient of the food for sale.

Standard 2.9.1-21 Declaration of nutrition information for infant formula products and Standard 1.2.8-6 nutrition information must include the name and average quantity of any other nutrient or biologically active substance expressed in units as appropriate. The proposed labelling “lactoferrin” used as a nutritive substance listed in the NIS expressed in weight/100mL (including any naturally occurring amount).

Standard 1.2.3 Information requirements – warning statements, advisory statements, and declarations, specifically 1.2.3-4 Mandatory declarations of certain foods means there is a requirement to include “**milk**” in the statement of allergens if bLf is added to any Infant formula products.

Standard 1.2.3-6(4) specifically addresses mandatory declarations for IFSDN, requiring the name of the food in accordance with subsections 1.2.3-6(4) and 1.2.3-6(5); for example, lactoferrin (**milk**).

It is important to note that Standard 1.2.7-4 prohibits health and nutrition claims on infant formula products. Furthermore, attention cannot be drawn to the addition of nutritive substances on pack, nor can the benefits be communicated, and specifically formula cannot be labelled with the word “humanised” or “maternalised” or any word or words having the same or similar effect (Standard 2.9.1-24).

An example of the ingredient listing, NIS and allergen statement for an infant formula product suitable for either 0-6 months or 6-12 is shown below (Figure 2-7).

Ingredients

Milk solids [lactose, demineralised whey powder, whole milk, whey protein concentrate, skim milk, lactoferrin], vegetable oils [soy, high oleic sunflower, coconut, canola, emulsifier (soy lecithin), antioxidant (mixed tocopherol)], galacto-oligosaccharide [GOS], minerals [sodium, calcium, phosphorus, potassium, chloride, magnesium, iron, zinc, selenium, copper, manganese, iodine], dried omega-3 and omega-6 oils [fish oil (tuna), arachidonic acid oil, sodium caseinate, emulsifier (soy lecithin)], vitamins [vitamins (A, B6, B12, C, D, E, K), thiamin, riboflavin, niacin, pantothenic acid, biotin, folic acid], acidity regulator [calcium hydroxide, citric acid], choline, taurine, inositol, nucleotides [cytidine 5'-monophosphate, uridine 5'-monophosphate, adenosine 5'-monophosphate, inosine 5'-monophosphate, guanosine 5'-monophosphate], l-carnitine. **Contains milk, soy and fish**

Nutrition information

	AVERAGE QUANTITY PER 100mL PREPARED FEED*
Energy	283kJ
Protein	1.5g
- Whey protein 60%	0.88g
- Casein protein 40%	0.59g
- A2 beta-casein	0.20g
Fat, total	3.5g
- Omega-3	76mg
- α-Linolenic acid (ALA)	64mg
- Docosahexaenoic acid (DHA)	12mg
- Omega-6	588mg
- Linoleic acid	576mg
- Arachidonic acid (ARA)	12mg
Carbohydrate	7.3g
Vitamins	
Vitamin A	68µg-RE
Vitamin B_6	56µg
Vitamin B_{12}	0.27µg
Vitamin C (ascorbic acid)	19mg
Vitamin D	0.94µg
Vitamin E	1.2mg α-TE
Vitamin K	6.3µg
Biotin	3.4µg
Niacin (Vitamin B_3)	536µg
Folic acid	13.4µg
Pantothenic acid (Vitamin B_5)	500µg
Riboflavin (Vitamin B_2)	160µg
Thiamin (Vitamin B_1)	76µg
Minerals	
Calcium	53mg
Copper	52µg
Iodine	9.4µg
Iron	0.75mg
Magnesium	6.7mg
Manganese	44µg
Phosphorus	35mg
Zinc	0.70mg
Selenium	2.5µg
Chloride	51mg
Potassium	73mg
Sodium	22mg
Other	
Galacto-oligosaccharide (GOS)	362mg
L-carnitine	1.1mg
Taurine	5.4mg
Choline	13mg
Inositol	4.42mg
Nucleotides, total	3.3mg
- Adenosine 5'-monophosphate	0.72mg
- Cytidine 5'-monophosphate	1.1mg
- Guanosine 5'-monophosphate	0.21mg
- Inosine 5'-monophosphate	0.46mg
- Uridine 5'-monophosphate	0.80mg
Lactoferrin	xxmg

* Refer to feeding guide

Figure 2-7 Example of ingredient list and nutrition information statement for an infant formula product containing bLf

2.3. Data related to the safety of bovine lactoferrin

2.3.1. Absorption, distribution, metabolism, and excretion of bLf

By the 24th week of gestation, the human foetal gut is sufficiently developed to enable the digestion and absorption of nutrients; hence, even premature infants are able to digest and absorb macronutrients (Lentze, 2015). Endogenous levels of lactoferrin exist in numerous organs of the human foetus, and is understood to be associated with maturity of the immune system (Reitamo *et al.*, 1981). Breastfed infants are exposed to dietary lactoferrin that has the capacity to exert a number of physiological functions including immunomodulation, antiviral and antibacterial activities (Lönnerdal, 2016).

The ability to study mechanisms of digestion, distribution, and metabolism in human infants is relatively limited; hence, various animal models are used as a proxy. Constable *et al.* (2017) reviewed the suitability of animal models for their use in the evaluation of safety and metabolism of food additives in early life. They concluded that rat and mouse animal models were of limited comparability for a wide range of tissues and organs including the gastrointestinal (GI) tract, liver, kidneys, reproductive systems, immune system, brain, central nervous system (CNS), neurodevelopment, and cognitive development (Constable *et al.*, 2017). In contrast, for human infants, the piglet is a more suitable model; postnatal gastrointestinal development and the nutritional requirements of piglets better reflecting that of the human infant (Alizadeh *et al.*, 2016; Constable *et al.*, 2017; Donovan, 2016; Miller & Ullrey, 1987; Moughan *et al.*, 1992). The digestion of lactoferrin has been extensively studied in piglet models, enabling some understanding of its absorption, distribution, metabolism, and excretion. Thus, due to the suitability of piglet and growing pig models for infant metabolism such studies are the primary focus in this section, with contribution from other animal models where appropriate. Where evidence from human infant studies is available this is also presented.

The biological activities of dietary lactoferrin from breast milk, or bLf supplemented formula can occur as either local effects in the gut lumen; e.g., bacteriostatic or bactericidal effects; or systemically mediated by the lactoferrin receptors (LfR) and transport into the systemic circulation, e.g., iron uptake, immunomodulatory effects, and epithelial growth and differentiation (Lönnerdal *et al.*, 2011).

2.3.1.1. Absorption

2.3.1.1.1. In vitro studies

Early *in vitro* digestion models suggested that lactoferrin was relatively resistant to digestion and intestinal degradation (Brock *et al.*, 1976). For lactoferrin to exert biological functions in the small intestines there is a requirement that it is, at least to some extent, resistant to digestion, and that it must interact with gastrointestinal tissue. Kawakami and Lönnerdal (1991) isolated human Lf receptors from foetal intestinal brush border membranes, finding the receptors demonstrated specific Lf binding, with similar affinity for deglycosylated Lf and partially digested Lf. Recognition of lactoferrin by its receptor does appear to be somewhat species specific, but not entirely (Kawakami & Lönnerdal, 1991). In contrast brush-border membrane vesicles from human intestines showed little binding with either hLf or bLf (Kawakami &

Lönnerdal, 1991). Using an *in vitro* model Lönnerdal *et al.* (2011) confirmed that bLf could be taken up by the human lactoferrin receptor (hLfR).

2.3.1.1.2. Animal studies

Gislason *et al.* (1994) found that lactoferrin binding occurred throughout the small intestine (duodenum, jejunum and ileum) in piglets (0 – 21 days of age), independent of age. Porcine Lf bound specifically, however porcine transferrin, hLf and bLf did not bind to the porcine Lf receptors, suggesting the potential for species specificity.

In contrast, Drescher *et al.* (1999) used radio-labelled proteins to study the precaecal digestibility of lactoferrin in comparison to casein in both suckling and adult miniature pigs. The 15N-digestibility of lactoferrin, both bovine (82.3 +/- 4.8%) and porcine (84.4 +/- 3.2%), was significantly lower than casein digestibility (97.6 +/- 0.5%) in the distal small intestine of suckling piglets ($P < 0.05$), with 4.5% of non- and partially digested lactoferrin found in the last third of the small intestine of piglets (Drescher *et al.*, 1999). These results suggest lactoferrin has relatively low digestibility, at least in piglets. In the adult pigs, no differences in the digestibility of lactoferrin and casein were observed, both being nearly completely digested (Drescher *et al.*, 1999).

2.3.1.1.3. Human studies

A proportion of the lactoferrin ingested by infants persists throughout the gastrointestinal tract (Dallas *et al.*, 2012; Davidson & Lönnerdal, 1987; Spik *et al.*, 1982). Sampling the gastric digesta of infants, Britton and Koldovsky (1989) and Chatterton *et al.* (2004), determined dietary lactoferrin may be partially degraded by preterm infant gastric fluid. At the prevailing postprandial gastric pH, hydrolysis is minimal, hence, both intact and bioactive fragments of lactoferrin are available for subsequent biological action within the infant (Liao *et al.*, 2012). Using isotopically labelled human milk, Hutchens *et al.* (1991b) showed that intact and DNA-binding lactoferrin was absorbed within the infant gastrointestinal tract. Substantial amounts of bovine lactoferrin (bLf) also survive the more challenging (low pH) gastric digestion in human adults (Troost *et al.*, 2001). Using proteomic techniques, Grosvenor *et al.* (2014) tracked the truncation and relative abundance of peptides released during time-course simulated gastric digestion of bLf, noting differences in the peptide patterns between pasteurised and unpasteurised samples. They concluded that the bioavailability of specific peptides may be influenced by thermal processing of the food prior to consumption, with some peptides becoming more available and others less available (Grosvenor *et al.*, 2014). The nutritional or clinical implications of such effects are not currently understood. Dallas *et al.* (2014) investigated the digestion of human milk in the infant stomach, analysing gastric aspirates of 4 to 12-day old neonates, sampled 2 hours after feeding. Peptide analysis was completed for both the digested and an undigested sample of the milk. There was a remarkable difference on the peptides present between the intact milk and gastric samples; 64 peptides were common to both sample points, 135 peptides were present only in the intact milk and not the digested sample; and 586 peptides were present only in the gastric samples. The

pattern of peptides suggested that degradation within the intact milk and stomach is protein selective (Dallas *et al.*, 2014). Peptides released from lactoferrin were not present in the intact milk but were present in significantly higher concentrations in the gastric samples (Dallas *et al.*, 2014). The authors concluded the increase in unique peptides from proteins in the stomach, including lactoferrin, has clinical relevance because the antibacterial, immunomodulatory, and other functions of these peptides are particularly relevant in the small bowel (Dallas *et al.*, 2014).

A certain proportion of lactoferrin and its peptides is absorbed within the intestinal lumen, and able to exert a range of systemic effects. Lactoferrin receptors occur throughout the intestine in the brush border membrane enabling the absorption of lactoferrin and potentially some large fragments such as a “nicked” but otherwise intact form of lactoferrin or lactoferricin that result from any proteolysis in the gut (Hutchens *et al.*, 1991a).

2.3.1.2.Distribution and metabolism

2.3.1.2.1. Animal Studies

In an investigation into the transport of lactoferrin from the intestinal lumen of piglets, Harada *et al.* (1999a) found that following oral administration in neonatal pigs, bLf appeared in the blood circulation and reached a peak level after 2 h. It was confirmed immunohistochemically that lactoferrin was transported by endocytosis via the epithelial cells. Lactoferrin absorbed into the blood was also detected in the bile and reached a peak value 12 h after oral administration. Transport of lactoferrin from the intestinal lumen into the bile via the bloodstream was also observed in weaning piglets. Lactoferrin transported into plasma and bile was confirmed to be the same substance as administrated lactoferrin by electrophoresis and immunoblotting methods. Lactoferrin transported into bile was re-absorbed into the blood in neonatal pigs. This suggests that orally administered lactoferrin is transported, at least partially, from the intestinal epithelium into the peripheral circulation, excreted into the bile and reabsorbed into the bloodstream of neonatal pigs, suggesting the presence of entero-hepatic circulation of bLf in neonatal pigs (Harada *et al.*, 1999a). Feeding formula containing physiologic concentrations of added bLf increased hepatic protein synthesis in newborn pigs, suggesting lactoferrin may have an anabolic function in neonates (Burrin *et al.*, 1996). Kitagawa *et al.* (2003) investigated the absorption and transport route of intestinally administered bLf in growing pigs and showed that the absorption of bLf was mediated by lactoferrin-binding factors on the epithelial cell membranes. Almost all of the absorbed bLf was transported via the lymphatics and the portal vein into the systemic circulation (Kitagawa *et al.*, 2003). The potential for lactoferrin to modify brain function was demonstrated by Harada *et al.* (1999b) after orally and intestinally administered bLf in neonatal pigs was detected in cerebrospinal fluid (CSF) and was matched to that appearing in the serum by electrophoretic and ELISA analysis.

In a further neonatal piglet study (Harada *et al.*, 2002) investigated the characteristic transfer of colostral components into CSF via serum after bLf administered directly infused into the intestinal lumen. Neonatal piglets were removed from their dams immediately following birth, without suckling for the non-suckling group. Blood was collected from the jugular vein and CSF from the cisterna magna at 0, 6- and 12-hours

post suckling/intestinal infusion. Following oral administration of bLf (1 g/kg BW), the Lf concentration in serum increased steeply, reached a peak value (2.1 ± 0.2 µg/ml) at 4 h, and then gradually declined. In contrast, the concentration of bLf in CSF gradually increased, reaching a peak value (59.0 ± 32.8 ng/ml) at 8 h. The study evaluated a range of colostral macromolecules, and interestingly not all followed the same pattern of absorption and distribution. This thus suggests that in the neonatal piglet the transport into CSF of Lf across the CSF-barrier or blood-brain barrier (BBB) in a time-dependent manner is selectively controlled, and therefore adds reason bLf may potentially modulate some physiological function in the immature brains of piglets and neonates (Harada *et al.*, 2002).

Using gene expression technology, together with a radial maze assay, Chen *et al.* (2015b) showed that neonatal piglets fed 0.6 g/L bLf showed improved neural development (as demonstrated by upregulation of canonical pathways associated with neurodevelopment and cognition; influence on multiple genes involved with cell migration and differentiation, the growth and targeting of axons; and upregulation of transcription factors associated with key pathways and signalling in neurodevelopment), together with enhanced cognition as measured in a maze test. Using a piglet model, Mudd *et al.* (2016) and Berding *et al.* (2016) determined that a novel combination of prebiotics, bovine-derived milk-fat-globule membrane phospholipid complex and bLf (0.3 g/100 g milk replacer powder) administered between days 2 to 31, was well tolerated, supported normal growth (Berding *et al.*, 2016), and positively influenced postnatal brain development in the piglet beyond that afforded by docosahexaenoic acid (DHA) and arachidonic acid (ARA) (Mudd *et al.*, 2016).

Talukder *et al.* (2002) investigated the transfer of bovine colostral macromolecules, including bLf, from the GI tract into the CSF of newborn calves, collecting blood and CSF from the jugular and cisterna magna respectively, at various time points. The study showed that bLf was absorbed into the systemic circulation, and also transported into the CSF in a time-dependent manner via the blood-CSF or BBB in newborn calves (Talukder *et al.*, 2002). These studies provide some evidence that the possibility exists for modification of immature brain functions by suckling colostrum (which includes high levels of Lf) in neonatal animals (Harada *et al.*, 2002).

In summary, Lf is transported into the circulatory system from the intestinal epithelium, excreted into bile, and may be re-absorbed into the blood stream suggesting the possibility of entero-hepatic circulation of Lf in neonatal pigs (Harada *et al.*, 1999a). In addition, Lf is transported into the cerebro-spinal fluid across the blood-CSF barrier and blood brain barrier in a time dependent manner (Harada *et al.*, 2002). Exposure of a range of tissues to Lf distributed to a range of organs throughout the body indicates Lf may be associated with a tissue specific physiological function.

2.3.1.2.2. Human studies

Bennett and Kokocinski (1979) investigated Lf turnover in human adults using radio-labelled Lf. Lf was rapidly eliminated from the plasma and shown to be rapidly taken up by the liver and spleen, persisting in these organs for several weeks before being slowly transferred to the bone marrow before appearing in circulating red blood cells. Graham *et al.* (2007) reviewed the metabolism of Lf in the liver, where two

lactoferrin binding sites have been reported on hepatocytes, although neither is specific for lactoferrin. The first is low-density lipoprotein receptor-related protein (LRP) and the second is the major (RHL-1) subunit of the asialoglycoprotein receptor. Lactoferrin appears to be cleared via receptor-mediated endocytosis regardless of its binding site. Most of the internalised lactoferrin is directed to lysosomes for degradation (Graham *et al.*, 2007).

2.3.1.3.Excretion

2.3.1.3.1. Animal studies

The persistence of Lf throughout the gastrointestinal tract is supported by the study of Reznikov *et al.* (2014) who observed that the levels of bLf excreted in the faeces of neonatal piglets through to 14 days were a function of the level of bLf in the piglet diets.

2.3.1.3.2. Human studies

The excretion of undigested lactoferrin and lactoferrin fragments in the urine and faeces of human infants is well documented. Spik *et al.* (1982), monitored lactoferrin in faecal extracts of breastfed infants, concluding that lactoferrin (both human and bovine in origin) are not completely destroyed during digestion, retain their ability to bind iron, and hence may supplement the bacteriostatic effects of endogenous lactoferrin in the intestinal tract. Using isotope labelled human milk proteins, Hutchens *et al.* (1991b) confirmed that intact (78kDa) lactoferrin of maternal origin is absorbed by the gut and excreted intact in the urine of preterm infants.

Further support is provided by alternate measures of amino acid digestibility, where the true digestibility of a number of amino acids in human milk protein were less digestible compared to others (Darragh & Moughan, 1998). Those amino acids found to be less digestible are present in greater proportions in the immune proteins, including lactoferrin, than other proteins of human milk (Darragh & Moughan, 1998). Goldman *et al.* (1990) identified similar fragments of lactoferrin in the stools and urine of very-low-birth-weight infants fed human milk that appeared to be produced by *in vivo* proteolysis and originating in the gastrointestinal tract. Davidson and Lönnerdal (1987) showed significant amounts of lactoferrin and secretory IgA were excreted by the infants in the faeces and this excretion decreased in a trend similar to the decreasing milk concentrations of these proteins.

Mastromarino *et al.* (2014) observed higher concentrations of faecal Lf at birth and 30 days after delivery in pre-term infants compared to that in full-term infants; concomitantly the level of faecal bifidobacteria and lactobacilli were significantly associated with the concentration of faecal Lf. This suggests a putative role of Lf in the promotion of a bifidogenic microflora in the gut in neonate and preterm infants. High levels of faecal Lf in in the first days of life contribute to a strong early host-microbe interaction that could be important for the composition of the neonatal gut microbiota and the development of these microorganisms

(Vega-Bautista *et al.*, 2019). Thus, physiological functions of Lf are related to the persistence of Lf through to the faeces.

Based on the totality of information, Synlait concludes there is compelling evidence that a substantial proportion of both intact lactoferrin and its peptides resist gastric digestion, persists throughout the gastrointestinal tract and is excreted in the faeces. This resistance to digestion is important for bLf to be able to exert some of its benefits, in particular its bacteriostatic effect on pathogens and beneficial effects on gut microbiota. Some lactoferrin is also absorbed in the intestinal lumen via lactoferrin receptor (LfR), exerting a range of systemic effects. This duplicity of fates affords it to play a range of different metabolic roles and manifest its bioactivity via a range of different mechanisms. This underlies the clinical benefits associated with the inclusion of bLf in milk-based infant formula products, as discussed in Section 3.2.3.

2.3.2. Information on the toxicity of bLf

The key toxicity studies for bLf have been completed by researchers for Morinaga Milk Industry Co. Ltd. and these studies have been used as the basis for toxicity evaluations for regulatory applications in the USA (GRN 67, 2001; GRN 77, 2001; GRN 130, 2003; GRN 464, 2014; GRN 465, 2014; GRN 669, 2016) and the European Union (EFSA Panel on Dietetic Products Nutrition and Allergies, 2012). It is acknowledged that the toxicity studies have been completed in adult animal models, and no guideline toxicity studies in juvenile animals have been identified. In the evaluation of bLf from manufacturers other than Morinaga, substantial equivalence was provided in each evaluation. In the GRAS notice of Synlait (GRN 669, 2016) data was provided showing the equivalence of Synlait bLf to that of Morinaga (Table 2-11). Furthermore the European Union has subsequently recognised the equivalence of bLf from various manufacturing sources in its assignment of bLf as a general class of novel food ingredient (European Commission, 2017). Based on the precedence of the toxicity studies of Morinaga Milk Industry Co. Ltd., being consistently accepted as sufficient to meet regulatory requirements in a range of international jurisdictions, that same data is used as the basis for this application. It is acknowledged that other than where published, the original toxicity study reports by Moringa Milk Industry Co. Ltd. are not available and cannot be sourced.

2.3.2.1.Acute toxicity

The acute toxicity of bLf was evaluated in Sprague-Dawley rats by Nishimura and colleagues (1991) of Morinaga Milk Industry Co. Ltd. The study was not openly published, remaining an internal report (Bozo Research Centre Inc., Setagaya-ku, Japan) commissioned by Morinaga Milk Industry Co. Ltd. The study report was submitted in confidence by the Morinaga Milk Industry Co. Ltd. and was the subject of a detailed review during safety evaluations of bLf ((GRN 465, 2014); and (EFSA Panel on Dietetic Products Nutrition and Allergies, 2012)). Only summarised information provided in the public documents published for those evaluations is available. Key original documents and study reports from Morinaga Milk Industry Co. Ltd are not available.

Both male and female Sprague-Dawley rats (Crj:CD(SD) SPF) were exposed to a single oral dose of 1,000 or 2,000 mg/kg BW of either standard bLf, or iron saturated bLf by gavage (GRN 465, 2014). The higher dose was reported as the highest technically possible to prepare for the test solution (Yamauchi *et al.*, 2000b). Control rats received the test vehicle (water) alone (2000 mg/kg BW). A 14-day observation period followed administration of the bLf or control vehicle, with animals observed for mortality, clinical signs, and any change in general condition, with body weight measured prior to bLf administration, and periodically during the observation period. Animals were euthanised following the 14-day observation period, to enable macroscopic examination of the cranial, thoracic and abdominal cavity organs (GRN 465, 2014).

A single oral dose of either 1,000 mg/kg BW or 2000 mg/kg BW of either standard or iron-saturated bLf resulted in no adverse effects or deaths in either the acute phase or over a 14-day follow-up period. No effects on the general condition of the animals or abnormal clinical signs were observed during the observation period, and no significant differences in the body weights of the test versus control rats. Post-mortem examination revealed no abnormal gross pathological findings (GRN 465, 2014).

Based on this study, the lethal dose of bLf was determined to exceed 2000 mg/kg BW (also cited in Yamauchi *et al.* (2000b).

2.3.2.2. Short-term toxicity

2.3.2.2.1. 4-Week sub-chronic oral toxicity in rats

The safety of bLf based on 4-week sub-chronic oral toxicity was evaluated in Sprague-Dawley rats by Nishimura and colleagues (1997) of Morinaga Milk Industry Co. Ltd. The study was not openly published, remaining an internal report (Bozo Research Centre Inc., Setagaya-ku, Japan) commissioned by Morinaga Milk Industry Co. Ltd. The study was the subject of a detailed review in safety evaluations of bLf ((GRN 465, 2014); and (EFSA Panel on Dietetic Products Nutrition and Allergies, 2012)), and is summarised based on information provided in those evaluations, and is also cited in Yamauchi *et al.* (2000b). Original documents and study reports from Morinaga Milk Industry Co. Ltd are not available.

Four-week-old male and female Sprague-Dawley rats were gavaged once daily with doses of 0 (water control), 200, 600 or 2,000 mg/kg BW of bLf for 4 weeks (28 days). All animals were observed daily for changes in appearance or behaviour. Body weight and feed consumption were measured prior to the start of treatment and twice weekly, every 3rd or 4th day, prior to receiving the allocated dose, for the duration of the study. At or prior to necropsy (at 4-weeks) ophthalmological examination, urinalysis, haematology and blood chemistry analyses were completed. At necropsy (on day 29) animals were observed for external abnormalities, organs and tissues in the cephalic, thoracic and abdominal cavities were examined and weighed. Histopathological examination of organs and tissues of animals on the control and high dose (2,000 mg/kg BW) were completed (GRN 465, 2014).

Across all doses of bLf administered there were no deaths or changes in the general condition, behaviour or appearance of any of the animals attributable to the administration of the doses of bLf. Body weight and feed consumption were similar in all groups throughout the study, with no significant differences observed

between groups. No changes in males or females or significant differences between test and control groups were observed in urinalysis (pH, protein, ketone body, glucose, occult blood, bilirubin, urobilinogen, colour, urinary sediments, 24-hour urine volume, osmolarity, sodium, potassium, chloride, or water intake); haematology (red cell count, haemoglobin, haematocrit, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, reticulocyte count, platelet count, white blood cell count, differential leukocyte count, prothrombin time, activated partial thromboplastin time, or fibrinogen), or blood chemistry parameters (GOT, GPT, LDH, ALP, total cholesterol, triglycerides, phospholipids, total bilirubin, glucose, blood urea nitrogen, creatinine, sodium, potassium, chloride, calcium, inorganic phosphorus, total protein, A/G ratio or protein fractions) (GRN 465, 2014).

A number of organ and histopathological observations were recorded in animals across all groups (GRN 465, 2014). On further investigation these were all considered incidental as they were not dose-related, nor did they occur consistently amongst the animals. The observed changes were cellular infiltration and focal haemorrhage of the lung; erosion in the glandular stomach; cellular infiltration of the cecum; microgranuloma in the liver; ectopic thymus; tubular basophilia, eosinophilic body in tubular epithelium, and cellular infiltration in interstitium of the kidney; degeneration and necrosis of spermatocyte; decrease of sperm in the epididymus duct; fibrosis in the muscle layer of the oesophagus; hyperplasia of ductal epithelium in the sublingual gland; and disarrangement of the retina. Gross morphological changes observed were slight to mild in severity, occurred sporadically in 1 or 2 animals, and were considered incidental (GRN 465, 2014).

Based on the outcomes of this study, the administration of 200, 600, and 2,000 mg/kg BW/day of bLf to male and female rats did not result in any mortality or treatment related changes in bodyweight, feed consumption, organ weight, ophthalmology, haematology, blood chemistry, urinalysis or gross pathology or histology upon observation. Accordingly, the NOAEL (no-observed-adverse-effect-level) of bLf was estimated to be in excess of 2,000 mg/kg BW/day (EFSA Panel on Dietetic Products Nutrition and Allergies, 2012; GRN 464, 2014; GRN 465, 2014).

2.3.2.2.2. 13-Week sub-chronic oral toxicity in rats

In a 13-week oral repeated administration toxicity study of bLf in male and female Sprague-Dawley rats, once daily doses of 0 (water), 200, 600 or 2,000 mg/kg BW of bLf (sourced from Morinaga Milk Industry Co. Ltd) were administered by oral gavage (Yamauchi *et al.*, 2000b). Full study details and results are presented in the appended publication by Yamauchi *et al.* (2000b), however original study data and reports are not available.

The 4 treatment groups each consisted of 12 male and 12 female rats, the study commenced when the animals were 4 weeks of age. Daily observations were made of the general condition of all animals. Food consumption and body weight were recorded on Day 1 of the study and twice weekly for the duration of the study. Ophthalmological examination was completed in 50% animals (both sexes) in the final week of the study. Urine samples were collected from all animals in week 6 and the final week of the study for urinalysis. Blood samples were collected from anaesthetised animals for haematological and biochemical

measures. Following euthanasia, necropsy was completed which included external inspection and gross examination of all organs and tissues.

Two animals died during the treatment period. On investigation, the deaths were not attributed to the consumption of the bLf. In week 10, one male in the 200 mg/kg BW group died because of an error in intubation. One female in the 2000 mg/kg BW group died (week 13) as a result of a spontaneous lymphoma, which is not uncommon in Sprague-Dawley rats (Yamauchi *et al.*, 2000b). Neither death was attributable to the bLf, and clinical signs of the surviving animals showed no changes, in either sex, attributable to the bLf or control.

In summary, no clinically relevant effects were observed in any of the 4 groups. There were no significant differences observed in body weight or feed consumption between the groups over the duration of the study. Furthermore, there were no changes in ophthalmological measures, blood chemistry or gross pathological examination outcomes that could be attributed to the consumption of bLf in any of the groups (Yamauchi *et al.*, 2000b). No changes in organ weights of animals in the 200 or 600 mg/kg BW groups were observed; however, females only in the 2000 mg/kg BW group had a slight but significant reduction in thyroid weights. The changes were not considered related to the bLf as they were related only to females and not correlated to any morphological findings on histopathological examination (Yamauchi *et al.*, 2000b).

A slight, but statistically significant reduction in urinary pH was observed for both males and females in the 2000 mg/kg BW bLf group. Lactoferrin was not detected in the urine (detection limit 0.1 µg/ml), Yamauchi *et al.* (2000b) however, suggested that the potential presence of undetected bLf fragments in the urine may influence urinary pH. Both intact lactoferrin and fragments of maternal lactoferrin have been detected in the faeces and urine of breastfed infants (Hutchens *et al.*, 1991b). Other observed urinalysis differences in male rats only included minor changes in urine volume and daily excretion of sodium, potassium and chloride ions. These differences were not related to bLf dose (Yamauchi *et al.*, 2000b). Histological examination of the kidneys revealed no abnormalities. In short, minor changes in urinalysis were not considered to be of any toxicological significance.

Islet fibrosis in the pancreas was observed in male rats, with the incidence and severity (slight to mild- control- 3/12; 200 mg/kg BW - 7/12; 600 mg/kg BW - 6/12 and 2000 mg/kg BW - 6/12) of the finding in each bLf administration group being slightly higher than for the control group. Islet fibrosis in the pancreas is known to occur at relatively high frequency as a phenomenon associated with aging and feed intake patterns in the Sprague-Dawley rat (Molon-Noblot *et al.*, 2001). This effect is supported by Imaoka *et al.* (2007), who reported the incidence of spontaneous pancreatic islet fibrosis in rats corresponding to the same age of rats used in the 13-week study of (Yamauchi *et al.*, 2000b). The islet fibrosis was not considered to be a consequence of bLf administration.

The overall conclusion of the 13-week oral toxicity study was that none of the observed differences were due to the administration of bLf, and that the NOAEL of bLf was 2,000 mg/kg BW per day, the highest dose tested.

2.3.2.3. Long-term toxicity and carcinogenicity

2.3.2.3.1. Chronic oral toxicity in rats

Tamano *et al.* (2008) completed 2 chronic feeding studies in male and female F344/DuCrj (Fisher) rats to determine if bLf (Morinaga Milk Industry Co. Ltd) and related compounds have any toxic effects in long-term feeding studies. The study was the subject of detailed review during the GRAS Notice of bLf for use in infant formula and other food uses (GRN 464, 2014; GRN 465, 2014). Two studies were completed to determine if bLf and related compounds had any toxic effects when fed long term.

In the first study, 15 male 6-week-old F344/DuCRj rats were fed either a basal control diet containing no bLf, or the basal diet containing 0.2% bLf for 40 weeks. Diet and water were available *ad libitum*. Animals were observed daily for general condition. Body weights and food consumption were recorded weekly for the first 4 weeks, then 4-weekly thereafter. At the end of the 40 weeks, following overnight fasting, all animals were euthanised, and blood samples taken were analysed for a range of biochemical markers. Gross examinations of organs and tissues were completed at necropsy. No adverse treatment-related clinical indications, effects on body weight or macroscopic changes were reported (Tamano *et al.*, 2008).

In the second experiment, 100 female and male F344/Crj rats (25/sex/group in control and high dose group; 10/sex/group in other groups) were fed a basal diet containing 0 (control), 0.02%, 0.2%, 2.0% or 5.0% bLf. Male rats underwent a 60-week trial commencing at 17 weeks of age, and females a 65-week trial starting at 11 weeks of age. Animals were observed daily for general condition and weight monitored at 8 intervals over the study period. Food consumption and water consumption were measured twice weekly for the first 16 weeks, and 4-weekly thereafter to completion of study. Gross examination was completed at necropsy and major organs weighed. Organ tissue samples and any large lesions were processed for histopathological examination. All incidences of histopathological alterations that were observed were noted to be within the range of spontaneously occurring lesions for F344 rats (Goodman *et al.*, 1979; Tamano *et al.*, 2008). No reported significant treatment-related adverse effects on final body weight, organ weight, gross or histopathology, including carcinogenicity, were evident for either sex (Tamano *et al.*, 2008). There was no evidence of long-term toxicity or carcinogenicity in animals fed bLf at 5.0% of diet.

The authors concluded that the studies provided subjective support for the safety of clinical studies of bLf for supplement use and suggested that based on the results of this study the NOAEL for bLf is at least 5.0% w/w of diet for both sexes (Tamano *et al.*, 2008).

2.3.2.3.2. Reproductive toxicity

No reproductive studies are known to have been completed for bLf.

Otsuki *et al.* (2014) reported on a single case study of a woman who consumed 700mg bLf orally and used a 150mg bLf vaginal tablet daily for 3 months prior to pregnancy and throughout the pregnancy. After a history of late term miscarriages, attributed to vaginal infection, the woman successfully delivered a healthy infant with no adverse effects to the infant (Otsuki *et al.*, 2014).

2.3.2.3.3. Developmental toxicity

Kruger *et al.* (2007) completed a teratogenicity study in Crj:CD (SD) IGS rats using a milk derived basic protein fraction that contained approximately 54% bLf manufactured by Snow Brand Milk Products Co., Ltd., Japan. Confirmed day of mating was gestational day 0 (G0). The bLf containing protein fraction was administered by gavage at a dose of 2000 mg/kg BW/day on gestational days 1-17 (G1-G17). Although not a purified form of bLf such as that manufactured by Synlait Milk Limited and Morinaga Milk Industry Co. Ltd., the use of the protein fraction equates to >1000 mg/kg BW/day of bLf and provides evidence on the potential effects of bLf consumption at these elevated rates reflective of a mixed food system.

Dams were observed daily at the time of test vehicle administration for mortality and clinical effects, from Day 0 to day 20 when they were euthanised (G20). Body weight was recorded for each animal on days 0, 3 and daily from day 7 to day 20 of gestation. Food consumption was monitored throughout the study period (Days 0, 3, 7, 9, 11, 13, 15, 17, and 20). Extensive analyses of dam organs and foetal measures was completed on necropsy on day 20 of gestation (G20), with selected organs and tissues preserved for examination at a later date. Ovaries and uteri were removed, and the gravid uteri were weighed. After observation of intrauterine, embryo-foetal, and placental conditions, and after the removal of live foetuses, the uteri and placentas were weighted. Implantation index, viability index of foetuses, incidence of dead or resorbed embryos and foetuses, and sex ratio were calculated. The foetal examination consisted of external examination, visceral examination, and skeletal examination. For visceral examination, all foetuses were fixed in Bouin's solution; for skeletal examination, all foetuses were fixed in 99.5% ethanol, stained with alizarin red S and cleared in 70% glycerin.

No adverse effects related to the protein fraction treatment were observed during the study (Table 2-14). No differences between treatment and control dams were reported in body weight, body weight gain, food consumption, numbers of corpora lutea, number of implantation sites, numbers of live and dead foetuses, numbers of resorbed embryos, viability indices of foetuses, sex ratio, placental weight, and body weight of foetuses. In live foetuses there were no significant protein fraction related external, visceral or skeletal anomalies (Kruger *et al.*, 2007).

Based on this study Kruger *et al.* (2007) concluded the protein fraction had no adverse effects on foetal development in Sprague-Dawley IGS rats at 2000 mg/kg BW/day. Extrapolation of these results suggests that bLf at 1000mg/kg BW/day has no adverse effects on foetal development in Sprague-Dawley IGS rats.

Table 2-14 Reproductive parameters of female rats in the teratogenicity study of high bLf milk protein (Kruger et al., 2007)

Parameter (units)	**High bLf milk protein dose groups[b]**	
	Control[a]	**2000 mg/kg[a]**
Number of corpora lutea	16.3 ± 2.0	16.3 ± 2.5
Number of implantation sites	15.8 ± 2.0	15.4 ± 2.2
Implantation index (%)[c]	97.01 ± 4.38	94.84 ± 7.70
Dead or resorbed embryos/foetuses		
Early[d]	1.0 ± 0.7	1.0 ± 1.1
Late[e]	0.0 ± 0.0	0.1 ± 0.3
Total	1.0 ± 0.7	1.1 ± 1.2
Incidence (%)[f]	6.10 ± 4.64	6.55 ± 6.84
Number of live foetuses	14.9 ± 2.0	14.4 ± 1.9
Viability index of foetuses (%)[f]	93.90 ± 4.64	93.45 ± 6.84
Live foetuses		
Sex ratio[h]	0.464 ± 0.153	0.490 ± 0.125
Body weight (g)		
Male	3.720 ± 0.234	3.698 ± 0.209
Female	3.552 ± 0.191	3.568 ± 0.213
Placental weight (g)	0.481 ± 0.038	0.478 ± 0.062

a Each value is the group mean ± SD.
b n = 20 per group.
c [Number of implantation sites/number of corpora lutea] x 100.
d Includes implantation sites and placental remnants.
e Includes macerated foetuses and dead term foetuses.
f [Number of dead or resorbed embryos and foetuses/number of implantation sites] x 100.
g [Number of live foetuses/number of implantation sites] x 100.
h Number of male live foetuses/number of live foetuses.

Lending further support to the safety of bLf during pregnancy on the developmental status of human infants, a significant number of studies in pregnant women have been undertaken. Artym *et al.* (2021) identified 14 clinical studies in pregnant women which investigated the oral consumption of bLf and the impact on iron homeostasis, and a further 5 studies investigating the potential prophylactic and therapeutic effects of bLf (administered vaginally) in pregnant women suffering from infection and inflammation. No studies in pregnant women administered bLf by either the oral or vaginal route have observed any adverse effects related to *in utero* infant development.

2.3.2.3.4. Genotoxicity

Yamauchi *et al.* (2000a) evaluated the genotoxic potential of bLf using the Ames mutagenicity test (Ames *et al.*, 1975). A total of 5 test strains including 3 base pair substitution-type strains, *Salmonella* typhimurium TA100, TA1535 and *Escherichia* coli WP2uvrA, and 2 frameshift-type strains, TA98 and TA1537, were used in the test. The test was performed by both the direct method and the metabolic activation method (provided by an Aroclor-induced, rat liver microsome fraction (S9mix)), with pre-incubation applied in each instance. The test bLf solution was tested at 6 concentrations: 160, 320, 630, 1250, 2500, and 5000 µg/plate, based on the results of a preliminary study to evaluate potential growth inhibition of the selected bacterial strains and to determine the dose levels (Yamauchi *et al.*, 2000a). Physiological saline was the negative control and was used to dissolve and dilute the bLf to the target concentrations. Testing was completed in duplicate.

Results from the positive and negative controls were used to establish whether the study was conducted appropriately – the number of revertant colonies induced by the positive control was more than twice (2x) that of the negative control for each test strain, and the number of colonies formed for each of the controls aligned with expected ranges based on other reverse mutation tests using the same controls (Yamauchi *et al.*, 2000a). At all concentrations of bLf tested, and across all bacterial strains both with and without activation, the number of revertant colonies was 1.4 times or less than that of the negative control. A factor of greater than 2 was required to denote a positive result.

Based on the results of this study the mutagenicity of bLf was judged negative. Bovine lactoferrin over a wide range of concentrations did not exhibit mutagenicity in the Ames test used (Yamauchi *et al.*, 2000a).

2.3.2.3.5. Special studies such as neurotoxicity or immunotoxicity

No additional studies addressing toxicological aspects of bLf have been identified.

2.3.2.4. Summary of toxicity and genotoxicity studies

Based on the results from the acute, sub-chronic and chronic animal toxicity studies, Synlait concludes that bLf is well tolerated with no significant adverse effects or toxicity at the concentrations tested. The NOAEL, based on these toxicity studies, is determined to be 2,000 mg/kg BW/day. The compound bLf is also non-genotoxic, as determined by the Ames mutagenicity test.

We note that while most toxicity studies were carried out in non-neonatal animals, and some toxicity areas have not been explored to date (notably reproductive toxicity), there is an abundance of evidence supporting the safe use of bLf in infant and other populations. Notably, as discussed in more detail in Section 3.2.2, no adverse effects were observed in intervention studies in infants, including the highly vulnerable group of preterm and very-low-birthweight (VLBW) infants, and addition of bLf has been shown to support normal growth and development. Levels used in preterm and VLBW infants were higher than the

maximum levels proposed in this application. Bovine lactoferrin is a protein naturally present in cow's milk, and so has a long history of safe consumption in humans, while high levels of Lf in human milk mean that there is also a history of safe consumption of large quantities of Lf in infants, noting that bLf shows many similarities with hLf in structure and function, as discussed in detail throughout this application. We also note that studies in neonatal animals presented in Section 3.2.3.1 do not report any adverse effects of bLf administration.

Overall, based on the toxicity data presented here and evidence of safe use in other studies, the evidence is convincing that bLf is safe for use in neonates, including the highly vulnerable group of preterm and VLBW infants.

2.3.3. Supporting studies

2.3.3.1. The direct effect of bLf on murine embryo development

Recently, Aya *et al.* (2021) reported the effects of bLf on embryo development and pregnancy in mice where sperm was pre-treated with lipopolysaccharide (LPS) to induce potential inflammatory damage reflective of that attributed to infertility caused by seminal bacterial infection.

Sperm was collected from 13 male B6D2F1/Jcl mice aged 8–16 weeks, and equally divided into one of four types of medium: TYH medium (LSI Medience, Tokyo, Japan; control group), TYH medium with 1.0 mg/ml bLf (NRL Pharma, Tokyo, Japan; bLf group), TYH medium with 1.0 mg/ml bLf and 1.0 × 10−3 mg/ml LPS from *E. coli* O111:B4 (Sigma-Aldrich, St. Louis, MO, USA; bLf/LPS group), and 1.0 × 10−3 mg/ml LPS (LPS group). After incubation under 5% CO_2 at 37°C for 3 hours, sperm were used for insemination.

Ova were collected from 47 female BDF1 mice following the induction of superovulation with CARD HyperOvaTM (Kyudo, Saga, Japan). Ova were divided into four groups (control group, bLf group, bLf/LPS group and LPS group), and co-incubated with 6 µl of sperm suspension from the four types of media in mHTF medium (Kyudo) under 5% CO_2 at 37°C for 3 hr, respectively. Fertilised ova were incubated in M16 medium (Sigma-Aldrich) under 5% CO_2 at 37°C for 16 hr, and only 2-cell stage embryos were used for embryo transfer to 15 female Jcl:ICR mice aged 8-22 weeks. Mice were divided into four groups (control group, bLf group, bLf/LPS group and LPS group) and 2-cell stage embryos, which were derived from sperm prepared. On day 12 post-embryo transfer, recipient mice were euthanised, and foetuses were removed with placentas.

The rate of the embryo development into the 2-cell stage were 56.4 ± 3.7%, 58.7 ± 4.8%, 53.6 ± 4.4% and 45.9 ± 4.9% in the control, bLf, bLf/LPS and LPS groups, respectively. These results suggest that bLf treatment not only rescued the LPS-affected sperm but also facilitated its embryogenesis. From the morphological observation on day 12 post-embryo transfer, the abnormal structures (small placenta-like tissues without foetuses), were frequently found in the uterus transferred the LPS-group embryo, but rarely in the control, bLf and bLf/LPS groups (*Figure 2-8*). Aya *et al.* (2021) concluded that the embryo abnormality that occurred in the LPS-affected sperm could be prevented by bLf treatment to the sperm.

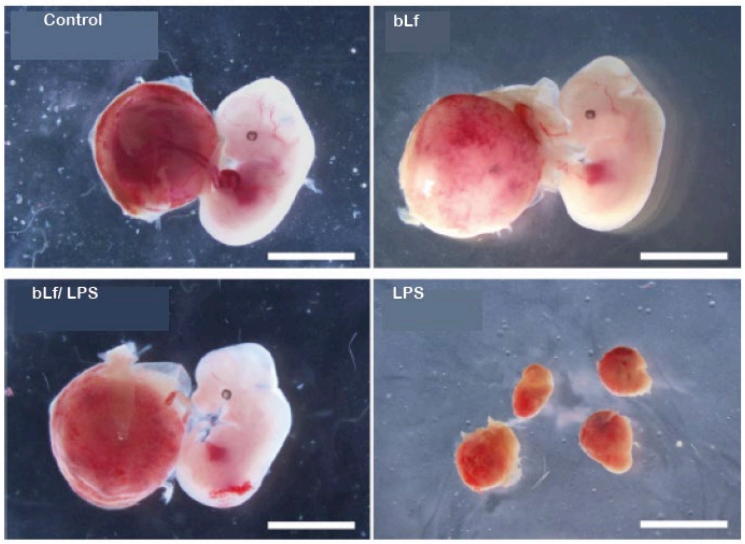

Figure 2-8 Effect of bLf on foetal development
(From Aya et al. (2021))

2.3.3.2. Effects of bLf on intrauterine growth restriction

Somm *et al.* (2014) investigated the effects of bLf in a dexamethasone (DEX)-induced intrauterine growth restriction (IUGR) Sprague-Dawley OFA rat model. The objective of the study was to assess the effect of maternal bLf supplementation (0.85% w/w in food pellet) in the IUGR model, with a special focus on pup growth and early brain metabolism/gene expression in the hippocampus (Somm *et al.*, 2014). IUGR was induced by subcutaneous infusion of 100 µg/kg BW/day DEX from gestational day 14 to 21. The study period covered from gestational day 0 (G0) through weaning, post-natal day (PND) 21. Maternal weight gain, food intake and food efficiency, together with haematological analysis was also investigated as a function of diet and the IUGR model. Four groups of dams were studied: CONTROL (normal diet and vehicle infusion); DEX (normal diet and DEX infusion [IUGR model]); LACTO (bLf enriched diet and vehicle infusion); DEX + LACTO (bLf enriched diet and DEX infusion).

Litter characteristics, pup birth weight and early postnatal growth were monitored. At PND 7 pups, under isoflurane sedation, underwent *in vivo* ^{1}H NMR spectroscopy for quantification of 17 metabolites in the hippocampus. Also, at PND 7, 10 pups from each group were euthanised. Hippocampal tissue was prepared for microarray analysis and PCR analysis, and brain tissue prepared for histological examination.

Dietary supplementation had no effect on maternal bodyweight over the gestational period. During gestation, feed intake and efficiency did not differ across groups until day 14 (G 14) when all dams were exposed to the DEX treatment. Food intake and efficiency was significantly retarded for all DEX treated dams post G 14. No effect of bLf supplementation was observed in any group. Haematology showed no

effect of bLf on haematocrit, red blood cell count or haemoglobin, however a slight increase in circulating immune cells was observed. Dam supplementation with bLf was not associated with any adverse effects during gestation and lactation to weaning.

At birth pups from LACTO dams were slightly (about 4%) but significantly heavier than control pups. Lactoferrin supplementation did not affect birth weight of pup born to DEX treated dams, which were significantly less than the CONTROL group. During early postnatal growth, the weight of DEX + LACTO pups caught up with the CONTROL group suggesting maternal bLf supplementation during lactation allowed improvements in the IUGR phenotype. Smaller litter sizes were observed in the DEX treated groups and no effect of maternal bLf supplementation was observed on litter size or sex ratio of pups. Extensive analysis of pup brain tissue showed no adverse effects of maternal bLf supplementation on the neurodevelopment of pups. Results suggested maternal bLf supplementation during lactation may reverse some of the defective hippocampal development effects of IUGR which typically result in long term cognitive impairment. More recently Ginet *et al.* (2016) found that bLf supplementation of maternal food during lactation reduces LPS-induced brain injury in rat pups, providing further supportive evidence for the safe consumption of bLf during lactation and no adverse effects on suckling neonates. Further support for the beneficial effects of bLf for the partial reversal of mild neurological damage due to a hypocaloric IUGR, was provided by van de Looij *et al.* (2019) in rat pups. Rat dam diets included bLf supplementation during pregnancy and lactation.

Together these results suggest no adverse effects of bLf on maternal and pup health over gestation through lactation to weaning.

2.3.3.3. Effects of bLf on motor activity, learning and behaviour

Shumake *et al.* (2014) conducted 2 studies in Holtzman rat pups with daily administration of bLf for 18 days from PND 16 coinciding with the pups' opening their eyes and onset of solid food consumption, through to weaning at PND 23, and a further 11 days post weaning. The studies were designed to investigate whether long-term feeding of bLf to nursing rat pups would lead to long-term changes in the behavioural response to stress (Shumake *et al.*, 2014). In the first study, a single bLf dose of 750 mg/kg BW was administered orally to avoid the stress of gavage. In the second study, which aimed to evaluate potential dose response 500 mg/kg BW, 1,000 mg/kg BW, and 2,000 mg /kg BW was administered daily. No growth information was reported in the publication of the studies. Shumake *et al.* (2014) found no adverse effects of bLf supplementation on the rats' general motor activity, behaviour, and/or learning. Major outcomes of the study lead to the authors concluding that bLf led to more cautious / less impulsive approach behaviour and improved learning under stress. Several dose-dependent behaviours were noted in male pups only – bLf dose-dependently accelerated stress-motivated discrimination learning, and improved passive-avoidance learning (Shumake *et al.*, 2014). The study provides support for no adverse effect of bLf on general motor activity, behaviour or learning.

2.3.3.4. Effect of bLf on the protection of the neonatal gastrointestinal tract

Preterm piglet models have been used to investigate the mechanism of how bLf may contribute to the protection of vulnerable infants from developing gastrointestinal inflammation and necrotizing enterocolitis (NEC) (Nguyen *et al.*, 2016; Nguyen *et al.*, 2014), and how it regulates the homeostasis of the immature intestine. One hundred and twenty-three different intestinal epithelial cell (IEC) proteins were altered by bLf. Low bLf doses (0.1-1 g/L) upregulated 11 proteins associated with glycolysis, energy metabolism and protein synthesis, indicating support for cell survival. In contrast, a high bLf dose (10 g/L) up-regulated three apoptosis-inducing proteins, down-regulated five anti-apoptotic and proliferation-inducing proteins and 15 proteins related to energy and amino acid metabolism and altered three proteins enhancing the hypoxia inducible factor-1 (HIF-1) pathway. In the preterm pig intestine, bLf at 10 g/L decreased villus height/crypt depth ratio and up-regulated the Bax/Bcl-2 ratio and HIF-1alpha, indicating several undesirable effects: elevated intestinal apoptosis and inflammation (Figure 2-9). The authors concluded, given that bLf dose-dependently affects IECs via metabolic, apoptotic and inflammatory pathways, it is important to select an appropriate dose when feeding vulnerable neonates with bLf to avoid detrimental effects brought about by excessive doses (Nguyen *et al.*, 2014).

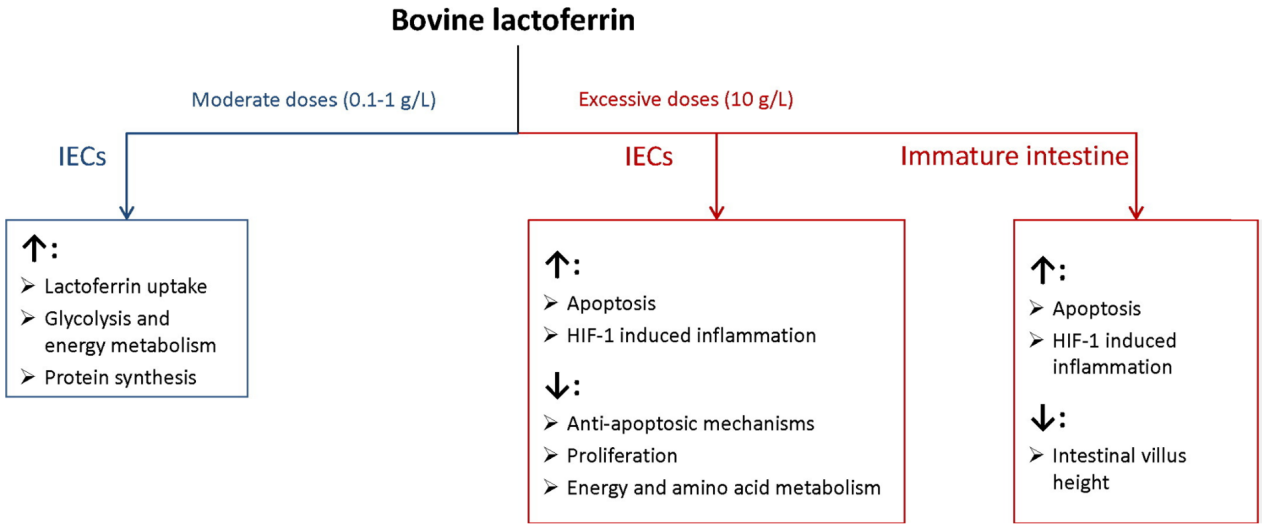

Figure 2-9 Effect of bLf on intestinal epithelial cells (from Nguyen et al. (2016)

Beneficial effects (increased crypt proliferation (60%), crypt depth and area and increased β-catenin mRNA expression) on IEC in neonatal piglets fed bLf up to 3.6 g/L were observed in a study by Reznikov *et al.* (2014), suggesting that undigested bLf can potentially affect intestinal proliferation through direct contact with IEC's. The same study investigated the effect of bLf on mucosal and systemic immune development

(Reznikov *et al.*, 2014), showing that dietary bLf can alter the capacity of the mesenteric lymph nodes (MLN) and spleen immune cells in response to stimulation. In piglets fed transgenic bovine milk containing recombinant human lactoferrin, a significantly reduced incidence of diarrhoea, enhanced humoral immunity, T helper (Th1 and Th2) cell responses, an improvement in the structure of the intestinal mucosa, no observed induction of food allergy led Li *et al.* (2014) to conclude that in neonatal piglets lactoferrin could improve both systemic and intestinal immune responses. In a piglet trial investigating the potential of bLf to improve immune function to reduce mortality in piglets during the stressful phase of weaning, Shan *et al.* (2007) found significant beneficial changes in several immune markers, a reduction in incidence of diarrhoea and improved growth and performance of the piglets fed bLf. Together these studies provide further evidence for a supporting role for lactoferrin in the initiation of protective immune responses via the gastrointestinal tract in neonates.

2.3.4. Potential allergenicity

Cow's milk allergy (CMA) is a hypersensitivity reaction to milk initiated by specific immunologic mechanisms. The main allergens of cow's milk are distributed among the whey and casein protein fractions, the 4 whey allergens including alpha-lactalbumin, beta-lactoglobulin, bovine serum albumin and the bovine immunoglobulins (Fiocchi *et al.*, 2010). Lactoferrin, present at approximately 0.1 g/L in cow's milk, is not listed as one of the milk allergens, and its clinical relevance as an allergen is unknown. Crittenden and Bennett (2005) reported the incidence of CMA is more prevalent in infants (2–6%) than in adults (0.1– 0.5%), and the dominant immunological mechanisms driving allergic reactions change with age.

In most children with CMA, the condition can be immunoglobulin E (IgE)-mediated and is thought to manifest as a phenotypical expression of atopy, together with (or in the absence of) atopic eczema, allergic rhinitis and/or asthma. A subset of patients, however, have non-IgE mediated (probably cell-mediated) allergy and present mainly with gastrointestinal symptoms in reaction to the ingestion of cow's milk (Fiocchi *et al.*, 2010).

The potential for IgE-mediated hypersensitivity and non-IgE-mediated hypersensitivity was extensively reviewed in the GRAS Notice (GRN 465) of bLf for use in infant formula. The bLf discussed in this document is essentially equivalent to the bLf discussed in GRN 465. In summary, that review, consistent with other reports (Natale *et al.*, 2004; Wal, 1998; Wal *et al.*, 1995) concluded that, although infants and individuals with CMA have anti-bLf IgE antibodies, there is no evidence to support a role for bLf as a causative agent for CMA (Goodman *et al.*, 2007). Importantly, given that oral administration reduces an antigen's immunoreactivity, providing small amounts of bLf may in fact contribute to the development of oral tolerance (GRN 465, 2014). Gaudin *et al.* (2008) concluded that based on IgE binding affinity bLf could be classified as a strong allergen to young children with CMA, however that the caseins are the main allergens in milk and that α_{S1}-casein is more allergenic than α_{S2}, β- and κ-caseins, which were recognised with almost a similar frequency by the sera of patients. Hence, as bLf is not one of the major cow milk proteins linked to

CMA (Fiocchi *et al.*, 2010), in the event an infant with CMA is fed a bLf fortified cow's milk-based formula, it is unlikely the bLf would be the primary causative agent of any immunologically driven hypersensitivity (Ahrens *et al.*, 2012; Gaudin *et al.*, 2008). Goodman *et al.* (2007) specifically investigated the potential allergenicity of a milk basic protein fraction, that contained >50% bLf. They concluded that based on molecular characteristics and expected exposure, the protein components in the protein fraction were unlikely to present any increased risk of allergy for milk allergic subjects or of cross-reactivity for other allergic subjects. Importantly, they noted that since the proteins are derived from milk, products containing the protein fraction will need to be labelled as containing milk proteins to warn milk allergic subjects of the potential risk of allergic reactions (Goodman *et al.*, 2007).

In Australia and New Zealand, the Food Standards Code (Standard 1.2.3 Information requirements – warning statements, advisory statements and declarations) mandates that the label of a food that contains an ingredient that is or contains milk declares the presence of the dairy component in the manner prescribed by the law. This necessitates a requirement for all milk-based formula and foods containing milk to be labelled and clearly identified as containing milk. Infants with CMA should not be fed cow's milk-based formula, and older children and adults that are sensitive to bovine protein, who are advised to avoid all dairy foods. Similarly, older infants, children and adults with confirmed CMA should not consume dairy products.

The use of bLf in non-dairy based formula would require the mandatory allergy warning for milk.

2.3.5. Hemochromatosis and the effect of bLf

Hereditary hemochromatosis (HH) is a genetic disorder that results in iron overload disease (Santos *et al.*, 1996). HH is characterised by abnormal iron absorption from the diet that leads to progressive iron overload causing tissue damage to organs, especially the liver, bone marrow and spleen (Carlson & Olsson, 2001; Graudal *et al.*, 1997). In healthy humans mobilisable iron reserves are predominantly located within cells of the reticulo-endothelial system (RES) in the bone marrow, liver and spleen (Graudal *et al.*, 1997). In contrast, in individuals with HH, iron accumulates in cells of the parenchymal organs (liver, pancreas, heart), and there is relatively low iron content in the RES (Graudal *et al.*, 1997).

HH is inherited recessively and is caused by defects in genes (HFE, T*f*R2, H*J*V, H*A*MP) that ultimately lead to the inefficient synthesis of hepcidin (Musci *et al.*, 2014). The HFE mutation accounts for about 80% of HH occurrence (Santos *et al.*, 2012) and other iron overload diseases (de Campos *et al.*, 2019). A mutation in the HFE gene removes an essential cysteine which normally participates in a disulphide bond, forming a structural conformation that can interact with β-2 microglobulin (Carlson & Olsson, 2001). Association of β-2 microglobulin to HFE is required for intracellular traffic and the incorporation of the HFE molecule in the cell membrane. A β-2 microglobulin mouse knockout model (β-2m$^{-/-}$) has been developed that characterises HH in humans and is suitable for the study of HH (Santos *et al.*, 1996).

Mice models are also used to study the essential acquisition of iron for the intracellular growth of *Mycobacterium tuberculosis* (Olakanmi *et al.*, 2007), and β-2m$^{-/-}$ knock-out mice are more susceptible to

tuberculosis than wild-type mice (Schaible *et al.*, 2002). Schaible *et al.* (2002) found that *M. tuberculosis* infection is exacerbated under iron overload, but that defect can be corrected by the administration of bLf which results in 100-fold lower bacterial loads in the β-2m$^{-/-}$ knock-out mice. They concluded that the iron binding property of bLf was responsible for the inhibition of mycobacterial growth in the β-2m$^{-/-}$ knock-out mouse. Although there is a known association between an increased risk of pulmonary tuberculosis and elevated dietary iron (Gangaidzo *et al.*, 2001), there is no known relationship between HH and tuberculosis (Schaible *et al.*, 2002).

In a murine model, Fransson et al. (1983) tested if iron bound to bLf is available to the young mouse; in addition, it was tested whether apo-bLf had an effect on the retention of dietary iron. Supplementation with iron chloride was evaluated for comparative purposes. The researchers found similar absorption of iron from bLf-bound iron and from iron chloride (whether apo-bLf was added or not), in iron-deficient mice, while absorption from iron chloride was higher than from holo-bLf in iron sufficient mice (Fransson *et al.*, 1983a). The findings by Fransson et al. (1983) suggest that bLf may play a role in regulating iron absorption by reducing it in a state of iron sufficiency.

In humans, Lf is produced in and released from neutrophils. As it binds to Lf receptors on macrophages and the associated iron is transferred to intracellular ferritin, a potential defect in the interaction of Lf with RES has been suggested as a putative mechanism that may contribute to iron overload in hemochromatosis (Graudal *et al.*, 1997). Moguilevsky *et al.* (1987) compared various properties of endogenous Lf from neutrophils of normal individuals and patients with familial haemochromatosis. No difference was found with respect to the Lf content of neutrophils, the molecular weight and isoelectric point of the protein, the dissociation of its complex with iron at acidic pH, its binding to isolated monocytes, and its uptake by the mouse RES. Macrophages from patients and controls were also found to be similar in their ability to bind and ingest lactoferrin and to process the iron provided by the protein. Moguilevsky *et al.* (1987) concluded that a defect in the interaction of Lf with the RES, related either to the protein itself or to the cells, seems unlikely. In a study with a single HH patient, Graudal *et al.* (1997) investigated the hypothesis that as a specific carrier for the removal of iron, Lf may be released from the neutrophils of HH individuals in order to bind circulating iron and transport it to the RES, thus reducing parenchymal iron overload. However, they found the plasma Lf levels in the HH patient were only half of those in healthy participants, and summarised it may be explained by a further theory based on *in vitro* data that iron overload may increase Lf uptake by the liver, hence at a constant rate of Lf production, the lower plasma levels observed in the patient (Graudal *et al.*, 1997). No consideration was given to the role of dietary Lf.

In summary there are no studies on the potential effects of dietary bLf in humans with HH. At a cellular level Lf is thought to play a role in the reduction of iron overload (Graudal *et al.*, 1997), and in a mouse model of hemochromatosis bLf has been shown to effectively reduce iron available to bacteria and reduce their proliferation (Schaible *et al.*, 2002). The findings by Fransson et al. (1983) suggest that bLf may play a role in regulating iron absorption by reducing it in a state of iron sufficiency. Together these studies suggest a putative role for Lf in maintaining iron homeostasis (Bonaccorsi di Patti *et al.*, 2018) including potentially in HH, and do not suggest any adverse potential for Lf to contribute to further iron overload.

2.3.6. Evidence for safe use of bLf from human intervention studies

Evidence from human intervention studies supporting the safe use of bLf in infants is covered in Section 3.2.2.

2.3.7. Safety assessment reports by international agencies or other national government agencies

Following an application to the European Commission (EC) for the use of bLf in a range of foods, the EFSA Panel on Dietetics, Nutrition and Allergies completed an assessment of bLf in the context of Regulation (EC) No 258/97 (Novel foods), and published their scientific opinion (EFSA Panel on Dietetic Products Nutrition and Allergies, 2012). The EFSA Panel concluded that as a novel food ingredient bLf is safe under the proposed uses and use levels of the original application. Further to the Opinion, the EC authorised bLf as a novel food: Commission Implementing decision 2012/727/EU: authorising the placing on the market of bovine lactoferrin as a novel food ingredient under Regulation (EC) 258/97 of the European Parliament and of the Council (FrieslandCampina) and, Commission Implementing decision 2012/725/EU: authorising the placing on the market of bovine lactoferrin as a novel food ingredient under Regulation (EC) 258/97 of the European Parliament and of the Council (Morinaga). Between 2012 and 2017 several companies applied for and were grated "substantial equivalence" enabling them to market bLf in the European market. In each instance the Competent Authority of the EC to whom the substantial equivalence applications were submitted, undertook at thorough safety review of the products.

As of 1 January 2018, the new Regulation (EU) 2015/2283[6] on novel foods (the new Regulation) is applicable (European Commission, 2017). It repeals and replaces Regulation (EC) No 258/97[7] and Regulation (EC) No 1852/2001[8] which were in force until 31 December 2017. Accordingly, bLf is now listed as an authorised novel food in the Union list of novel foods[9].

Note: Synlait did not apply for substantial equivalence with the EU, as of 2018 this mechanism was replaced by the updated regulation allowing any bLf that meets the specification under Regulation (EU) 2015/2283 to be legally used as within the EU.

Bovine lactoferrin has been the subject of several GRAS Notices submitted to the FDA in the USA, each of which have received a "no questions" letter. A summary of the GRAS Notices, which cover a range of foods and food applications, is shown in Table 2-15.

A copy of the FDA response to the Synlait GRAS Notice (GRN 669) is provided in Appendix 5 (A5:2).

[6] https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32015R2283
[7] https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:31997R0258
[8] https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32001R1852
[9] http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32017R2470&from=EN

Table 2-15 Lactoferrin GRAS Notifications

GRN No.	Substance	Date of closure	Applicant	Purpose / Use
669	Cow's milk-derived lactoferrin	Mar 9, 2017	Synlait Milk Lit.	Intended to be used as an ingredient in milk-based term infant formula, excluding exempt formulas as defined in 21 CFR Part 107.3, and to toddler formulas at levels of up to 100 milligrams per 100 grams of formula solids.
465	Cow's milk-derived lactoferrin	Feb 18, 2014	Morinaga Milk Industry Co. Ltd	As an ingredient in cow's milk-based term infant formulas at levels of 100 milligrams (mg) per 100 grams (g) powdered formulas, 26 mg per 100 millilitres (ml) liquid concentrates, and 13 mg /100ml ready-to-feed formula
464	Cow's milk-derived lactoferrin	Feb 18, 2014	Morinaga Milk Industry Co. Ltd	As an ingredient in cow's milk-based food products, including yogurt (100 milligrams (mg) per 100 grams (g)), powdered milk (400 mg/100 g), and ice cream and sherbets (200 mg/100 g), and in chewing gum (30 mg/g)
130	Bovine milk-derived lactoferrin	Aug 21, 2003	aLF Ventures	Antimicrobial spray for meat carcasses
77	Milk-derived lactoferrin	Aug 14, 2001	DMV International	Sports & functional foods at 100mg / serve
67	Milk-derived lactoferrin	Oct 23, 2001	Farmland National Packaging Co.	Use as antimicrobial in meat packing

2.4. Information on dietary intake of the nutritive substance

2.4.1. Foods and food groups proposed to contain bovine lactoferrin

This application proposes the permission for the addition of bLf to Special purpose foods as regulated under Part 2.9 Special purpose foods, of the Food Standards Code, specifically Standards 2.9.1 Infant formula products, including infant formula (birth to 6 months), follow-on formula (6 to 12 months) and infant formula for special dietary use (birth to 12 months) (see Table 2-16).

2.4.2. Proposed maximum levels permitted in Infant formula products

The proposed maximum levels of bLf permitted in infant formula and follow-on formula, and infant formula for special dietary use within Standard 2.9.1 Infant formula products, are presented in Table 2-16. The maximum levels were set based on levels found in human breastmilk, levels used in human intervention studies included in this application and aligned with international standards (see Section 2.4.5). Since lactoferrin levels in human breastmilk drop significantly during the first weeks post-partum, but then drop at a much slower rate, it is proposed that levels in milk of well-nourished Australian women >15 days post-partum (1230-1420 mg/L) is used as a reference for setting maximum levels of bLf in Infant formula products. The proposed maximum permitted levels are also based on levels that have been used and found to be safe in clinical studies in term and preterm infants. Table 2-17 provides a comparison of proposed maximum permitted levels with levels found in human breastmilk, with levels used in clinical studies and levels permitted in China and the European Union. For more details on the data presented in Table 2-17 refer to Section 3.1.1.

Table 2-16 Proposed maximum permitted levels of bLf in foods defined within Standard 2.9.1 Infant formula products

Standard	Target population	Specific category	Maximum permitted levels
2.9.1 Infant formula products	Infants 0-12 months	Infant formula	40 mg/ 100kJ
		Follow-on formula	40 mg/ 100kJ
		Infant formula for special dietary use	40 mg/ 100kJ

Table 2-17 Comparison of maximum permitted levels with levels in human milk, levels used in clinical studies, and levels permitted in other countries

Standard	Infant formula	Follow-on formula
Proposed maximum permitted levels	40 mg/ 100kJ	40 mg/ 100kJ
Equivalent levels in made-up formula[a]	1130 mg/L (1000-1260 mg/L)	1210 mg/L (1000-1420 mg/L)
Average concentration in human milk of Australian women[c] (mg/L)	1230-1420 mg/L	
bLf levels used in intervention studies in term infants	60-1000 mg/L	
Estimated equivalent bLf levels used in preterm and low-birth weight infant studies	370 – 1960mg/L	
Permitted levels in the European Union	1000 mg/L	1000 mg/L
Permitted levels in China	1000 mg/L	1000 mg/L

[a]Based on energy requirements for infant formula (2500kJ/L to 3150kJ/L) and follow-on formula (2500kJ/L and 3550kJ/L) as per Standard 2.9.1; levels are presented for mid-point energy requirement, and minimum and maximum levels in brackets.

2.4.3. Information on the likely levels of consumption of infant and follow-on formula

Typical consumption levels of infant formula products have been identified previously by FSANZ (Food Standards Australia New Zealand (FSANZ), 2016) as 0.8 L/day for infants from birth to ≤ 6 months and 0.6 L/day for infants 6 to ≤12 months, which is based on typical consumption of breastmilk of 0.8 L/day and 0.6 L/day, respectively (National Health and Medical Research Council, 2006). This assumes that an infant no longer receives breastmilk and is solely fed formula. In addition, infants 6 to ≤ 12 months would typically consume 200g/day of complementary food (Ministry of Health, 2012).

The 2016 NZ Total Diet Survey-suggested consumption rates of infant and follow-on formula among New Zealand infants are presented in Table 2-18 (Ministry for Primary Industries, 2018), as are the levels used for modelling of infant (9-month old) food consumption patterns in Australia (Food Standards Australia New Zealand (FSANZ), 2014).

Table 2-18 Intake of infant and follow-on formula in Australian and New Zealand infants

Food Group	New Zealand Infants[a] (g/day)	Australian 9-month-old infants[b] (g/day)
Infant / follow-on formula	400	544[c]

[a] *Ministry for Primary Industries (2018)*
[b] *Food Standards Australia New Zealand (FSANZ) (2014)*
[c] *Non-soy formula*

It is not expected that consumption of infant formula products has significantly changed since the release of the latest data presented above.

For information on exposure to the nutritive substance if bLf is added to infant formula products, including exposure to iron from bLf, refer to Section 3.3.

2.4.4. Percentage of food group to which use of bLf is proposed or the percentage of the market likely to use the nutritive substance

Several manufacturers and brand owners have expressed an interest to add lactoferrin to their products. Forecasted ANZ market trends could be extrapolated from other markets where bLf permission in Infant formula products has resulted in many brands now including bLf in their formulas. For example, in China, where bLf permission was given in 2013, of the 845 infant formula products launched between November 2013 and March 2022 (Mintel Global NPD Database), 231 products (27%) were with lactoferrin.

2.4.5. Information relating to use of bLf in other countries

Bovine lactoferrin was first used in infant formula (50 mg bLf /100 g formula powder) manufactured by Morinaga Milk Industry Co. Ltd in Japan in 1986 (GRN 465, 2014, p. 39 (pdf)). Since that time it has been used extensively in infant formula products for both domestic and export markets (GRN 465, 2014, p. 40 (pdf)). In Japan, lactoferrin is listed on the "List of Existing Food Additives" (2014): Additive 341 "Lactoferrin concentrates: a substance composed mainly of lactoferrin obtained from mammals' milk"[10]. The specification for "Lactoferrin Concentration" in the existing food additives list in Japan, as submitted in GRN 465, is provided in Appendix 4 (pg. A4:48), and a copy of the "List of Existing Food Additives" in Appendix 4 (pg. A4: 38). There is no specific restriction of use for bLf in Japan because it is a natural substance.

In addition to Japan, the use of bLf in infant formula products is widely accepted throughout a number of other Asian jurisdictions and is considered a desirable addition to infant formula products by many consumers. In Taiwan, the use of bLf (additive code 08112) has been approved since 2000, with addition rate in infant formula prescribed "as practically needed" (Standards for Specification, Scope, Application and Limitation of Food Additives (Appendix 4, pg. A4: 40). In Korea, bLf is listed as an approved Food Additive for use in infant and follow-up formula (Standards for Manufacturing and Preparation: General Standards for Food Additive use in Foods (infant foods); Appendix 4, pg. A4: 42) with no specific restrictions on level of addition. Considered a natural additive, the Korean Food Additives Code contains a specification and identity testing criteria for lactoferrin (Appendix 4, pg. A4: 50). In 2017, an update to the Food Regulations in Singapore permits the use of bovine lactoferrin in infant formulas at levels up to 100 mg/100 ml of formula[11].

[10] http://www.ffcr.or.jp/zaidan/FFCRHOME.nsf/pages/list-exst.add

[11] https://sso.agc.gov.sg/SL/SFA1973-RG1?DocDate=20210913&ProvIds=pr252-&ViewType=Within&Phrase=lactoferrin&WiAl=1

In the European Union, bLf is an authorised novel food and is permitted in a range of foods, including infant and follow-on formula and foods on dairy basis intended for young children (Appendix 4, A4:2) (Table 2-19). The specified designation of bLf on the labelling of foods containing it is “Lactoferrin from cows’ milk”.

Table 2-19 Permitted levels of bLf in infant and follow-on formula in the European Union and China

	Specified food category	**Maximum levels permitted**
European Union	Infant formula and follow-on formula as defined in Regulation (EU) No 609/2013 (ready to drink)	100 mg/100 mL
China	Baby formula foods (Food category 13.01; which includes Infant formula, formula for older infants and young children, and infant formula for special medical purposes)	≤ 1.0 g / L as ready-to-consume

In China, the addition of bLf to foods is regulated under GB14880-2012 (Appendix 4, A4:16), as detailed in Table 2-19. A 2013 update (Announcement No. 11, November 15, 2013)) by the National Health and Family Planning Commission (NHFPC) extended the use and dosage of bLf in category 13.01 (Baby formula foods) to 1.0 g/L as ready to consume (see Appendix 4, A4: 34 for original announcement in Chinese and English reference to announcement in Appendix 4, A4:37). This applies to both powdered formula, levels of bLf adjusted to comply as reconstituted, and liquid ready-to-feed formula.

In the USA, several GRAS Notifications have addressed the use of bLf in a ranges of food applications, as well as infant formula products Table 2-15.

2.4.6. Information on likely current food consumption for foods where consumption has changed in recent years

There have been no reported or observed significant changes in intakes of infant formula products in Australia and New Zealand.

2.5. Information related to the nutritional impact of bLf

2.5.1. Information related to the nutritional purpose of the use of bLf

The nutritional purpose of adding bLf to Infant formula products is discussed in detail in Section 3.1.1. In brief, the purpose of the use of bLf in Infant formula products is due to the evidence for the reduced risk of infection in formula-fed infants receiving bLf-fortified formula compared to standard formula not fortified with bLf. Adding bLf to Infant formula products helps ensure infants who cannot be breastfed do not miss out on the benefits of lactoferrin.

2.6. Information related to the potential impact on the consumer understanding and behaviour

2.6.1. Information to demonstrate the level of consumer awareness and understanding of bLf in foods

Based on the general availability of bLf as a dietary supplement, there is likely already a level of awareness of bLf and its purported benefits among some consumers in Australia and New Zealand. Among the relevant population group for this application (parents or expecting parents), there is also a certain level of awareness of lactoferrin as an ingredient. There is anecdotal evidence from brand owners that purchasers of infant formula products in ANZ have frequently enquired about bLf as an ingredient. A 2021 consumer study carried out in Australia and including 609 participants, reported that 28% of those surveyed showed awareness of lactoferrin as an ingredient (Figure 2-10) (McMillan, 2021). This is remarkably high seeing that lactoferrin is not currently permitted in infant formula in Australia and New Zealand. Once lactoferrin is permitted and used in products for infants, awareness is expected to increase.

A likely source of information that is responsible for a certain level of awareness of lactoferrin among consumers, and likely among health care professionals, are articles or guidelines that talk about the benefits of breastfeeding. These often refer to lactoferrin as being one of the components in breastmilk that provides key benefits to breastfed infants, especially relating to immune protection[12,13,14]. For example, the Eat for Health Infant Feeding Guidelines (Information for health workers)[15] note lactoferrin as an immune protection component in breastmilk stating that “lactoferrin makes iron unavailable to micro-organisms that require iron for growth (e.g. Escherichia coli, candida albicans) and releases a peptide with bactericidal properties”.

[12] https://journal.nzma.org.nz/journal-articles/supporting-mothers-protecting-babies-for-long-term-health-establishing-a-pasteurised-human-milk-bank
[13] https://www.womenshealthcouncil.org.nz/Womens+Health+Issues/Breastfeeding/Mothers+Milk+precious+protection.html
[14] https://www.breastfeeding.asn.au/bfinfo/breastmilk-composition
[15] https://www.eatforhealth.gov.au/sites/default/files/files/the_guidelines/n56_infant_feeding_guidelines.pdf

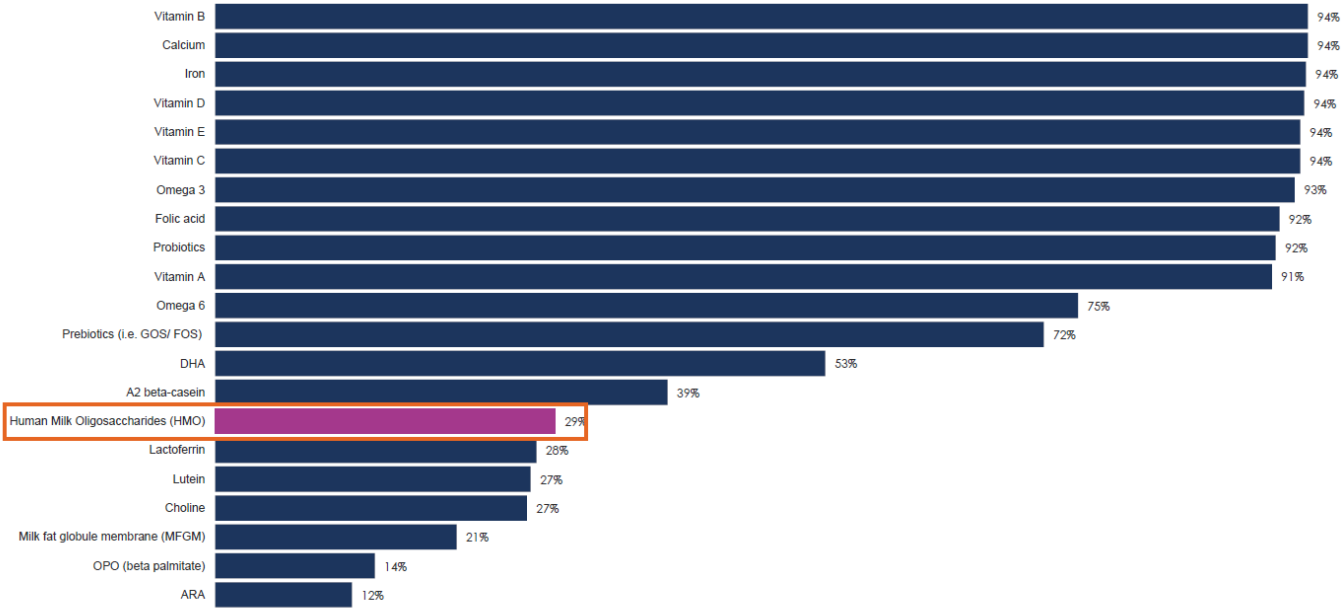

Figure 2-10 Australian market awareness of various ingredients (McMillan, 2021)

Evidence on consumer awareness of lactoferrin can also be obtained from overseas. To date, the most common food application for bLf has been infant formula products. Many mothers, when researching formula, find information relating to lactoferrin and understand it is one of the important (bioactive) proteins in human milk. In a 2016 review completed by Mintel (www.mintel.com) on behalf of Synlait, the awareness of consumers in China of bLf and its potential benefits was presented. In a survey of 1,910 female users of infant milk formula aged 20-39 years, 64% have heard of lactoferrin and know about its benefits. Of those who are aware of lactoferrin, 50% associated it with enhancing baby's immunity (Figure 2-11). This is consistent with a more recent survey of 2,659 Chinese aged 20-39 who have fed babies with infant milk formula where 64% recognises lactoferrin and know its function and an additional 30% have heard of lactoferrin as an ingredient (McMillan, 2021).

Chinese consumers associated the use of bLf with a range of benefits for infants and in general were aware of a range of health functions for bLf, mostly related to support of the immune system (Figure 2-11).

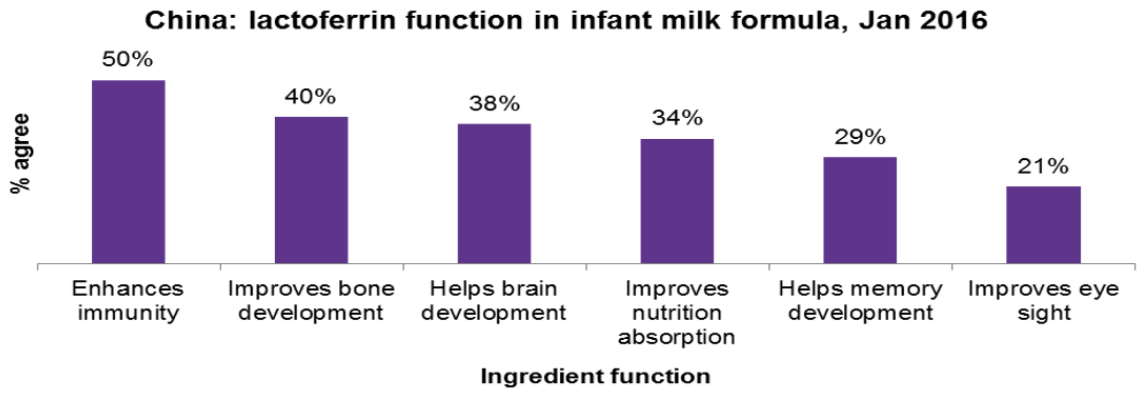

Figure 2-11 Awareness of health functions of bLf amongst Chinese formula users (report by Mintel for Synlait)

In countries such as the USA, where bLf is added to infant formula products, brand owner websites often provide top level information providing consumers with a simple understanding of the ingredient benefits e.g. Enspire® Infant Formula by Mead Johnson[16].

Recent market reviews suggest continuing significant growth (compound annual growth rate [CAGR] of 8.4%) in the global lactoferrin market, which is forecast to reach USD167.9 million by 2025. The market is forecast to grow across a wide range of product applications but is primarily driven by infant formula products[17].

Infant formula segment is expected to have a steady growth rate over the next eight years with an estimated CAGR of 8.1%, driven by a growing awareness of the need to maintain child health, particularly in Asia-Pacific region.

2.6.2. Information on the actual or potential behaviour of consumers in response to proposed foods

Bovine lactoferrin will be listed as an ingredient in the ingredient list and will also be included in the Nutrient Information Statement. Parents who have chosen to formula-feed and are aware of lactoferrin may choose a formula containing bLf and thereby replace a similar formula not containing bLf with one containing bLf. Synlait does not anticipate any nutritional concerns with this replacement seeing that any Infant formula products sold in Australia and New Zealand must meet strict regulatory standards.

Synlait does not anticipate that parents who are breastfeeding choose to switch to formula because of addition of bLf to formula. Human breastmilk is the natural source of nutrition for infants and is rich in many

[16] https://www.meadjohnson.com/pediatrics/us-en/product-information/products/infants/enspire#product-features
[17] https://www.grandviewresearch.com/press-release/global-lactoferrin-market

bioactive components that provide unique benefits to infant growth and development. Adding one single ingredient to formula will unlikely be perceived as making formula close to, let alone superior to, breastmilk. Research in Australian mothers found that the most frequently cited reasons for mothers to stop breastfeeding are perceived breastmilk insufficiency (not producing any/enough milk); resuming work; mastitis, nipple soreness and pain on feeding; and mothers felt it was the right time to stop (Magarey *et al.*, 2016). This research did not suggest that mothers stop breastfeeding because they believe formula is equivalent or superior to breastmilk.

It is important to note that Standard 1.2.7-4 prohibits health and nutrition claims on infant formula products. Furthermore, attention cannot be drawn to the addition of nutritive substances on pack, nor can the benefits be communicated, and specifically formula cannot be labelled with the word “humanised” or “maternalised” or any word or words having the same or similar effect (Standard 2.9.1-24). Hence the inclusion of bLf in Infant formula products is only likely to be noted by those caregivers who pay attention to product composition when making a choice in Infant formula product selection, not a driver to initiate formula feeding.

Therefore, we do not anticipate that addition of bLf will change overall consumption of Infant formula products, rather, it provides consumers with more choice.

2.6.3. Information to demonstrate consumption of foods containing bLf will not adversely affect any population groups

The addition of bLf to Infant formula products as per this application means there will be bLf containing formulas available for consumption by infants. Unlike general purpose foods, these foods are better defined in terms of intake or serve size and are typically consumed for specific purposes and in defined quantities. There is no evidence to support any adverse implications in population groups that may consume Infant formula products containing bLf. Further information on the safe use of the foods containing bLf can be found in Sections 3.2.1.2, 3.2.2 and 3.3.

3. Special purpose food – Infant formula products (3.6.2)

3.1. Information related to the composition

3.1.1. Purpose of the compositional change

The purpose of the use of bLf in Infant formula products is due to the evidence of a beneficial effect on health, more specifically a reduced risk of infection in formula-fed infants receiving bLf-fortified formula versus those receiving formula that does not contain bLf. Lactoferrin is present at significant levels in human milk, which suggests Lf being an important component in infants. Lactoferrin provided by human milk is known to exert immunoregulatory, antibacterial, and antiviral activity, and is involved in iron homeostasis (Demmelmair *et al.*, 2017; Lönnerdal, 2016). Bovine and human Lf are not identical, but show a 69% amino acid sequence identity, which is associated with some differences in tertiary structure (see Section 2.2.2 for more detail). However, this results in only minor differences in cellular uptake and bovine and human Lf have similar functions (Demmelmair *et al.*, 2017).

Bovine milk on the other hand is naturally low in bLf, and consequently standard Infant formula products on the market that have no bLf added contain significantly lower levels of bLf compared to hLf levels in human milk, meaning that infants that cannot be breastfed miss out on the beneficial health effects of Lf. The levels in unfortified formula vary, which is due to different milk ingredients used with varying bLf contents (e.g. milk, protein concentrates, protein isolates) and differences in processing conditions. Pang *et al.* (2020) measured bLf levels in infant formula products available at retailers in China, and found varying levels in formula. Of the 5 formulas tested, one was significantly higher in bLf, which was likely due to fortification, while the other samples showed levels that would be expected in unfortified formula (see Table 3-1).

Table 3-1: Levels of bLf in commercial infant formula compared to average concentration in human milk

Infant formula samples (sample No)	**Detected bLf content in IF samples[a] (mg/100g powder)**	**Likely bLf fortification status**	**Estimated bLf content in made-up formula[b] (mg/L)**	**Average concentration in human milk of Australian women[c] (mg/L)**
4	7.9±0.6	Unfortified	10.6	1230-1420
5	7.9±0.9	Unfortified	10.6	
6	77.3±2.5	Fortified	104	
7	20.4±2.1	Unfortified	27.3	
8	11.3±1.1	Unfortified	15.1	

[a] (Pang *et al.*, 2020), authors did not record fortification or ingredients

[b] Assuming 13.4g powder/100mL

[c] Based on typical levels in human milk from Australian women postnatal day >15 (Houghton *et al.*, 1985)

Adding bLf to Infant formula products allows parents that are unable to breastfeed to choose a product for their baby that provides benefits similar to those provided by human Lf. For exclusively breast-fed infants (0 - ≤ 6 months) the average mean daily intake of lactoferrin in Australia is estimated to be approximately 1000-1100 mg/day, based on the typical values for human milk intake rates of 0.8 L/day (EFSA Panel on Dietetic Products Nutrition and Allergies, 2013, 2014; Food Standards Australia New Zealand (FSANZ), 2016; National Health and Medical Research Council, 2006) and the average values of lactoferrin in human breast milk of Australian women (Table 3-2). No data on bLf levels in breastmilk in New Zealand women is available, but similar levels as in Australian women can be expected in New Zealand women. For older infants (6 - ≤ 12 months), whose diet includes complementary foods as well as breast milk, the mean volume of breast milk intake is approximately 0.6 L/day (Food Standards Australia New Zealand (FSANZ), 2016; National Health and Medical Research Council, 2006), providing approximately 800 mg/day of lactoferrin (Table 3-2).

Table 3-2: Estimated of intake of lactoferrin in breast-fed infants and infants fed formula (unfortified and fortified with bLf)

Human Milk	**Average concentration in source food (mg/L)**	**Mean Lf intake[b] (mg/day)**
Infants fed human milk, birth to ≤ 6 months	1230-1420[a]	984-1136
Infants fed human milk, 6 to ≤12months		738-852
Infants fed unfortified formula, birth to ≤ 6 months	~15[c]	12
Infants fed unfortified formula, 6 to ≤12months		9
Infants fed bLf-fortified formula, birth to ≤ 6 months	1130[d]	904
Infants fed bLf-fortified formula, 6 to ≤12months	1210[d]	726

[a] Based on typical levels in human milk from Australian women postnatal day >15 (Houghton *et al.*, 1985)
[b] Based on typical human milk intakes (birth to ≤ 6 months: 0.8L/day; 6 to ≤12months: 0.6L/day) (Food Standards Australia New Zealand (FSANZ), 2016)
[c] Based on findings by Pang *et al.* (2020) of average bLf content in commercial formula (excluding one formula that was likely fortified with bLf)
[d]Based on maximum proposed level of 40mg/100kJ and using the energy-midpoint per litre infant formula (2,825kJ/L) and follow-on formula (3,025kJ) as per Standard 2.9.1

Formula-fed infants on the other hand have much lower intakes of lactoferrin when consuming formula that did not have lactoferrin added. Assuming an average concentration in standard infant formula not fortified with bLf of ~15mg/L and assuming formula intake levels similar to mean breastmilk intakes, the intake of lactoferrin in infants fed exclusively unfortified formula would be around 10mg/day, significantly lower than lactoferrin intakes of breastfed infants (Table 3-2). Addition of bLf to infant formula can help bring intakes of lactoferrin in formula-fed infants closer to intake levels seen in breastfed infants.

Table 3-3 provides a comparison of proposed maximum permitted levels with levels found in human milk, levels used in clinical studies, and levels permitted in other countries. Table 3-4 provides a detailed view on bLf levels used in clinical studies in term and preterm infants.

Table 3-3: Comparison of maximum permitted levels with levels in human milk, levels used in clinical studies, and levels permitted in other countries

Standard	**Infant formula**	**Follow-on formula**
Proposed maximum permitted levels	40 mg/ 100kJ	40 mg/ 100kJ
Equivalent levels in made-up formula[a]	1130 mg/L (1000-1260 mg/L)	1210 mg/L (1000-1420 mg/L)
Average concentration in human milk of Australian women[c] (mg/L)	1230-1420 mg/L	
bLf levels used in intervention studies in term infants	60-1000 mg/L	
Estimated equivalent bLf levels used in preterm and low-birth weight infant studies	370 – 1960mg/L	
Permitted levels in the European Union	1000 mg/L	1000 mg/L
Permitted levels in China	1000 mg/L	1000 mg/L

[a]Based on energy requirements for infant formula (2500kJ/L to 3150kJ/L) and follow-on formula (2500kJ/L and 3550kJ/L) as per Standard 2.9.1; levels are presented for mid-point energy requirement, and minimum and maximum levels in brackets.

Table 3-4 bLf levels per L formula used in human intervention studies included in this application

Term infants	**bLf levels**	**Preterm infants**	**Estimated equivalent levels based on daily dose given[a]**
King et al. (2007)	850 mg/L	Manzoni et al. (2009); Manzoni et al. (2014)	370 – 1316 mg/L
Chen et al. (2016)	60 mg/L	Akin et al. (2014)	593 – 1961 mg/L
Chen et al. (2021)	60 mg/L 120 mg/L	Ochoa et al. (2015)	1111 – 1316 mg/L
Schulz-Lell et al. (1991)	1000 mg/L	Kaur and Gathwala (2015)	444 – 954 mg/L
Chierici et al. (1992)	1000 mg/L	Barrington et al. (2016)	556 – 667 mg/L
Hernell and Lönnerdal (2002)	1000 mg/L	ELFIN Trial Investigators Group (2019)	833 –987 mg/L
Johnston et al. (2015)	600 mg/L 1000 mg/L	Tarnow-Mordi et al. (2020)	1109 – 1331 mg/L
Björmsjö et al. (2022)	1000 mg/L	Ochoa et al. (2020)	1111 – 1316 mg/L

[a]See Section 3.2.2.2 and Table 3-8 for further detail on how values were calculated

3.1.2. General data requirements for supporting evidence

See Section 2.1.2 for details on general data requirements for supporting evidence.

3.2. Specific information requirements for the nutritional safety, tolerance and efficacy of the proposed compositional change

3.2.1. Characterisation of proposed substance or the comparable substances in breast milk

Human milk is a dynamic and complex substance consisting of thousands of constituents such as immune factors, hormones, and live cells in addition to macronutrients, vitamins, and minerals. There are marked differences in the protein composition of cow's milk-based infant formula products and human milk, notably human milk contains significantly higher concentrations of lactoferrin (Demmelmair *et al.*, 2017). Breastfeeding is the best way to feed a baby, however, where breastfeeding is not possible Infant formula products are the only alternative to human milk in infants. Increasing the lactoferrin content of formula is one way of making the protein composition of formula more similar to that of human milk, ensuring infants who cannot be breastfed do not miss out on the benefits of lactoferrin.

3.2.1.1. Lactoferrin content in human milk

The biggest influence on lactoferrin content in human milk is stage of lactation, with lactoferrin levels being highest right after birth (Rai *et al.*, 2014). Lactoferrin levels are also influenced by gestation (Trend *et al.*, 2016), while influence of nutritional status is less clear, with one study reporting no association between lactoferrin levels with maternal BMI, age, mode of delivery, parturition and protein intake (Yang *et al.*, 2018), while a study in Australian women suggests that women with low body weight may produce milk with lower levels of lactoferrin (Houghton *et al.*, 1985).

A range of concentrations of lactoferrin in human milk has been reported both as a function of the stage of lactation, and geography (Lien *et al.*, 2004; Mehta & Petrova, 2011; Rai *et al.*, 2014; Trend *et al.*, 2016). Lien *et al.* (2004) found a range of mean concentrations of between 1.37 g/L in Mexico to 2.12 g/L in China, with an overall mean of 1.83 ± 0.67g/L. The decline in lactoferrin concentration over the duration of lactation was typified by the pattern observed in Canada, falling from a little over 2 g/L at birth through to 1.5 g/L at 1 year (Lien *et al.*, 2004). In a systematic review of the longitudinal changes in lactoferrin concentration in human milk, Rai *et al.* (2014) found that across the 52 studies from around the world, the unweighted mean of means (+/-standard error of mean) concentrations of lactoferrin in early milk (<28 days lactation) was 4.91 +/- 0.31 g/L (range of means 0.34-17.94 g/L; median 4.03). For mature milk (≥28 days lactation), the mean of means was 2.10 +/- 0.87 g/L (range of means 0.44-4.4 g/L; median 1.91) (Rai *et al.*, 2014).

Similar trends in lactoferrin levels across stages of lactation were observed for different regions, including those representative for Australia and New Zealand (Europe and North America) (see Figure 3-1).

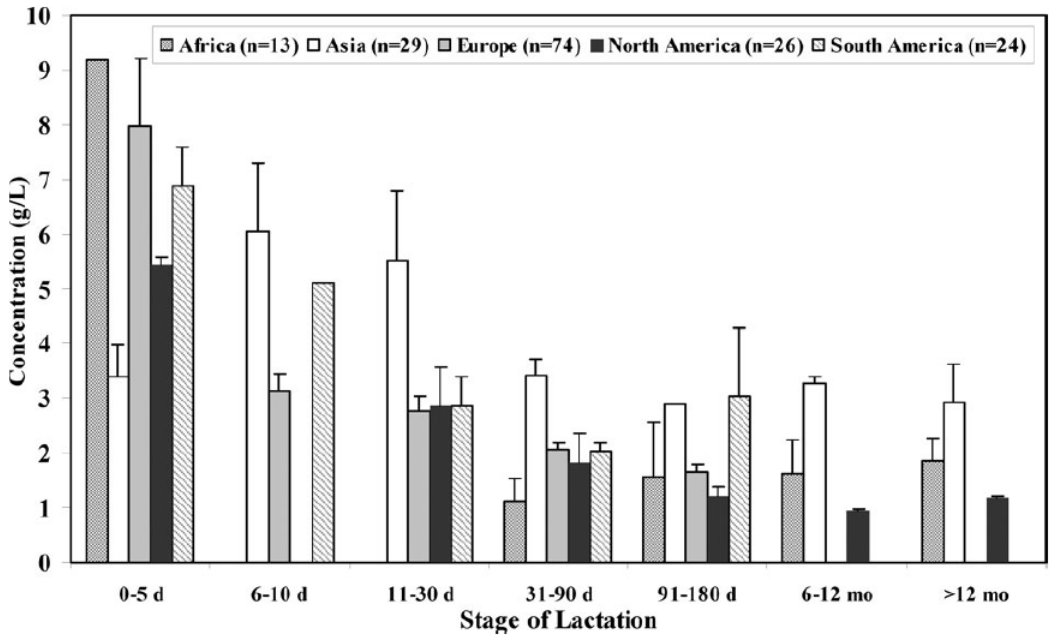

Figure 3-1 Lactoferrin concentration by stage of lactation presented according to region (values in g/L)
From: Rai *et al.* (2014)

Data on specific levels of lactoferrin in human milk of Australian women are presented in Table 3-5. Lactoferrin levels in Australian women show similar trends to those reported by Rai *et al.* (2014), with levels being higher in the first 15 days post-partum (2820-3490mg/L) than after 15 days post-partum (1230-1420mg/L). While there were numerical differences between Caucasian and Aboriginal women, there was no statistically significant effect of race. Lactoferrin levels in Australian women were higher when they were well-nourished compared to when they were malnourished (defined as 90% or less weight for height ratio) (Houghton *et al.*, 1985). Data on lactoferrin levels in New Zealand lactating women is not available, but levels are expected to be comparable to those seen in Australian women.

Table 3-5: Lactoferrin concentrations in breastmilk of Australian women (g/L)				
%Weight for height of mother*	**≤15 days post-partum**		**>15 days post-partum**	
	Caucasian	Aboriginal	Caucasian	Aboriginal
≥90	2.82 (0.24)** n=12	3.49 (0.24) n=15	1.42 (0.16) n=16	1.23 (0.23) n=8
<90	-	2.89 (0.27) n=6	0.66 (0.14) n=6	0.87 (0.18) n=16
*Used as proxy for nutritional status of mothers, with <90% considered undernourished **Standard error of mean (SEM)				

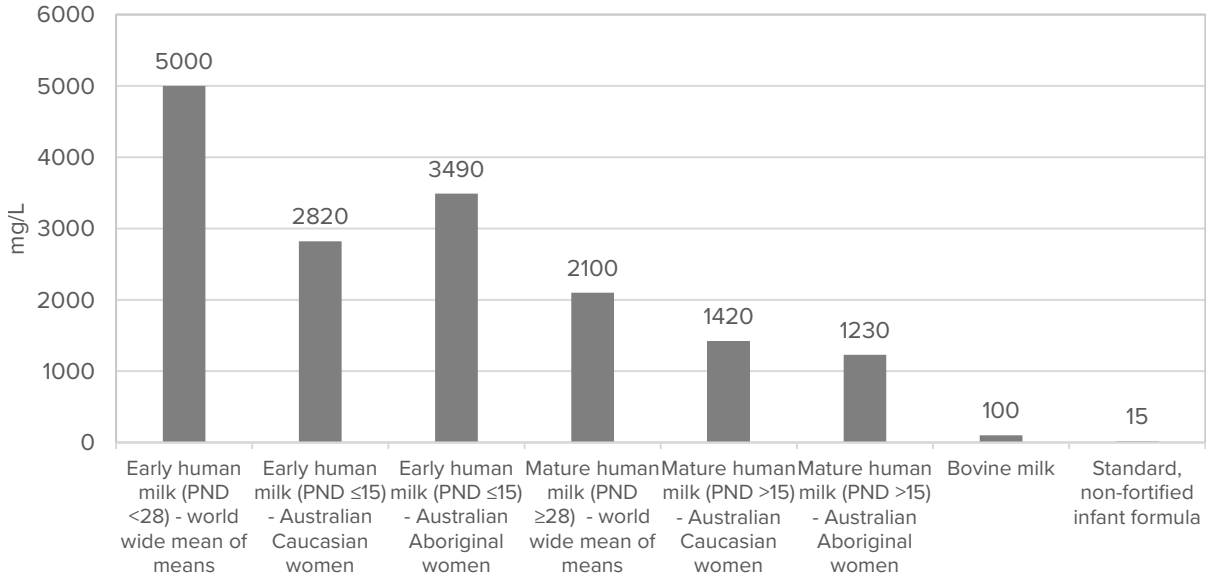

Figure 3-2 Lactoferrin content in human milk, bovine milk, and standard non-fortified infant formula
PND = Postnatal day
(Barth & Behnke, 1997; Houghton et al., 1985; Korhonen & Pihlanto, 2007; Pang et al., 2020; Rai et al., 2014)

More recently, Trend *et al.* (2016) identified the concentration of lactoferrin in breastmilk varied as a function of gestation (extremely preterm, very preterm, preterm and term deliveries), with a trend for increased concentrations of lactoferrin in the colostrum (days 2-5 post-partum) and transition milk (days 8-12) in the milk of mothers who delivered extremely and very preterm infants (Trend *et al.*, 2016).

In contrast to the relatively high levels of Lf found in human milk, a typical value of 100mg/L in mature bovine milk has been reported, although levels among individual cows and across stage of lactation can vary considerably (Barth & Behnke, 1997; Cheng *et al.*, 2008; Rainard *et al.*, 1982). The natural level of bLf in milk-based infant formula is very low, partly due to the relatively low levels in cow's milk and also due to losses during processing. As discussed earlier, one study found that four out of five infant formula products tested contained levels between 7.9 and 20.4mg/100g (~10-27mg/L); while one product contained significantly higher levels at 77.3mg/100g (~100mg/L), this product was likely fortified with lactoferrin as other milk ingredients would have contributed less than this (Pang *et al.*, 2020). Therefore, the content of standard non-fortified formula is estimated to typically be between 10-27mg/L (see Table 3-1).

Since lactoferrin levels drop significantly during the first weeks post-partum, but then drop at a much slower rate, it is proposed that levels in milk of well-nourished Australian women >15 days post-partum (1230-1420 mg/L) is used as a reference for setting maximum levels of bLf in Infant formula products.

Structural similarities and differences between human and bovine lactoferrin are discussed in Section 2.2.

3.2.1.2.Information on bLf and mineral homeostasis

Bovine lactoferrin has a high affinity to ferric iron (i.e. non-haeme iron), and can also bind to zinc and copper. This property of bLf does not impact iron and other mineral homeostasis, as discussed in detail in the following.

3.2.1.2.1. Absorption of iron bound to bLf

Infant formula products are fortified with iron to reduce the risk of ID and anaemia (Hernell *et al.*, 2015). Lactoferrin, including bLf, has a high affinity to ferric iron (Demmelmair *et al.*, 2017). When adding bLf to formula, it binds iron, making it unavailable to pathogens and thereby exerting a bacteriostatic effect (see Section 3.2.3.1.1 for further detail). At the same time, iron remains available to the infant via absorption through specific receptors for human lactoferrin in brush-border membrane cells of human infants (Kawakami & Lönnerdal, 1991; Suzuki *et al.*, 2001). Suzuki *et al.* (2001) found that both the protein and any iron bound to human lactoferrin can be taken up by Caco-2 cells. Lönnerdal *et al.* (2011) also found that bLf was able to bind to human Caco-2 cells and both the protein and iron were taken up by the cells, although to a lesser extent than hLf; iron-saturation did not affect binding to cells (Lönnerdal *et al.*, 2011). In a more recent study, Lönnerdal *et al.* (2020) confirmed their earlier results that commercial bLf samples, including Synlait bLf, and iron bound to bLf are taken up by Caco-2 cells (Lönnerdal *et al.*, 2020).

In a murine model, Fransson *et al.* (1983a) found similar absorption of iron from bLf-bound iron and from iron chloride (whether apo-bLf was added or not) in iron-deficient mice, while absorption from iron chloride was higher than from holo-bLf in iron sufficient mice (Fransson *et al.*, 1983a). The findings by Fransson *et al.* (1983a) suggest that bLf may play a role in regulating iron absorption by reducing it in a state of iron sufficiency. Fransson *et al.* (1983b) also tested absorption and retention of iron from iron-saturated bLf in

suckling pigs using ^{59}Fe-labelled iron and compared it to $FeSO_4$. The researchers found that bLf-bound iron was absorbed and incorporated into red blood cells to the same extent as sulphate iron. Net iron retention, measured by whole body counting of radioactivity one week after administration of a tracer dose also showed a similar uptake of bLf-bound iron and sulphate iron (Fransson *et al.*, 1983b). Davidson *et al.* (1990) found that in rhesus monkeys bLf addition to iron fortified formula led to similar iron retention than that seen from human milk or from milk-based formula without bLf. Further support for the bioavailability of iron bound to bLf comes from human studies.

Fairweather-Tait *et al.* (1987) tested the effect of iron-saturated (holo) bLf on iron absorption in newborn infants. Infants received either ^{58}Fe-labelled iron-saturated bLf or ^{58}Fe-labelled ferric chloride plus ascorbic acid. The authors found no difference in faecal iron concentration or total iron excreted during a 3-day period after feeding. Mean percent iron retention from ferric chloride was 44.4% (SD 25.8) and from bLf 46.2% (SD 23.9). The researchers concluded that bLf-bound iron was absorbed at a similar rate to ferric chloride given with ascorbic acid (Fairweather-Tait *et al.*, 1987).

In another iron balance study, 16 healthy term infants 3 weeks of age were either given non-iron-fortified infant formula with bLf (100mg/100mL) or the same formula without bLf for a duration of 14 weeks. Addition of bLf led to a higher iron-content of the formula compared to standard formula (1.06 mg/L v. 0.77 mg /L). Iron content in faeces was measured at several time points. Due to higher iron levels in bLf fortified formula, the supplemented group received 169 µg iron/kg BW/day and retained 63 µg/kg BW/day (36%), while infants fed standard formula received 118 µg/kg BW/day and retained 43 µg/kg BW/day (28%) (Schulz-Lell *et al.*, 1991).

A more recent study in Kenyan infants investigated the effect of apo-bLf (0.56% saturation) and holo-bLf (47% saturation) on iron absorption. The single-blind, randomised, controlled, cross-over trial included 25 healthy full-term babies aged 3-6 months with no chronic or acute illness and already receiving complementary food. All infants received three different test meals on days 1, 4 and 7. The test meals were made of maize flour, sugar and water and contained either: 1) 1.5mg of iron as ^{54}Fe-labelled ferrous sulphate; 2) 1.42mg of iron as ^{58}Fe-labelled ferrous sulphate and 1.41 g apo-bLf (containing 0.08mg of unlabelled native iron); and 3) fortified with intrinsically ^{57}Fe-labelled holo-bLf, containing 1.5mg iron and 1.41g bLf. At 21 days after the third test meal (day 28) whole blood for iron isotopic analysis was collected. No significant difference in fractional iron absorption (FIA) and total iron absorption (TIA) between meals containing holo-bLf and ferrous sulphate only was found. Interestingly, FIA and TIA were significantly higher from the meal containing apo-bLf and ferrous sulphate compared to the meal containing holo-bLf and the meal containing ferrous sulphate only (Mikulic *et al.*, 2020).

3.2.1.2.2. Iron, zinc and copper homeostasis

Several studies provide evidence that bLf supports normal iron homeostasis in infants, of which two provide evidence for no effect on zinc homeostasis and one provides evidence for no effect on copper homeostasis.

The first study by Chierici *et al.* (1992) was done in Italian healthy, naturally delivered, full-term newborns recruited after birth. Infants received either non-iron-fortified formula without bLf or with bLf fortification at two different doses (10mg/100mL or 100mg/100mL) for a period of 150 days; a breastfed group was also included as a reference group. Iron status parameters were measured at days 0, 7, 30, 90 and 150 (the breastfed group was not assessed at day 150). At day 30, serum ferritin was significantly higher in the human milk group compared to all formulas. At day 90, significantly higher serum ferritin values were observed in human milk group compared to unsupplemented formula (p=0.0024), while serum ferritin levels of infants receiving bLf-supplemented formulas were not significantly different from breastfed infants. At day 150, the high-bLf formula group (100mg/L) had significantly higher levels than the unsupplemented formula group (p=0.02). The researchers found no statistically significant differences in haemoglobin levels or iron serum levels between feeding groups. Serum zinc levels were not affected by bLf supplementation (Chierici *et al.*, 1992).

In a study by Hernell and Lönnerdal (2002) in healthy term infants in Sweden, infants received formula with varying iron levels with or without bLf: control group (n=11): infant formula with 4mg iron/L as ferrous iron; low iron group (n=12): infant formula with 1.6mg iron/L as ferrous iron; bLf group (n=10): infant formula with added holo-bLf (~1000mg/L) and 1.8mg iron (1.3mg from Lf); nucleotide group (n=10): infant formula with added nucleotides and 2.2mg iron/L from ferrous iron. No significant differences in any of the haematologic indexes were observed between groups at age 4 and 6 months when there was a correction for the initial difference in serum iron concentration. There were also no differences in serum zinc and copper status at 4 and 6 months of age (Hernell & Lönnerdal, 2002).

King et al. (2007) also investigated impact of long-term feeding of bLf-enhanced formula on haematologic parameters. The study included healthy term or near-term (≥34 weeks' gestation) bottle-fed infants aged 0-4 weeks when recruited. Infants received either an iron-fortified formula supplemented with 85mg bLf/100mL or the same iron-fortified formula without added bLf. Infants fed bLf-enhanced formula had significantly higher haematocrit levels at 9 months of age, while haemoglobin (Hb) and mean corpuscular volume (MCV) were numerically but not significantly higher. No difference in haematocrit, Hb or MCV were observed at 12 months of age (King *et al.*, 2007).

Another clinical trial investigating the effect of bLf on haematological parameters was a double-blind, randomised, controlled trial carried out in China. Infants received formula fortified with bLf at levels of 38mg/100g powder, or the same formula without bLf. Both formulas contained 4mg iron/100g powder. A group of exclusively breastfed term infants was also enrolled as a reference group. Overall, 316 infants were included in the analysis (115 in bLf group, 98 in control group, 103 in breastfed reference group). Supplementation with bLf led to significantly higher haemoglobin levels vs. unsupplemented formula (125.5±15.4 vs. 116.9±13.1 g/L; p=0.000; no sign. difference at baseline). bLf supplementation also led to significantly higher serum ferritin levels vs. unsupplemented formula (44.7±17.2 vs. 31.6±18.4 µg/L; p=0.000; no sign. difference at baseline). Detection rate of ID and anaemia was significantly lower in bLf vs. control group (ID: 13.9% vs. 24.4%, p=0.049, no significant difference at baseline; anaemia: 4.1% vs. 7.5%, p<0.001, no significant difference at baseline). Prevalence of IDA was numerically but not statistically significantly different (1.7% vs. 6.1%, p=0.094). bLf supplementation also led to significantly higher total body iron content

(6.12±0.78 vs. 5.26±0.55 mg/kg body weight; p=0.000), but no significant effect on serum transferrin receptor (p=0.218) was found (Chen *et al.*, 2015a).

In a more recently published double-blind randomised controlled study, Chen *et al.* (2020) investigated the effect of bLf fortification on haemoglobin levels and other iron parameters in 6-9 month old infants with anaemia, but otherwise healthy. Infants received either unfortified infant formula (control); the same formula fortified with 38mg bLf/100g powder (low bLf group); or the same formula fortified with 76mg bLf/100g powder (high bLf group). All three formulas contained 4mg/100g of iron. Duration of intervention was 3 months. bLf supplementation at the higher level (76mg/100g) led to significantly higher Hb levels after 3 months intervention compared to lower bLf levels and no bLf (121.5±5.1 vs. 116.58±6.4 vs. 116.49±8.0, p=0.0059; no significant difference at baseline). No significant effect of bLf supplementation on serum ferritin or any other iron status parameters were found (Chen *et al.*, 2020).

The most recent study investigating the effect of bLf on haematological parameters and iron status was done in healthy, term infants in Sweden. Infants were recruited at 6±2 weeks of age, and intervention lasted until 6 months of age. Infants were randomised to receive either low-iron formula (2mg/L) supplemented with bLf (1000mg/L), low-iron formula without bLf, or high-iron formula (8mg/L) without bLf. A group of breastfed infants were also included as a reference group. The researchers found no effect of bLf addition on any haematological and iron status parameters (Björmsjö *et al.*, 2021).

3.2.2. Nutritional safety and tolerance of the proposed compositional change

3.2.2.1. Safety and tolerance of bLf in term infants

A significant body of evidence supports the safety of bLf for infants and provides support for the safe consumption of bLf under the intended use in Infant formula products. Five clinical trials in healthy term infants provide the most important data to support the safe use of bLf in infant formula, delivering results on key anthropometric parameters, as well as on tolerability (Table 3-6). In addition to these five studies, studies presented in Sections 3.2.2.2 and 3.2.3.2 provide further support for the safe use of bLf in the target population.

In a study by Hernell & Lönnerdal (2002), which was carried out in healthy term infants in Sweden, infants received formula with varying iron levels with or without bLf: control (n=11): infant formula with 4mg iron/L as ferrous iron; low iron (n=12): infant formula with 1.6mg iron/L as ferrous iron; bLf group (n=10): infant formula with added holo-bLf (~1000mg/L) and 1.8mg iron/L (1.3mg from Lf); nucleotide group (n=10): infant formula with added nucleotides and 2.2mg iron/L from ferrous iron. A breast-fed group was added as a reference group. Infants were recruited at 4±2 weeks of age, and intervention went until infants were 6 months of age. All formulas were well tolerated. No significant differences in weight or length at birth were observed among the groups. After adjustment for initial weight and height, height was significantly greater in the bLf group than in the nucleotide group at 4 and 6 months, and at 6 months the weight of infants in the bLf group was significantly greater than that of infants in the nucleotide group. No significant differences in

weight and height were observed at any time point between the bLf group and any other group, including the breastfed group (Hernell & Lönnerdal, 2002). A limitation of the study was that the study population was relatively small and may not be appropriately powered to detect statistically significant differences. However, the average weight and height of infants in the bLf group was numerically the highest among all groups at 4 and 6 months, giving confidence in the results supporting healthy growth of infants consuming bLf.

The second study was a double-blind, randomised controlled trial based in the United States (King *et al.*, 2007). The study included healthy term or near-term (≥34 weeks' gestation) bottle-fed infants aged 0-4 weeks when recruited. Infants received either an iron-fortified formula supplemented with 85mg bLf/100mL or the same iron-fortified formula without added bLf. Both formulas contained 0.3mg/100mL elemental iron. The bLf used in this study contained 120µg iron/g bLf powder. Duration of intervention was 12 months. Of 79 infants enrolled in the study, 52 completed the full-year study period, with similar drop-out rates in both study group. Of the 27 dropouts, 19 withdrew because of parental perception of infant intolerance (10 in the bLf formula group), 3 withdrew consent without further explanation, and 5 infants were lost to follow-up. Of the 52 infants included in analyses, 26 were in the bLf group and 26 in the control group. No statistically significant differences in growth parameters were noted, although a trend toward a greater increase in weight in the first six months was observed in the bLf group (p=0.06); this trend disappeared after 6 months of age. There was no difference in serious adverse events (SAE) (hospitalisation) between groups, and no formula tolerance issues were reported (King *et al.*, 2007). Limitations of the study was the lack of a breastfed reference group. While criteria for human trials have changed and matured over time, and many clinical trials in infants nowadays include a breastfed reference group, that approach has not always been considered necessary for gold standard intervention study protocols. This study was primarily set up to investigate the benefit of adding bLf to formula vs. not adding bLf to formula; the approach taken is appropriate for the primary purpose of the study. The study nevertheless provides valuable evidence for the safe use of bLf in formula compared to feeding formula not containing bLf.

The third study was a large growth study carried out in US infants and was set up specifically to assess the safety and tolerability of two intervention formulas containing bLf at two different levels (0.6 g/L and 1.0 g/L) (Johnston *et al.*, 2015). The intervention formulas were similar to the control formula, an already existing product on the market not containing bLf, but differed in two additional aspects: the intervention formulas had a prebiotic mix of polydextrose (PDX) and galactooligosaccharides (GOS) added, and had lower levels of arachidonic acid (ARA) compared to the control formula. No statistically significant group differences by gender in the primary outcome, weight growth rate from day 14–120, were detected. No statistically significant differences were observed for weight, length, or head circumference growth rates by gender for any measured age range among study groups with the exception of lower weight growth rate for females in the bLf-1.0 compared to the Control group from day 14–60 (29.7 ± 0.9 vs 32.4 ± 1.0 g/day; $P < 0.05$). This small difference within a single measured age range at less than 3 g/day was not considered clinically significant. In addition, no other statistically significant differences were observed for mean achieved weight, length, or head circumference at any measured time point up to 365 days of age. Parent-reported mean study formula intake increased from day 30–120 for all groups by gender, indicating normal intake for

bLf-0.6 and bLf-1.0 groups when compared to the control group for this time period. Parent-reported gassiness and fussiness were similar among groups at all study time points. Statistically significant differences in stool consistency were detected between control and intervention formula groups from day 30 through 180; this is most likely due to addition of the prebiotic blend. Of the 55 participants with formula-related discontinuation, formula intolerance as determined by the study investigator was the most common reason (control: 13; bLf-0.6: 14; bLf-1.0: 15) with fussiness (control: 5; bLf-0.6: 8; bLf-1.0: 10) and gas (control: 6; bLf-0.6: 3; bLf-1.0: 6) as the most common symptoms (Johnston *et al.*, 2015). A limitation of this study is that it does not contain a breastfed reference group. The study most likely was carried out to support a New Infant Formula Notification (NIFN) to the US Food and Drug Administration (FDA); FDA does not require a breastfed group to be included in growth studies supporting the safety of a new infant formula. Nevertheless, this study supports the safety and tolerability of bLf addition to infant formula seeing that no impact on growth or tolerance was seen with bLf addition to formula.

The fourth study providing data on safety of bLf addition to infant formula was carried out in 451 healthy term infants in China (Fei *et al.*, 2019). Infants were recruited at age 10-14 days and received formula containing bLf (0.6g/L) and milk fat globule membrane (MFGM), or the same formula not containing bLf or MFGM. No significant differences in growth rate (weight, length, head circumference) were detected between intervention and control group at any time point from 30 to 120 days, with the exception of slightly larger length growth rate at day 60 and 90 in females consuming bLf/MFGM formula. Formula intake increased at a similar rate in both groups, indicating normal intake in the intervention group. No differences in fussiness, gassiness, mean stool frequency or stool consistency were seen. Overall incidence of adverse events (AEs), categorised by respiratory and gastrointestinal system, were significantly lower in the intervention group. No other differences in other AEs are reported (Fei *et al.*, 2019). The key limitation of this study was that no breastfed infants were included as a reference group. This study was primarily set up to investigate the benefit of adding bLf and other components to formula vs. not adding these components to formula; the approach taken is appropriate for the primary purpose of the study. The study nevertheless provides valuable evidence for the safe use of bLf in formula compared to feeding formula not containing bLf.

The fifth study providing data on safety of bLf addition to infant formula was done in 246 healthy Swedish term infants. While recruitment age was 6±2 weeks and therefore does not strictly meet the requirements for safety studies outlined in Guideline 3.6.2 A3.1. b) (i) of the Application Handbook (infants recruited before age 1 month), recruitment age was only slightly above 1 month and therefore the study provides valuable support for the safe use of bLf in infants. Formula-fed infants received either low-iron formula (2mg/L) supplemented with bLf (1000mg/L), low-iron formula without bLf, or high-iron formula (8mg/L) without bLf. A reference group of breastfed infants was also included. No adverse or beneficial effects on gastrointestinal parameters were observed in infants receiving bLf fortified formula compared to other formula groups. All formula groups had significantly lower frequency of stools per day (1.36 [1.0-1.8] vs. 1.77 [1.2-2.8] in bLf and breastfed group, respectively, $p<0.05$), significantly fewer soft stools per day (1.2 [0.8-1.6] vs. 1.57 [0.8-2.5] in bLf and breastfed groups, respectively, $p<0.05$), but no differences in watery or hard stools between bLf and breastfed groups were observed. No significant differences in days with abdominal

pain was observed between formula-fed and breastfed infants, however, use of Simeticone (anti-bloating medication) was higher in all formula-fed compared to breastfed infants (20%, 15.9%, 18.8% vs. 2.9% in bLf, Group2, Group3 and breastfed group, respectively, $p<0.05$). No significant differences between groups were found for weight, length and head circumference at baseline. At 4 months of age, no significant differences in any anthropometric parameters across groups were observed. At 6 months of age, all formula-fed infants were significantly heavier than infants in the breastfed group (8.14±0.80 vs. 7.78±0.85 in bLf and breastfed group, respectively, $p<0.05$), with no significant difference between the formula groups. Length at 6 months was also significantly different between all formula-fed groups and breastfed group (67.5±2.1 vs. 66.3±2.3 in bLf and breastfed group, respectively, $p<0.05$), with length being greater in formula-fed groups; no significant difference in length across formula-fed groups was observed. There were no significant differences in head circumference at 6 months across formula-fed and breastfed groups, however, head circumference gain (mm/day 6w-6mo) was statistically significantly higher in formula-fed compared to breastfed groups (0.37±0.05 vs. 0.35±0.04 in bLf, low-iron no bLf, high-iron no bLf and breastfed group, respectively, $p<0.05$), but was not different across formula-fed groups (Björmsjö *et al.*, 2021). Other than the age of infants at recruitment being slightly above 1 month of age, no other limitations are noted. This study supports tolerance and normal growth of infants receiving infant formula with bLf.

Overall, these studies provide convincing evidence for the safe use of bLf up to levels of 1000mg/L in infants. While not all studies met all the criteria outlined in the FSANZ Application Handbook, taken together the evidence provided by these studies consistently supports the safe use of bLf in infant formula, with no adverse effects seen compared to formula-fed infants not receiving additional bLf, and also no adverse outcomes compared to breastfed reference groups.

Table 3-6 Intervention studies assessing the safety of bovine lactoferrin in healthy term infants

Reference	Study design	Country	bLf source	Study population, age at baseline and number	Study groups and intervention	Summary of findings relating to safety	Study limitations
Hernell and Lönnerdal (2002)	Single-blind, controlled intervention study (researchers were blinded, parents were able to choose formula)	Sweden	*SMR*; bLf was saturated with iron by researchers (1.24mg iron/g bLf protein)	Healthy, term infants, 4±2 weeks old N=59	Infants received either a control formula with 4mg iron/L, or one of 3 experimental formulae with 2mg/L iron (targeted level; actual levels varied - see below). Group 1 (reference group, n=16): Breastfed infants Group 2 (control, n=11): infant formula with 4mg iron/L as ferrous iron Group 3 (n=12): infant formula with 1.6mg iron/L as ferrous iron Group 4 (n=10): infant formula with holo-lactoferrin (~1000mg/L) and 1.8mg iron (1.3mg from lactoferrin) Group 5 (n=10): infant formula with added nucleotides and 2.2mg iron/L from ferrous iron Intervention duration: Until 6 months of age	All formulas were well tolerated. No significant differences in weight or length at birth were observed among the groups. After adjustment for initial weight and height, height was significantly greater in the bLf group (Group 4) than in the nucleotide group (Group 5) at 4 and 6 months. At 6 months the weight of infants in the bLf group was significantly greater than that of infants in the nucleotide group. No significant differences in weight and height were observed at any time point between bLf group and any other group, including the breastfed group.	The study population was relatively small and may not be appropriately powered to detect statistically significant differences. However, the average weight and height of infants in the bLf group was the highest among all groups at 4 and 6 months, giving confidence in the results suggesting healthy growth of infants consuming bLf. Some infants may have been older than 1 month at recruitment.
King *et al.* (2007)	Double-blind, randomised controlled trial	USA	*DMV (Friesland Campina)*; iron content of 120µg/g powder (estimated iron saturation: <10%)	Healthy, bottle-fed infants, ≥34wk gestation, >2kg at birth, 0-4 weeks of age N enrolled = 79 N incl. in analysis = 52 13 of 27 dropouts were in bLf group; 19 withdrew because of parental perception of intolerance (10 in bLf group), 3 withdrew	Infants received either formula supplemented with 85mg bLf/100ml or non-supplemented cow's milk-based formula, which the manufacturer claims contains naturally 10.2mg bLf/100mL [however, it is important to note that bLf from milk ingredients was likely denatured during the infant formula production process]. Group 1 (n=26): bLf supplemented formula at 85mg/100mL	No statistically significant differences in growth parameters were noted between the treatment and control groups. However, there was a trend toward a greater increase in weight over time for the bLf group for the first 6 months (p=0.06), a trend that disappeared after 6 months of age. No difference in serious adverse events (hospitalisation) between groups. No formula tolerance issues reported.	The study population was relatively small. No breastfed group included in study.

Table 3-6 Intervention studies assessing the safety of bovine lactoferrin in healthy term infants

Reference	Study design	Country	bLf source	Study population, age at baseline and number	Study groups and intervention	Summary of findings relating to safety	Study limitations
				consent without further explanation, 5 infants lost to follow up.	Group 2 (n=26): unsupplemented formula Intervention duration: 12 months		
Johnston *et al.* (2015)	Multi-centre, double-blind randomised controlled trial	USA	*DMV (Friesland Campina)*, standard bLf	Healthy, term infants, 12-16 days old N recruited = 480 N completed study = 353 4 did not consume study formula, 54 discontinued due to formula-related reasons, 69 discontinued due to reasons unrelated to formula; drop-out rates were similar across study groups	Infants received either an existing product on the market (Enfamil, Mead Johnson) or one of two bLf fortified formulae; as well as bLf the intervention formulae also contained a prebiotic blend of polydextrose (PDX) and galactooligosaccharides (GOS), and had lower levels of ARA compared to the control formula. Group 1 (control, n=110): Enfamil, which does not contain bLf Group 2 (n=127): 600mg/L bLf, as well as PDX/GOS blend, and lower ARA levels, otherwise no difference Group 3 (n=116): 1000mg/L bLf, as well as PDX/GOS blend, and lower ARA levels, otherwise no difference Duration of intervention: until 1 year of age	No statistically significant group differences by gender in the primary outcome, weight growth rate from day 14–120, were detected. No statistically significant differences were observed for weight, length, or head circumference growth rates by gender for any measured age range among study groups with the exception of lower weight growth rate for females in the bLf-1.0 compared to the Control group from day 14–60 (29.7 ± 0.9 vs 32.4 ± 1.0 g/day; $P < 0.05$). This small difference within a single measured age range at less than 3 g/day was not considered clinically significant. In addition, no other statistically significant differences were observed for mean achieved weight, length, or head circumference at any measured time point up to 365 days of age. Parent-reported mean study formula intake increased from day 30–120 for all groups by gender, indicating normal intake for bLf-0.6 and bLf-1.0 groups when compared to the control for this time period. Parent-reported gassiness and fussiness were similar among groups at all study time points. Statistically significant differences in stool consistency were detected between control and intervention formula groups from day 30 through 180; this is most likely due to addition of the prebiotic blend. Of the 55 participants with formula-related discontinuation, formula intolerance as determined by the study investigator was the most common	This was the only study specifically designed to assess safety of bLf-fortified formula. However, a limitation of this study is that it does not contain a breastfed reference group. The study most likely was carried out to support a New Infant Formula Notification (NIFN) to the US Food and Drug Administration, which does not require a breastfed group to be included in a growth study to support a NIFN. Nevertheless, this study supports the safety and tolerability of bLf addition to infant formula.

Table 3-6 Intervention studies assessing the safety of bovine lactoferrin in healthy term infants

Reference	Study design	Country	bLf source	Study population, age at baseline and number	Study groups and intervention	Summary of findings relating to safety	Study limitations
						reason (control: 13; bLf-0.6: 14; bLf-1.0: 15) with fussiness (control: 5; bLf-0.6: 8; bLf-1.0: 10) and gas (control: 6; bLf-0.6: 3; bLf-1.0: 6) as the most common symptoms.	
Fei *et al.* (2019)	Multi-centre, double-blind randomised controlled trial	China	*DMV (Friesland Campina)*, standard bLf	Healthy full term infants, 10-14 days of age. N recruited = 451 N completed study through day 365 = 292 For anthropometric and safety assessment all infants at a certain timepoint were included, and n will vary by month.	Infants received either formula with or without bLf and milk fat globule membrane (MFGM). Formulae used in intervention and control group were the same otherwise. Group 1 (n=223): formula + 0.6g/L bLf and 5g/L MFGM Group 2 (n=228): same formula without bLf or MFGM (control) Intervention duration: 1 year	No significant differences in growth rate (weight, length, head circumference) were detected between intervention and control group at any time point from 30 to 120 days, with the exception of slightly larger length growth rate in females consuming bLf/MFGM at day 60 and 90. Formula intake increased at a similar rate in both groups, indicating normal intake in intervention group. No differences in fussiness, gassiness, mean stool frequency or stool consistency. Overall incidence of AEs categorised by respiratory and gastrointestinal system were significantly lower in intervention group. No other differences in other AEs reported.	A limitation of this study is that it does not contain a breastfed reference group.
Björmsjö *et al.* (2021)	Double-blind randomised controlled trial	Sweden	*Hilmar*, standard bLf	Healthy full term babies, 6±2 weeks of age. Formula-fed infants and a breastfed reference group were included. N recruited = 180 formula fed and 72 breastfed N analysed = 176 formula fed and 70 breastfed 3 participants were considered to be poor compliers, 3 infants switched formula due to gastrointestinal	Formula-fed infants received either low-iron formula (2mg/L) supplemented with bLf (1000mg/L), low-iron formula without bLf, or high-iron formula (8mg/L) without bLf. Group 1 (n=72): low-iron + 1000mg/L bLf Group 2 (n=71): low-iron, no bLf Group 3 (n=33): high-iron, no bLf Group 4 (n=70): breastfed (reference group) Intervention duration: Until 6 months of age	No adverse or beneficial effects on gastrointestinal parameters were observed from adding bLf compared to other formula groups. All formula groups had significantly lower frequency of stools per day (1.36 [1.0-1.8] vs. 1.77 [1.2-2.8] in bLf and breastfed groups, respectively, $p<0.05$), significantly lower soft stools per day (1.2 [0.8-1.6] vs. 1.57 [0.8-2.5] in bLf and breastfed groups, respectively, $p<0.05$), but no differences in watery or hard stools between bLf and breastfed groups were observed. No significant differences in days with abdominal pain was observed between formula-fed and breastfed infants, however, use of Simeticone (anti-bloating medication) was higher in all formula-fed compared to breastfed infants (20%, 15.9%, 18.8% vs. 2.9% in Group 1, Group2, Group3 and breastfed group, respectively, $p<0.05$). No significant differences between groups were	Age of infants recruited in this study was above 1 month of age, although some infants may have been 1 month or less at recruitment; this means the study does not strictly meet the requirements outlines in Guideline 3.6.2 A3.1. b) (i) of the Application Handbook, however, this study nevertheless provides valuable support for the safe use of bLf in infants.

Table 3-6 Intervention studies assessing the safety of bovine lactoferrin in healthy term infants							
Reference	**Study design**	**Country**	**bLf source**	**Study population, age at baseline and number**	**Study groups and intervention**	**Summary of findings relating to safety**	**Study limitations**
				symptoms and excessive eczema		found for weight, length and head circumference at baseline. At 4 mo of age, no significant differences in any anthropometric parameters across groups were observed. At 6 mo of age, all formula-fed infants were significantly heavier than infants in the breastfed group (8.14±0.80 vs. 7.78±0.85 in bLf and breastfed group, respectively, $p<0.05$), with no significant difference between the formula groups. Length at 6mo was also significantly different between all formula-fed groups and breastfed group (67.5±2.1 vs. 66.3±2.3 in bLf and breastfed group, respectively, $p<0.05$), with length being greater in formula-fed groups; no significant difference in length across formula-fed groups was observed. There were no significant differences in had circumference at 6 mo across formula-fed and breastfed groups, however, head circumference gain (mm/day 6w-6mo) was statistically significantly higher in formula-fed than breastfed groups (0.37±0.05 vs. 0.35±0.04 in bLf and breastfed group, respectively, $p<0.05$), but was not different across formula-fed groups.	

Further supporting evidence for the safe use of bLf comes from many other clinical trials carried out in infants. None of the studies covered in Sections 3.2.1.2 and 3.2.3.2 reported adverse events related to consumption of bLf and consistently reported that the use of bLf is well tolerated and supported normal growth.

Overall, there are a substantial number of studies in term infants that provide convincing and consistent evidence for the safe consumption and tolerance of bLf for the intended use in infant formula and follow-on formula.

3.2.2.2. Evidence on safe use of bLf in preterm infants

There is significant evidence for safe use of bLf in preterm and low-birthweight (LBW) infants, which are a highly vulnerable group, with no safety concerns reported in any of the studies (see Table 3-7). Studies include a wide range of exposure levels to bLf, with none of the studies reporting any safety concerns at any of these levels.

Manzoni *et al.* (2009) studied the effect of 100mg bLf per day administered orally for 30 days in 472 very-low-birth-weight (VLBW) infants (500-1500g) in Italy and New Zealand. The researchers reported no intolerances or adverse events related to bLf administration. There was also no discontinuation of bLf administration due to presumed adverse effects, intolerance, or potentially dangerous interactions with other drugs. In an extension of the trial, Manzoni *et al.* (2014) included 743 VLBW infants from Italy and New Zealand, receiving the same treatment, and again, no adverse effects or treatment intolerance was reported.

Akin *et al.* (2014) investigated the effect of oral bLf at a dose of 200mg/day in 50 VLBW (<1500g BW) or premature (<32 weeks gestation) infants. bLf was given throughout hospitalisation. No adverse effects relating to bLf supplementation were reported.

Ochoa *et al.* (2015) investigated the effect of oral bLf administration at a dose of 200mg bLf/kg BW/day for a duration of 4 weeks in 190 infants with low birth weight (<2500g). The researchers reported no signs of allergy or treatment intolerance. Medical and surgical complications were similar between groups, as were growth (weight) measurements at 1 and 3 months. None of the severe adverse events were attributable to the intervention.

In a randomised, controlled, double-blind study in 139 neonates with a birth weight of <2000g, Kaur and Gathwala (2015) orally administered varying levels of bLf depending on weight of infants (BW 1000-1249g: 100 bLf mg/d; BW 1250-1499g: 150 bLf mg/d; BW 1500-1749g: 200 bLf mg/d; BW 1750-1999g: 250 bLf mg/d) for a period of 4 weeks starting with first day of life. No adverse effects relating to bLf supplementation were observed, and there were no discontinuations of treatment due to intolerance

Barrington *et al.* (2016) investigated tolerability and acceptability of adding bLf (100mg/day) to milk (mother's milk or preterm formula) in 79 infants born <31 weeks of gestation. Intervention was up to 36 weeks

postmenstrual age. There was no effect of bLf on feeding tolerance, and no adverse outcomes were reported.

The ELFIN trial, a large UK-based trial in 2182 very preterm infants (<32 weeks gestation), investigated the effect of administering bLf enterally (150mg/kg BW/day). Of 16 serious adverse events, 6 were in bLf group and 10 in control (sucrose) group; two serious adverse events (1 blood in stool and 1 death after intestinal perforation) in bLf group were assessed as being possibly related to trial intervention; other serious adverse events were considered unrelated (ELFIN Trial Investigators Group, 2019).

Ochoa *et al.* (2020) investigated the effect of adminstering 200mg/kg BW/day in 414 LBW infants (500-2000g). Signs or symptoms of allergic reactions or intolerance were closely monitored, with no differences in vomiting, abdominal circumference increase and diarrhoea reported. No serious adverse events were attributed to the intervention.

The LIFT trial carried out in Australia and New Zealand included 1542 VLBW (<1500g) who received milk (mother's milk or other) either with bLf (200mg/kg BW/day) or without bLf up to 34 weeks' post menstrual age. No safety concerns were reported, and adverse events and death/morbidity was similar between bLf and intervention group (Tarnow-Mordi *et al.*, 2020).

In conclusion, there are a substantial number of studies in preterm and LBW infants that provide convincing and consistent evidence for the safe consumption and tolerance of bLf for the intended use in Infant formula products, including infant formula for special dietary needs.

Table 3-7 Studies in pre-term infants supporting safety of bLf supplementation

Reference	Study population	bLf dose and duration of intervention	Safety and tolerance-related outcomes
Manzoni *et al.* (2009)	472 VLBW infants (500-1500g BW)	Orally administered bLf 100mg/day, birth until 30 days	No intolerances or AEs related to bLf were recorded. No discontinuation due to presumed adverse effects, intolerance, or potentially dangerous interactions with other drugs.
Manzoni *et al.* (2014)	743 VLBW infants (<1500g BW)	Orally administered bLf 100mg/day, birth until 30 days	No adverse effects or treatment intolerance occurred.
Akin *et al.* (2014)	50 VLBW (<1500g BW) or premature (<32 weeks) infants	Orally administered bLf 200mg/day, throughout hospitalisation	Adverse effects were monitored; no adverse effects reported.
Ochoa *et al.* (2015)	190 infants with BW <2500g	Orally administered bLf 200mg/kg BW/day, 4 weeks	No signs of allergy or treatment intolerance. Medical and surgical complications were similar between groups, as were growth (weight) measurements at 1 and 3 months. None of the severe AEs were attributable to the intervention.
Kaur and Gathwala (2015)	136 neonates with BW <2000g	Orally administered bLf BW 1000-1249g: 100 bLf mg/d; BW 1250-1499g: 150 bLf mg/d; BW 1500-1749g: 200 bLf mg/d; BW 1750-1999g: 250 bLf mg/d; 4 weeks	No adverse effects observed. No discontinuations due to intolerance.
Barrington *et al.* (2016)	79 infants <31 weeks gestation	Orally administered bLf 100mg/day added to milk, up to 36 weeks postmenstrual age	There was 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)	2203 very preterm infants <32 weeks	Enterally administered bLf 150mg/kg BW/day up to max. of 300mg/day, up to 34 weeks postmenstrual age	Of 16 serious AEs, 6 were in bLf group and 10 in control (sucrose) group; two serious AEs (1 blood in stool and 1 death after intestinal perforation) in Lf group were assessed as being possibly related to trial intervention; other serious AEs considered unrelated.
Tarnow-Mordi *et al.* (2020)	1542 VLBW with BW <1500g	Orally administered bLf 200mg/kg BW/day, up to 34 weeks postmenstrual age (median treatment 29 days)	No safety concerns reported; AEs and death/morbidity similar between bLf and intervention group.
Ochoa *et al.* (2020)	414 neonates with BW 500-2000g	Orally administered bLf 200mg/kg BW/day, 8 weeks	Signs or symptoms of allergic reactions or intolerance were closely monitored – no significant difference in vomiting, abdominal circumference increase and diarrhoea. No serious AEs attributed to intervention.

VLBW = very low birth weight; BW = body weight; AE = adverse event

To enable an estimate of what levels of bLf in formula may be safe for use in vulnerable population groups such as preterm and LBW infants, we calculated equivalent bLf concentrations per litre formula based on supplemented levels used in studies, and using estimated formula range intakes based on proposed range of milk feeding for preterm infants by the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) (see Table 3-8).

Table 3-8: Estimated equivalent bLf per L formula used in studies in preterm and low birth weight infants

Reference	Body weight	Intervention	Estimated formula intake range, based on ESPGHAN proposed range for milk feeding for preterm infants of 150-180mL/kg/day (Agostoni *et al.*, 2010)	Equivalent bLf per L formula, based on ESPGHAN proposed range for milk feeding for preterm infants of 150-180mL/kg/day
Manzoni et al. (2009); Manzoni et al. (2014)	500-1500 g	100mg bLf/d	76 – 270 mL/day	370 – 1316mg/L
Akin et al. (2014)	680-1870g	200mg bLf/d	102 – 337 mL/day	593 – 1961mg/L
Ochoa et al. (2015)	<2500g (500-2500g)	200mg bLf/kg BW/d (100-500mg/day)	76 – 450mL/day	1111 – 1316mg/L
Kaur and Gathwala (2015)	<2000g	BW 1000-1249g: 100mg bLf /d BW 1250-1499g: 150mg bLf/d BW 1500-1749g: 200mg bLf/d BW 1750-1999g: 250mg bLf/ d	150 – 225mL/day 188 – 270mL/day 226 – 315mL/day 262 – 360mL/day	444 – 667mg/L 556 – 798mg/L 635 – 885mg/L 694 – 954mg/L
Barrington *et al.* (2016)	~1000g (mean)	100mg/d	150 – 180mL/day	556 – 667mg/L
ELFIN Trial Investigators Group (2019)	500-2000g	150mg bLf/kg BW/day up to max. of 300mg/d (75-300mg/d)	76 – 360mL/day	833 –987mg/L
Tarnow-Mordi *et al.* (2020)	<1500g (~1065g mean)	200mg bLf/kg BW/d (~213mg/d mean)	~160 – 192mL/day (based on mean	1109 – 1331mg/L
Ochoa *et al.* (2020)	500-2000g	200mg bLf/kg BW/d (100-400mg bLf/d)	76 – 360mL/day	1111 – 1316mg/L
			Total range of equivalent bLf per 100mL formula	**370 – 1960mg/L**

3.2.2.3. After-marketing surveillance

Synlait currently manufactures infant formula products containing bLf destined for the Chinese market, where addition of bLf is permitted. Synlait has a system in place to ensure effective response to any customer complaints and concerns. All complaints received are recorded and investigated, and their root cause is identified. Actions appropriate to the seriousness and frequency of the complaint are taken promptly and effectively. The complaint data is analysed and used to implement ongoing improvement to

avoid recurrence of complaints. To date no complaints have been logged that were related to the presence of bLf in the product, providing confidence that addition of Synlait bLf to Infant formula products is safe.

Synlait also has been selling bLf as an ingredient to large international infant formula brand owners for use in their Infant formula products for many years, none of which have reported any adverse events related to bLf addition.

Globally there are no known recalls related to products due to presence of bLf.

3.2.3. Efficacy of the proposed compositional change – Reduced risk of infection

Breastfeeding is the best way to feed an infant. This is because human breastmilk not only provides all of the essential nutrients needed for growth and development, but also an array of bioactive components that support infant digestion, absorption, gastrointestinal functions, growth, immune development, and neurodevelopment, which leads to better developmental outcomes in breastfed compared to formula-fed infants (Demmelmair *et al.*, 2017; Lönnerdal, 2014).

Compared to formula-fed infants, breastfed infants are at lower risk of diseases such as diarrhoea and other infectious diseases, and it is thought that this is partly due to the presence of bioactive components in human breastmilk that are not present in infant formula, or present at much lower levels compared to human breastmilk. Lactoferrin is one of the components in human milk that has been shown to have beneficial effects on the infant's resistance to disease (Demmelmair *et al.*, 2017).

This section outlines evidence that supports a benefit in formula-fed infants of adding bLf to infant formula products compared to infant formula not containing bLf, notably reduced risk of infection and mechanisms supporting this reduced risk.

3.2.3.1. Mechanistic action of bLf relating to risk of infection

Lactoferrin's activity is related, in part, to its ability to resist proteolysis, as discussed in Section 2.3.1. The activity of lactoferrin can be divided into local effects in the gut lumen, and systemic effects mediated by lactoferrin receptors (LfR) present in the apical membrane of the small intestine (Lönnerdal *et al.*, 2011). Lactoferrin (including bLf) is able to bind to intestinal LfR's and be internalised by a clathrin-mediated endocytic mechanism (Liao *et al.*, 2012; Suzuki *et al.*, 2005), as discussed in more detail in Section 3.2.1.2. Once internalised lactoferrin will localise to the nucleus, and is able to act as a transcription factor (Liao *et al.*, 2012), and influence the expression of cytokines and growth factors (Lönnerdal, 2014). Non-LfR mediated activity of lactoferrin is related to its structure, and in particular its high affinity to ferric iron (Brock, 2012). Lactoferrin actively sequesters iron, resulting in a bacteriostatic effect by withholding iron from iron-requiring pathogens by forming localised iron-deficient regions (Vogel, 2012). The cationic N-terminal

region may act directly on bacteria, also resulting in antibacterial effects (Elass-Rochard *et al.*, 1995). Mechanisms supporting benefits of bLf in infants are discussed in more detail in the following sections.

3.2.3.1.1. Anti-bacterial effect

Several mechanisms have been proposed to support the effect of bLf on risk of infection, including an anti-bacterial effect which has been studied extensively. Lactoferrin's anti-bacterial effect is two-ways: its ability to bind iron removes iron as food from pathogens, thereby limiting their growth (bacteriostatic effect), and lactoferrin can also directly bind to, and inactivate, pathogens (bactericidal effect).

Early research focused on the bacteriostatic effect of lactoferrin by binding free iron and thereby removing it from pathogenic bacteria. Pathogenic bacteria require iron for their growth and survival (Ratledge & Dover, 2000) and thereby removing iron from the environment has a bacteriostatic effect. This was first shown by Bullen *et al.* (1972) who found that human lactoferrin was a potent inhibitor of *Escherichia coli* growth. However, the bacteriostatic effect on *E. coli* disappeared when iron was added and thereby lactoferrin iron-saturated (Bullen *et al.*, 1972), supporting the hypothesis that the iron-sequestering effect of lactoferrin contributes to the observed antibacterial effect. Aguila *et al.* (2001) found that human lactoferrin exerted a strong growth inhibitory effect against *Staphylococcus aureus*, even when the strains were antibiotic resistant. The growth inhibitory effect disappeared when iron was added, once again supporting the bacteriostatic effect of bLf dependent on its iron-binding properties.

Perhaps the most important aspect of bLf relating to its bacteriostatic effect is that the bound iron remains available to the infant whilst it is unavailable to pathogens. Infant formula products are fortified with iron to reduce the risk of iron deficiency and anaemia (Hernell *et al.*, 2015). When adding bLf to formula, it binds iron, making the bound iron unavailable to pathogens and thereby exerting a bacteriostatic effect, while at the same time iron bound to bLf remains available to the infant via absorption through specific receptors for human lactoferrin in brush-border membrane cells of human infants (Kawakami & Lönnerdal, 1991; Suzuki *et al.*, 2001). A detailed discussion and evidence supporting the bioavailability of iron bound to bLf is presented in Section 3.2.1.2.

While early research focused on the bacteriostatic effect of bLf by binding iron and removing it from pathogens, subsequent research led to the hypothesis that lactoferrin may also have bactericidal effects that are not due to removing iron from the pathogen's environment. Arnold *et al.* (1980) demonstrated the bactericidal effect of apo-lactoferrin (human), but not holo-lactoferrin, on *Streptococcus mutans*. Addition of iron did not reverse or prevent lactoferrin's ability to kill *S. mutans*, providing support for a bactericidal effect of lactoferrin that is independent of simple iron deprivation. Ellison *et al.* (1988) demonstrated that human lactoferrin (3-10% iron saturation) produced significant lipopolysaccharide (LPS) release from, and therefore damage to, the gram-negative outer membrane of *E. coli* and *Salmonella typhimurium* (Ellison *et al.*, 1988). This effect was not observed with iron-saturated lactoferrin (101-102% saturation). The researchers were able to replicate their findings in a follow-up study (Ellison *et al.*, 1990).

The ability of lactoferrin to bind LPS is key for its bactericidal property. Elass-Rochard *et al.* (1995) identified two LPS-binding sites on human lactoferrin, and found that bovine lactoferrin exhibited the same behaviour towards LPS, also containing two LPS-binding sites (Elass-Rochard *et al.*, 1995). The bactericidal effect of lactoferrin is now well established, and was recently confirmed in a study by Lönnerdal *et al.* (2020) where 10 commercial bLf samples, including a sample of Synlait bLf, were found to have bactericidal effects on *E. coli* similar to human lactoferrin. Numerous other *in vitro* studies support the antibacterial effect of bLf, as summarised by Jenssen and Hancock (2009)

Two animal studies investigated the antibacterial effect of bLf on *Enterobacteriaceae* and *Clostridium* species in the gut of mice. The first study investigated the effect of varying levels of native bLf, apo- (2.3% iron saturation) and holo-bLf (97.6% iron saturation) on faecal *Enterobacteriaceae* and found a significant decrease independent of the degree of iron saturation of bLf, with a dose-response effect from 0.5% to 2% bLf content of milk, after which it plateaued (Teraguchi *et al.*, 1993). The finding that holo-bLf also showed an antibacterial effect *in vivo* may suggest that partial digestion of holo-bLf may expose binding sites necessary to attach to the cell wall of bacteria, thereby enabling its bactericidal effect. In another animal model using mice, Teraguchi *et al.* (1995) found a bacteriostatic effect of native bLf on several *Clostridium* strains, and again found a dose-response effect from 0.5% to 2% bLf content of milk.

Overall, there is significant evidence to support the antibacterial effect of lactoferrin, including bLf.

3.2.3.1.2. Anti-viral effect

Lactoferrin has also been shown to have anti-viral effects in a large number of *in vitro* studies (Berlutti *et al.*, 2011). For example, Hasegawa *et al.* (1994) showed that bLf has anti-viral activity against human cytomegalovirus (CMV) and human herpes simplex virus-1 (HSV-1), significantly reducing replication of both viruses in a dose-dependent manner, and inhibiting adsorption and penetration of the virus to the host cells (Hasegawa *et al.*, 1994). Portelli *et al.* (1998) investigated the effect of human lactoferrin (hLf) on respiratory syncytial virus (RSV) and CMV and found that hLf inhibited growth of both RSV (80-85%) and CMV (90-95%). The researchers also tested inhibition of virus of hLf fortified formula and found that levels of 0.5-1 mg/mL inhibited RSV to a lesser degree (30%) than isolated hLf while it remained at a similar level for CMV (95-98%); lower levels of hLf in formula showed less effectiveness *in vitro* (Portelli *et al.*, 1998).

Yamamoto *et al.* (2010) investigated the effect of bLf on human parainfluenza virus type 2 (hPIV-2) and found that bLf was able to reduce virus RNA synthesis and virus protein synthesis but was not able to inhibit these completely. However, the researchers found that virus entry into cells was considerably inhibited by bLf binding to the cell surface, while cell-to-cell spread was not inhibited. The number of viruses produced by cells was found to be significantly reduced by bLf (Yamamoto *et al.*, 2010).

Arnold *et al.* (2002) investigated the effect of bLf in three different forms (native, apo [iron-depleted], holo [iron-saturated]) on adenovirus activity, which is a common virus leading to infections in infants. Results showed that all forms of bLf were able to inhibit adenovirus replication in a dose-dependent manner, with native bLf showing a somewhat larger effect than holo- and apo-bLf. The researchers further tested four

different scenarios: cells were incubated with bLf before infection; bLf was added together with the virus inoculum during the adsorption step; bLf was incubated with the cells after the viral adsorption step; and bLf was present during the whole experiment. bLf was effective in preventing viral replication when added both before or during the viral adsorption step and when it was present during the entire replicative cycle, but it was not effective when it was added after viral adsorption (Arnold *et al.*, 2002). In a follow-up study by the same research group, the researchers showed that preincubation of cells with bLf produced an inhibition of viral infection of about 60%, whereas when bLf and adenovirus were mixed together for 1 hour and then added to cells, viral antigen synthesis was inhibited by about 95%. The researchers showed that bLf was able to bind to the virus via two adenovirus proteins (Pietrantoni *et al.*, 2003).

Another common type of virus causing illness in early life are rhinoviruses. Clarke and May (2000) tested the effect of hLf on three rhinovirus strains and found that it failed to inhibit their growth when cells were incubated with hLf before adsorption of the viruses; hLf did inhibit growth of CMV (Clarke & May, 2000), confirming results by Portelli *et al.* (1998). Denani *et al.* (2021) recently investigated the effect of bLf on rhinovirus RV-B14, adding bLf at different steps of the infection cycle (pre-adsorption, adsorption, post-adsorption, all steps). The strongest effect was observed when bLf was presented during adsorption with ~52% plaque reduction and throughout all steps (60% plaque reduction). Pre-adsorption and post-adsorption addition of bLf was significantly less effective with only around 30% plaque reduction (which might explain the lack of benefit in the study by Clarke and May (2000)). The substantial reduction observed when bLf was present at the start of the infection, comparable to its continuous presence at all steps, indicates that it predominantly interfered with an early step of the viral life cycle. Further investigation showed that the presence of bLf at that step resulted in a decrease in RV-B14 entry by around 44%. Since virtually no surface-associated virus was detected, the authors proposed that this confirmed that bLf prominently acts during cell entry by significantly blocking virus attachment (Denani *et al.*, 2021).

Rotaviruses are common pathogens leading to gastrointestinal illness. Superti *et al.* (1997) found that both apo- and holo-bLf were able to inhibit replication of rotavirus SA-11 in a dose-dependent manner, with apo-bLf being the most effective. The researchers also found that apo-bLf hinders virus attachment to cell receptors by binding to viral particles and preventing both rotavirus haemagglutination and viral binding to susceptible cells. Further, bLf markedly inhibited rotavirus antigen synthesis and yield when added to cells during viral adsorption or when it was added within the first hour of infection (Superti *et al.*, 1997).

Another area that has gained increasing attention recently is the effect of bLf on common human coronaviruses and SARS-CoV-2. A recent in vitro study found that bLf has broad-spectrum antiviral activity against SARS-CoV-2 and other coronaviruses in cell culture, with bLf being more potent than hLf. The antiviral mechanism of action of bLf was found to be mediated through binding to heparan sulfate proteoglycans (HSPGs) on the host cell surface, thereby preventing viral attachment to the host cells (Hu *et al.*, 2021).

Overall, there is significant evidence to support anti-viral activity of lactoferrin, including bLf.

3.2.3.1.3. Immunomodulatory effect

Oral lactoferrin intake also has direct immunomodulatory effects and has been suggested to be able to both up- and down-regulate immune response (Demmelmair *et al.*, 2017). This has been shown in several animal studies.

Several studies in mice support the immunomodulatory effect of bLf. Debbabi *et al.* (1998) fed mice bLf for a period of four weeks, giving them either a low dose (0.05mg bLf/g BW/day) or a high dose (1mg bLf/g BW/day). bLf feeding led to significantly increased IgA and IgG levels in intestinal fluid and saliva, while no effect on serum IgA or IgG levels was seen. IgA and IgG secretion was enhanced in Peyer's patches and spleen from bLf-fed mice compared to control. The authors concluded that bLf could act as an immune-stimulating factor on the mucosal immune system (Debbabi *et al.*, 1998).

Wakabayashi *et al.* (2006) investigated the effect of acute bLf administration on number of leukocytes in peripheral blood and spleens of mice 24 hours after administration of 2.5g bLf/kg BW. bLf supplementation led to changes in the number of cells in leukocyte subsets in the peripheral blood and spleens, confirming immunomodulatory effects. bLf supplementation also modulated expression of genes closely related to the host defence in the small intestine. Takakura *et al.* (2006) found that bLf enhanced both interferon (IFN)-γ and interleukin (IL)-10 production in intestinal intraepithelial lymphocytes and mesenteric lymph-node (MLN) cells. Kuhara *et al.* (2006) showed that bLf increases natural killer (NK) cell populations in peripheral blood and spleen in a dose-dependent manner, and enhances IFN-γ production by NK cells. Oral bLf administration also produced an increase in IL-18 levels in the portal circulation, and increased expression of IFN-α and IFN-β in Peyer's patches and MLN.

Three additional studies in mice by research groups in Mexico provided further support for an immunomodulatory effect of bLf in the intestine (Arciniega-Martinez *et al.*, 2016; Godinez-Victoria *et al.*, 2017; Ynga-Durand *et al.*, 2021).

Two studies were in neonatal piglets, which is a well-accepted model for human infant gastrointestinal function, growth, and metabolism (Comstock *et al.*, 2014), and therefore provides particularly relevant support for a beneficial effect in a human infant population. The first study in piglets investigated the effect of bLf on NK cells, components of the innate immune defence system. NK cell levels are known to differ between breastfed and formula-fed infants. Piglets were either sow-reared or given formula with or without bLf. Addition of bLf to formula was able to increase NK cell population size similar to those found in sow-reared piglets, and significantly more compared to unsupplemented formula. Levels in the MLN were higher in sow-reared and bLf-supplemented piglets compared to those fed standard formula ($p<0.0097$). Levels in the spleen were also higher in sow-reared and the bLf formula groups compared to the standard formula group, but this did not reach statistical significance. Levels in peripheral blood mononuclear cells were significantly higher in sow-reared piglets compared to the standard formula group, with the bLf group being in the middle with no significant difference to either sow-reared or standard formula group. The researchers found no increase in NK cytotoxicity with bLf supplementation (Liu *et al.*, 2013).

In the second piglet study, researchers investigated the effect of bLf supplementation on several immune parameters. Neonatal piglets were removed from the sow immediately after birth and were given either:

control formula without addition of bLf (but innate bLf levels at 360mg/L); formula with bLf at 1000mg/L; or formula with bLf at 3620mg/L. The two lactoferrin concentrations were selected to reflect the dose of Lf consumed by breastfed human infants or 5 times that dose on a mg/kg BW basis. Lymphocyte populations (cluster determinant (CD)4, CD8, and NK cells) developed normally and were unaffected by dietary bLf. Piglets fed the higher bLf levels tended to have 1.4 to 2 times more serum IgG than control piglets (p=0.07) or piglets fed lower bLf levels (p=0.03), but IgA in ascending colon contents was unaffected by bLf. Spleen cells from high bLf fed piglets produced 2 times more IL-10 and tumour necrosis factor (TNF)-α *ex vivo* than those from control or lower bLf piglets. MLN cells from low bLf and high bLf piglets produced 40% more IL-10 and tended to produce 40% more IL-6 (P = 0.05) than those from control piglets. The authors concluded that dietary bLf alters the capacity of MLN and spleen immune cells to respond to stimulation, supporting a role for bLf in the initiation of protective immune responses in these immunologically challenged neonates (Comstock *et al.*, 2014).

Further support for the immune modulatory effect also comes from *in vitro* studies. For example, Kong *et al.* (2020) found that when rat intestinal epithelial cells were challenged with lipopolysaccharides, co-stimulation of bLf significantly decreased inflammatory markers IL-6 and TNF-α, decreased mRNA level of IL-1β, IL-6 and TNF-α, and inhibited activation of a key signalling pathway induced by LPS (Kong *et al.*, 2020). Lönnerdal *et al.* (2020) investigated the effect of bLf samples on IL-18 and transcription of the TGF-β1 gene in Caco-2 cells, and found a modest effect on both parameters.

Overall, evidence from animal studies confirm an immunomodulatory effect of bLf.

3.2.3.1.4. Preventative effect of bLf on infections in animals

Gastrointestinal infections

Four animal studies, three in mice and one in rats, investigated the preventative effect of oral bLf administration on common pathogens causing gastrointestinal infections. In the first study, bLf with an iron saturation of 30% was given to four-week-old germfree male and female mice. bLf was provided as a 10mg/mL bLf solution in place of water. On day 7, mice were exposed to two enterotoxigenic *Escherichia coli* (ETEC) strains using a stomach probe. bLf administration did not reduce viable ETEC cell count in faecal specimens and therefore did not show a bacteriostatic effect. However, adhesion to intestinal tract of both ETEC strains was reduced by oral administration of bLf, and this effect was seen very soon after exposure. The authors speculated that bLf's anti-adhesive effect is likely a key mechanism for reducing risk of gastrointestinal infections since adhesion to the intestinal tract is the first step in an infection (Kawasaki *et al.*, 2000).

Mosquito *et al.* (2010) investigated the effect of bLf on *Salmonella* ser. Typhimurium infection in female BALB/c mice 6 to 8 weeks old. The mice were given bLf with an iron saturation of 15% at a concentration of 10mg/ml, which is approximately the human Lf concentration present in colostrum. Mice in the bLf group received 200µL of the bLf solution two hours before infection, and then for seven days. Administration of

bLf led to significantly lower mortality (1/29 compared to 8/29 in the control group; $p<0.05$), less clinical signs of infection, less bacteraemia as a consequence of infection, and less histopathologic abnormalities. Further, mice given bLf did not lose weight, compared to the control group that showed significant weight loss from day 3 to 5 post infection. The blood culture was positive for *Salmonella* for all mice studied in the control group (17/17), but was positive in only 6/17 animals in the bLf group ($p<0.05$). Overall, bLf showed a protective effect against *Salmonella* ser. Typhimurium in mice (Mosquito *et al.*, 2010).

Drago-Serrano *et al.* (2010) also investigated the effect of bLf supplementation on *Salmonella* ser. Typhimurium in mice. Male BALB/c mice 8 to 12 weeks old were given a low (5mg) and high (100mg) dose of bLf (3% iron saturation) daily for 21 days. Mice were infected with *Salmonella* ser. Typhimurium on day 7 with either a lethal or sub-lethal dose. Survival of mice given the lethal dose was significantly greater in both bLf groups compared to the control group, with 80% surviving from days 9-14 post-infection vs. 40% in the control group. All mice given the sub-lethal dose survived. Intestinal bacterial load was examined in mice given the sub-lethal dose, with bLf significantly reducing bacterial load in faeces; no difference between high and low bLf groups was found. Bacterial colonisation at the Peyer's patch was also significantly lower in mice treated with bLf, with no significant differences between low and high bLf groups. bLf administration also reduced systemic bacterial translocation and enhanced levels of IgA, IgG and IgM antibodies. The authors proposed that the effect of bLf against infection of *Salmonella* ser. Typhimurium in mice may have been the result of an antimicrobial activity linked with its modulatory effect on immunocompetent cells (from intestinal and peripheral organs) involved in antibody production (Drago-Serrano *et al.*, 2010).

Perez-Cano *et al.* (2008) investigated the effect of whey protein concentrate (WPC) given with our without bLf on rotaviral infection in suckling rat pups. Rat pups were supplemented from day 3 of life and received either WPC, WPC plus bLf, standard infant formula or no supplemental feed (control). Rats were infected with rotavirus at day 8 of life at a level that induced mild diarrhoea but did not provoke weight loss or death. WPC and WPC+bLf supplementation led to lower incidence of rotavirus infection compared to standard infant formula and control, with no difference between the two groups. However, only WPC+bLf supplementation led to significantly lower diarrhoea index and diarrhoea severity compared to standard formula and control groups, suggesting a benefit of bLf supplementation. bLf supplementation also appeared to reduce the duration of diarrhoea, although this did not reach statistical significance. Interestingly, viral load in the WPC+LF group was significantly higher compared to the control group, although statistical significance was not achieved. The authors speculate that this increased viral shedding may be due to bLf reducing viral adsorption (i.e. bLf is 'flushing out' the virus) (Perez-Cano *et al.*, 2008).

Respiratory infections

One study investigating the effect of bLf supplementation against respiratory syncytial virus (RSV) in a mouse model failed to find any effects on RSV loads, lung inflammation or any of the immune parameters assessed. There was also no effect on weight loss, degree of airway obstruction and disease severity scores. Since Lf exerts antiviral activity against RSV *in vitro*, the authors proposed that the lack of antiviral

activity may have been attributed to a possible lack of absorption from the small bowel to the systemic circulation and therefore lungs (Gualdi *et al.*, 2013). It is noteworthy that bLf administration started only 48 hours before infection, which may not have been enough time for an immunomodulatory effect that may have influenced the outcome.

Another study investigated the effect of bLf administration on influenza virus in a mouse model. Specific-pathogen-free female BALB/c mice 6 weeks old were administered 62.5mg bLf once daily, with administration starting one day before infection and until day 6 after infection. Initially 18 animals per group were included, and a non-significant effect on consolidation score was detected between mice given bLf and control mice; after including an additional 20 animals per group to investigate the effect further, a significantly lower consolidation score was observed in mice given bLf compared with control mice ($p=0.040$). No significant effect on body weight loss and virus yield in bronchoalveolar lavage fluid (BALF) was found. bLf administration led to significantly lower total numbers of infiltrated cells in BALF on day 6 ($p = 0.004$) but not yet on day 4. Mice given bLf had significantly lower numbers of macrophages and neutrophils in infiltrated cells on day 6 compared to the control mice ($p=0.041$). Suppression of the infiltrated inflammatory cells due to bLf administration was consistent with the reduction in the lung consolidation score. The authors concluded that bLf administration beneficially affected influenza-virus-infected mice and attenuated pneumonia by suppressing the infiltration of inflammatory cells in the lung (Shin *et al.*, 2005).

Staphylococcus aureus

Two animal studies, one in mice and one in piglets, investigated the effect of bLf administration on *Staphylococcus aureus* infection, which is a fatal bacterial infection for neonates. The objective of the mouse study was to examine the effect of bLf administration on kidney infections induced by *Staphylococcus aureus.* Animals were given bLf in water or whole bovine milk one day prior to infection and for a period of 14 days. bLf administration reduced the number of kidney infections by 40-60% and bacterial counts in kidneys 5-12 fold, with no significant difference across different iron saturation levels of bLf (Bhimani *et al.*, 1999).

The second study investigating the effect of bLf on *Staphylococcus aureus* infection was done in 49 female neonatal pigs. Piglets were fed sow-milk replacer formula with either 4g supplemental protein/L as whey (control) or as bLf. Piglets were fed 360mL formula/kg BW/day, resulting in a mean supplemental protein intake of 4g/day at day 7, and 4.8g/day at day 12. On day 7, piglets were infected with *Staphylococcus aureus.* Piglets supplemented with bLf had significantly lower numbers of *Staphylococcus aureus* in the kidneys ($p=0.02$) and tended to have lower numbers in the lungs ($p=0.07$) and heart ($p=0.06$). bLf supplementation also led to more weight gain over the study period. The authors proposed that the lower bacterial count in organs showed improved bacterial clearance in the presence of bLf, which they argue was likely due, in part, to an enhanced Th1 immune response in bLf-supplemented pigs, which is supported by their observation of increased IFN-γ mRNA expression in the lungs after infection. Furthermore, bLf-supplemented pigs had less serum IL-10 on day 7 postpartum just before *Staphylococcus aureus* infection; a high concentration of IL-10 is known to inhibit Th1 immune response (Reznikov *et al.*, 2018).

Overall, evidence from animal studies support a benefit of bLf administration of reduced risk of infections, with evidence being particularly strong for gastrointestinal infections.

Summary of mechanistic studies

Evidence supports several mechanisms of how bLf can reduce risk of infections. bLf shows a strong antibacterial effect, which it exerts in two ways: a bacteriostatic effect by removing iron and a bactericidal effect by directly binding to the cell walls of bacteria and killing them. *In vitro* studies also support an anti-viral effect of bLf, and animal studies confirm that bLf exerts immunomodulatory effects, upregulating immune parameters in the intestine and the body. Several animal studies found a reduced risk of infection or better clinical outcomes when an infection occurred, providing further support for bLf's beneficial effect on risk of infection.

3.2.3.2. Evidence from intervention studies in term infants

Four human intervention studies in healthy term infants were identified that investigated the effect of oral bLf on risk of infection. The details of these studies are presented in Table 3-9 and will be briefly discussed in the following.

The first study by King *et al.* (2007) found a significantly reduced risk of respiratory infections in infants receiving bLf-fortified formula versus standard formula. This double-blind, randomised controlled trial based in the United States aimed to assess the impact of long-term feeding of bLf-enhanced formula on growth, haematologic and immune parameters, and the evaluation of common childhood illness. The study included healthy term or near-term (≥34 weeks gestation) bottle-fed infants aged 0-4 weeks when recruited. Infants received either an iron-fortified formula supplemented with 85mg bLf/100mL or the same iron-fortified formula without added bLf, which according to the manufacturer naturally contains 10.2mg bLf/100mL (however, any bLf naturally present in milk-based ingredients were likely denatured during processing[18]). Both formulae contained 0.3mg/100mL elemental iron. The bLf used in this study contained 120µg iron/g bLf powder, which means the estimated iron saturation was less than 10%. Duration of intervention was 12 months. Of 79 infants enrolled in the study, 52 completed the full-year study period, with similar drop-out rates in both study group. Of the 27 dropouts, 19 withdrew because of parental perception of infant intolerance (10 in the bLf formula group), 3 withdrew consent without further explanation, and 5 infants were lost to follow-up. Of the 52 infants included in analyses, 26 were in the bLf group and 26 in the control group. Supplementation with bLf led to significantly fewer episodes of lower respiratory tract infections (0.15 vs. 0.5 episodes per child-year, $p<0.05$). No significant differences were seen for upper respiratory infections, acute otitis media or diarrhoea. No significant differences in duration of any illnesses were noted. Laboratory results showed no significant differences in antibody levels to diphtheria, tetanus, human

[18]It is not clear if this was a calculated value based on the theoretical contribution from milk ingredients, or if this was a measured value; if it was the earlier, then based on the conditions used in manufacturing of infant formula powders it is reasonable to assume that most of the lactoferrin present would be in a denatured state.

influenzae B, or hepatitis B (King *et al.*, 2007). Effect on haematological parameters is discussed in Section 3.2.1.2. The study had several strengths, most notably that it was randomised, controlled and double-blind. However, a limitation of the study was the small sample size and large number of variables studied with no pre-defined primary outcome. King *et al.* (2007) did not include a breast-fed reference group. While criteria for human trials have changed and matured over time, and many clinical trials in infants nowadays include a breastfed reference group, that approach is not always considered necessary for gold standard intervention study protocols. While for studying the safety of new components the inclusion of a breastfed reference group may provide valuable information, for the purpose of studying the differential *benefit* of adding a component to an infant formula versus not adding that component to formula, the approach taken by King *et al.* is appropriate for the investigation of efficacy, on a differential basis, in nutritional intervention studies. Therefore, the lack of a breastfed group does not impact the objective or conclusions of this study on efficacy of adding bLf to infant formula versus not adding bLf to formula.

The second clinical trial by Chen *et al.* (2016) was also a double-blind, randomised, controlled trial carried out in China, and again found a reduced risk of respiratory infections in formula-fed infants receiving bLf, and also found a reduced risk of diarrhoea-related illness. The study included healthy term infants previously exclusively breastfed but weaned, aged 4-6 months. Approximately 260 infants were recruited and randomised to either receive formula fortified with bLf at levels of 38mg/100g powder, or the same formula without added bLf. Both formulas contained 4mg iron/100g. A group of 130 exclusively breastfed term infants was also enrolled as a reference group. Thirty-one infants were excluded due to parents' refusal to participate. The primary study outcome was morbidity of diarrhoea and respiratory tract infections during the intervention (3 months). Secondary outcome was the effect of intervention on the duration of respiratory- and diarrhoea-related illnesses. Of the 359 infants randomised, 43 dropped out: 5 were rejected for using another formula, 22 for loss of data, 1 for formula allergy, and 15 for adding formulae due to insufficiency of breastmilk. No information on drop-out by group is given. Overall, 316 infants were included in the analysis (115 in bLf group, 98 in control group, 103 in breast-fed reference group). Supplementation with bLf led to significantly less morbidity of respiratory-related illness and diarrhoea-related illness compared to the control group ($p<0.05$), while no significant difference between bLf-fortified group and breast-fed reference group was found. Significant beneficial effects of bLf fortification was also found for running nose, cough, wheezing and diarrhoea. In addition, duration of respiratory- and diarrhoea-related illnesses were shorter in the bLf and breast-fed groups compared to the control group ($p<0.05$) (Chen *et al.*, 2016).

Further evidence for a reduced risk of both respiratory- and diarrhoea-related illness comes from another more recently published double-blind randomised controlled study by Chen *et al.* (2021). This study investigated the effect of bLf fortification on diarrhoea and respiratory tract infections in previously breastfed but weaned infants with anaemia, but otherwise healthy. Infants were aged 6-9 months when recruited, and had anaemia diagnosed according to World Health Organization (WHO) criteria (haemoglobin <100g/L for those aged 4-6 months or <110g/L for those aged ≥6 months) (Chen *et al.*, 2021). According to the WHO, around 15% of infants and young children aged 6-59months in Australia and New Zealand have haemoglobin levels <110g/L and are therefore considered anaemic (World Health

Organization, 2015). Chen *et al.* (2021) randomly assigned infants to one of three groups: unfortified infant formula (control); the same formula fortified with 38mg bLf/100g powder (low bLf group); or the same formula fortified with 76mg bLf/100g powder (high bLf group). All three formulae contained 4mg/100g of iron. Duration of intervention was 3 months. Primary endpoints of this study were morbidity of diarrhoea and respiratory tract infections, and secondary endpoints were effect on duration of respiratory and diarrhoea-related illness and the immune parameters measured in faecal samples. Overall, 108 infants were assessed, and following exclusion of three infants due to refusal to accept written informed consent, 105 infants were randomised across the three groups (n=35 in each group). Subsequently nine infants were excluded owing to a lack of haemoglobin data before, during and/or after the intervention (2 in the low bLf group, 7 in the high bLf group). For respiratory-related illness, significantly less morbidity in the high bLf group vs. control group were reported (3.33 vs. 5.56 morbidity events per 100 child days, $p<0.05$). While morbidity events of respiratory-related illness were also lower in the low bLf group vs. control, this did not reach statistical significance (4.44 vs. 5.56, $p>0.05$). Wheezing was significantly lower in both the low- and high-bLf groups vs. control group (2.29 and 2.22 vs. 5.56, respectively; $p<0.05$). Diarrhoea-related events (2.42 vs. 5.56, $p<0.05$) and diarrhoea (2.22 vs. 5.56, $p<0.05$) were also significantly lower in the high-bLf vs control group. Morbidity events for diarrhoea-related events (4.44 vs. 5.56, $p>0.05$) and diarrhoea (4.48 vs. 5.56, $p>0.05$) were numerically lower in low-bLf vs. control group, but once again did not reach statistical significance. Morbidity of nausea and vomiting was significantly lower in low-bLf and high-bLf vs. control groups (vomiting: 3.84 and 2.58 vs. 6.67, $p<0.05$; nausea: 4.14 and 2.82 vs. 6.67, $p<0.05$). Except for duration of rhinorrhoea, no significant differences were observed in duration of illness. A significant beneficial effect on faecal biochemical indexes was also found, with significant effects found for both doses vs. control formula across all biomarkers ($p<0.05$), and with the higher dose showing significantly more beneficial effects compared to the lower dose across all markers ($p<0.05$). No serious adverse events in any of the intervention groups were observed (Chen *et al.*, 2021). Chen *et al.* (2021) did not include a breastfed reference group in this study, noting that they had included a breastfed group in their earlier study. Of particular note in this study is the study population group was infants ≥6 months. At this age infants start having a more diverse diet and therefore comparison of formula versus breastfeeding becomes more challenging, and the rationale to include an exclusively breastfed group is nullified. As noted above, for the purpose of studying the *benefit* of adding a component to an infant formula versus not adding that component to formula, the approach taken by Chen *et al.* is appropriate. Therefore, the lack of a breastfed group does not impact the conclusions of this study on efficacy of adding bLf to infant formula versus not adding bLf to formula

The most recent study was carried out in healthy, term infants in Sweden. This double-blind, randomised controlled trial was primarily done to evaluate the effect of lowering iron levels in infant formula on haematological parameters (see Section 3.2.1.2) and on immunological development; in addition, the researchers investigated the effect of adding bLf to lower-iron infant formula. Infants were recruited at 6±2 weeks of age, and intervention lasted until infants were 6 months of age. Infants were randomised to receive either low-iron formula (2mg/L) supplemented with bLf (1000mg/L), low-iron formula without bLf, or high-iron formula (8mg/L) without bLf. A group of breastfed infants were also included as a reference group. The primary immunological development-related endpoint was cytokine profile, and secondary endpoints

were prevalence of infections and other infection-related morbidity and treatments. There were no significant differences in cytokine levels across all groups throughout study duration, with breastfed infants showing similar levels to all formula-fed groups. TGF-β2 was significantly lower in the pooled low-iron group compared to the high-iron group at 6 months, but not at other time points. No other significant differences were found. For the secondary endpoints, no significant effect of bLf supplementation was observed (Björmsjö *et al.*, 2022). This study supports the safe addition to formula in a healthy, well-nourished population group. All study groups had similar blood immune parameter levels to breastfed infants, which is likely an explanation for why no additional effects of bLf supplementation were found in this well-nourished population group. While no significant effect on prevalence of infections was found, it is important to note that this was not the primary endpoint of the study, and neither was the primary objective of the study to assess the efficacy of bLf, but to assess the safety of reduced iron levels.

Overall, these randomised controlled trials support a beneficial effect of bLf on reduced risk of gastrointestinal and respiratory infections, with no safety issues reported in any of the studies.

Table 3-9 Intervention studies assessing the effect of bovine lactoferrin on risk of common infections in healthy term infants (≤12 months)

Reference	Study design	Country	bLf source	Study population, age at baseline and number	Study groups and intervention	Summary of findings	Significance of findings
King *et al.* (2007)	Double-blind, randomised controlled trial	USA	*DMV (Friesland Campina)*, iron content of 120µg/g bLf powder (estimated <10% saturation)	Healthy, bottle-fed, ≥34wk gestation, >2kg at birth, 0-4 weeks of age N enrolled = 79 N incl. in analysis = 52 13 of 27 dropouts were in bLf group; 19 withdrew because of parental perception of intolerance (10 in bLf group), 3 withdrawal of consent without further explanation, 5 infants lost to follow up.	Infants received either formula supplemented with 85mg bLf/100ml or non-supplemented cow's milk based formula, which the manufacturer claims contains naturally 10.2mg bLf/100mL [however, it is important to note that bLf from milk ingredients was likely denatured during the production process of infant formula powder]. **Group 1** (n=26): bLf supplemented formula at 85mg/100mL **Group 2** (n=26): unsupplemented formula Intervention duration: 12 months	Significantly fewer lower respiratory tract illness (0.15 vs. 0.5 episodes/infant-year in bLf vs. control, p<0.05), primarily wheezing. No difference in incidence of other illnesses, or duration of any illnesses. Serious adverse events requiring hospitalisation: n=8 (n=4 treatment; n=4 control); no difference between groups. No tolerance issues of bLf addition due to roughly equal numbers of dropouts in both treatment arms. No other adverse events reported.	Evidence for beneficial health effect of bLf by reducing risk of lower respiratory tract infections.
Chen *et al.* (2016)	Double-blind, randomised controlled trial	China	Not disclosed	Infants previously exclusively breastfed but weaned, ages 4-6 mo; breastfed reference group was included N recruited = 260 formula-fed and 130 breastfed N incl. in analysis = 213 formula-fed and 103 breastfed 31 excluded due to parental rejection; 5 rejected for using another formula, 22 for loss of data, 1 for formula allergy, and 15 for adding formulas	Formula-fed infants received either formula with 38mg bLf/100g powder or formula without bLf; both had iron content of 4mg/100g. Breastfed infants were exclusively breastfed. **Group 1** (n=115): bLf fortified formula **Group 2** (n=98): standard unfortified formula (control) **Group 3** (n=103): breastfed infants (reference group) Intervention duration: 3 months	Formula-fed infants receiving bLf fortified formula vs. standard unfortified formula had significantly fewer respiratory-related illness (given as incidence per 100 child days; 2.01 vs 2.94, p<0.05), running nose (1.72 vs 2.31, p<0.05), cough (1.05 vs 2.02, p<0.05), wheezing (0.2 vs 0.4, p<0.05), diarrhoea-related illness (0.60 vs 0.92, p<0.05) and diarrhoea (0.57 vs 0.92, p<0.05). Breastfed infants vs. control group also had significantly lower respiratory illness, running nose, cough, wheezing, diarrhoea related illness and diarrhoea (p<0.05), while no significant difference between breastfed infants and formula-fed infants receiving bLf was found in any of the examined parameters. Duration of respiratory-related and diarrhoea-related illnesses were significantly shorter in the	Evidence for beneficial health effect of bLf by reducing risk and duration of respiratory tract infections and diarrhoea-related illness.

Table 3-9 Intervention studies assessing the effect of bovine lactoferrin on risk of common infections in healthy term infants (≤12 months)

Reference	Study design	Country	bLf source	Study population, age at baseline and number	Study groups and intervention	Summary of findings	Significance of findings
				due to insufficiency of breast milk.		bLf group (5.2 and 5.6 days, respectively) and the breastfed group (5.4 and 5.5 days, respectively), compared to the control group (7.6 and 7.7 days, respectively; p<0.05). No adverse effects related to bLf.	
Chen *et al.* (2021)	Double-blind, randomised controlled trial	China	*Hilmar*, standard bLf	Anaemic but otherwise healthy infants who were previously breastfed but weaned and formula-fed at 6-9 months; Hb <110g/L and >60g/L. N recruited = 108 N enrolled = 105 N incl. in analysis = 96 3 excluded for refusing to give consent; 9 dropouts for loss of Hb data (2 in Group 1, 2 in Group 2)	Infants received either formula fortified with 38mg bLf/100g powder, formula fortified with 76mg/100g or a formula not fortified with bLf. All formulae had the same nutrient composition and contained 4mg iron/100g. **Group 1** (n=33): bLf fortified formula 38mg/100g **Group 2** (n=28): bLf fortified formula 76mg/100g **Group 3** (n=35): standard unfortified formula (control) Intervention duration: 3 months	Formula-fed infants receiving bLf fortified formula at 76mg/100g vs. standard unfortified formula had significantly less respiratory illness (given as morbidity per 100 child days; 3.33 vs. 5.56, p<0.05), wheezing (2.22 vs. 5.56, p<0.05), diarrhoea-related illness (2.42 vs. 5.56, p<0.05), diarrhoea (2.22 vs. 5.56, p<0.05), vomiting (2.58 vs. 6.67, p<0.05) and nausea (2.82 vs. 6.67, p<0.05). Formula-fed infants receiving bLf fortified formula at 36mg/100g vs. standard unfortified formula also had less wheezing (2.29 vs 5.56, p<0.05), vomiting (3.84 vs. 6.67, p<0.05) and nausea (4.14 vs. 6.67, p<0.05), but did not have significantly less respiratory-related illness, diarrhoea-related illness or diarrhoea. They did have fewer episodes or rhinorrhoea (4.65 vs. 6.67, p<0.05), which was not observed in the group receiving 76mg/100g. Significant beneficial effect on faecal biochemical indexes was also found, with significant effects found for both bLf doses vs. control formula across all biomarkers (p<0.05), and with the higher dose showing significantly more beneficial effects compared to the lower dose across all markers (p<0.05) No serious adverse events in any of the intervention groups were observed.	Evidence for beneficial health effect of bLf by reducing risk and duration of respiratory tract infections and gastro-intestinal illness. Evidence for dose-response effect, with higher doses being more effective.

Table 3-9 Intervention studies assessing the effect of bovine lactoferrin on risk of common infections in healthy term infants (≤12 months)

Reference	Study design	Country	bLf source	Study population, age at baseline and number	Study groups and intervention	Summary of findings	Significance of findings
Björmsjö *et al.* (2022)	Double-blind randomised controlled trial	Sweden	*Hilmar;* standard bLf	Healthy full-term babies, 6±2 weeks of age. Formula-fed infants and a breastfed reference group were included. N recruited = 180 formula-fed and 72 breastfed N analysed = 176 formula fed and 70 breastfed No infants excluded, 3 participants were considered to be poor compliers, 3 infants switched formula due to gastrointestinal symptoms and excessive eczema	Formula-fed infants received either low-iron formula (2mg/L) supplemented with bLf (1000mg/L), low-iron formula without bLf, or high-iron formula (8mg/L) without bLf. Reference group was breastfed. **Group 1** (n=72): low-iron + bLf **Group 2** (n=71): low-iron, no bLf **Group 3** (n=33): high-iron, no bLf (control) **Group 4** (n=70): breastfed (reference group) Intervention duration: Until 6 months of age	No sign. differences in cytokine levels across all groups throughout study duration, with breastfed infants showing similar levels. For secondary endpoints (prevalence of infections and other infection-related morbidity and treatments), no significant effect of bLf supplementation was observed.	Evidence to support safe addition to formula in a healthy, well-nourished population group. All study groups had similar blood immune parameter levels to breastfed infants. Morbidity was similar across all formula groups in this well-nourished study population.

Equivalence of Synlait bLf with bLf ingredients used in human intervention studies

Bovine lactoferrin from different manufacturers were used in human intervention studies presented here. While none of the clinical trials included in this application used Synlait manufactured bLf, conclusions of the studies can still be extrapolated to support the benefit of Synlait's bLf for several reasons:

- Bovine lactoferrin is a protein naturally present in bovine milk; it is a well-defined component as outlined in Section 2.2.
- While the manufacture of lactoferrin differs somewhat between manufacturers and is likely to lead to some differences in iron saturation, level of denaturation, and protein content and purity of the final product, these differences are generally small and evidence suggests that all commercial bLf confer similar effects. The relatively consistent findings relating to efficacy and safety of bLf in both animal and human studies, despite products from different manufacturers being used, highlights that bLf products with similar specifications provide similar benefits. While Synlait's bLf was to the best of our knowledge not used in the above cited animal or human studies, Synlait's bLf has been tested *in vitro* and was found to be comparable to other commercial bLf samples, including those used in clinical trials (e.g. Tatua, Hilmar, Friesland Campina, Milei/Morinaga).
- This was recently supported by independent research by Lönnerdal et al. (2020), where 10 commercial samples of bLf were tested and compared to human Lf and bLf extracted by the researchers. The researchers found some differences in purity and endotoxin levels of lactoferrin samples, with Synlait lactoferrin showing high purity. They found that using *in vitro* assays, all commercial samples were taken up by Caco-2 cells, with Synlait lactoferrin being similar to most other commercial lactoferrin samples (Figure 3-3), including some of those used in human clinical trials (e.g. Tatua, Hilmar, Friesland Campina). The same is true for iron uptake from bLf, with Synlait lactoferrin being similar to other commercial samples including Tatua, Hilmar and FrieslandCampina. The effect on cell proliferation and differentiation was also similar across most commercial samples, with Synlait bLf showing a similar or larger effect compared to other commercial products. The effect of Synlait bLf on immune markers was also comparable with other commercial products, and all lactoferrin samples tested also showed comparable antibacterial effects. All commercial samples partially resisted digestion (Lönnerdal et al., 2020). This independent set of data supports that biological effects of commercial bLf samples are comparable and this allows drawing conclusions from studies using bLf from different manufacturers.
- One mode of action of bLf to exert a bacteriostatic effect is by binding iron. Synlait bLf contains no more than 15mg iron per 100g bLf, which equals an iron saturation of no more than 10.7%[19], leaving sufficient iron-binding capacity to support a bacteriostatic effect.
- This data shows that findings from clinical trials using bLf ingredients from different manfuacturers supports the benefit of the well-defined compound of bLf, including that manufactured by Synlait.

[19] One molecule of lactoferrin can bind two ferric ions. Iron saturation is calculated using the molecular weights of ferric iron (56g/mol) and lactoferrin (80,000g/mol), whereby the maximum amount of ferric iron bound to lactoferrin (100% saturation) is 112g/80,000g, which equals 140mg/100g. The following formula can be used to calculate iron saturation: iron saturation = iron content per 100g (mg) x 100 / 140. E.g. 15mg iron per 100g bLf equals 10.7% (15x100/140 =10.7).

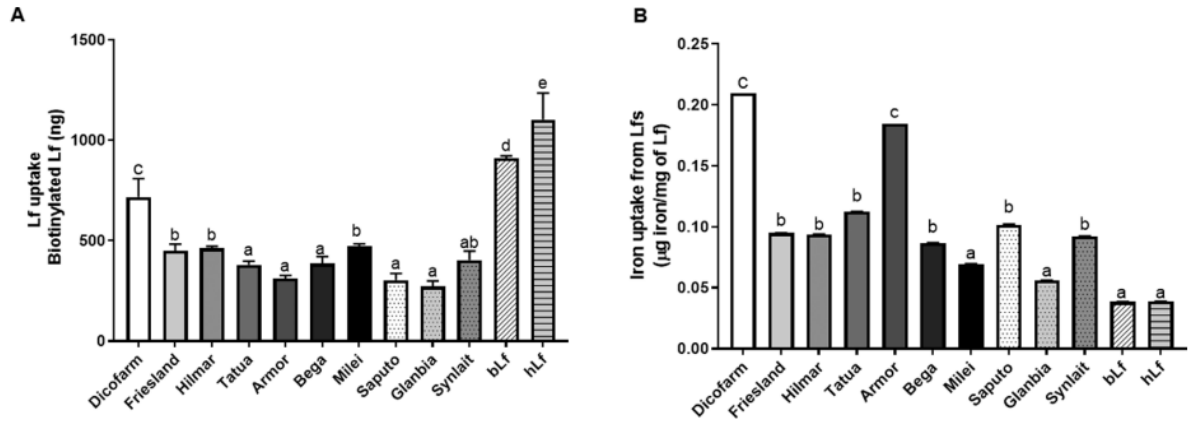

Figure 3-3 Uptake of bLf (A) and iron from bLf (B) by Caco-2 cells
(From Lönnerdal et al. (2020).

3.2.3.3. Supporting evidence from studies in preterm and low-birthweight infants

Some of the strongest evidence on the beneficial effect of bLf comes from the highly vulnerable group of pre-term infants and low-birthweight (LBW) infants. This population group has a particularly high risk of adverse health outcomes, meaning that effective dietary interventions are of particular importance. While studies in preterm and LBW infants often administer bLf in the form of supplements (sometimes added to milk or formula by the researchers) rather than premade formula supplemented with bLf, these studies nevertheless provide valuable supporting evidence for the benefit of bLf on risk of infection.

Neonatal sepsis is the most common cause of neonatal death worldwide and is a particular problem in very low birth weight (VLBW) infants (birth weight <1500g); early-onset sepsis (EOS) (sepsis in infants <72 hours of life) occurs in about 1.5% and late-onset sepsis (LOS) in about 21% of VLBW infants (Pammi & Suresh, 2017). Another common and debilitating health issue in infants is necrotizing enterocolitis (NEC), which occurs in 1% to 5% of admissions to the neonatal intensive care unit, with the most consistent risk factors being prematurity and low birth weight (Pammi & Abrams, 2015). Lactoferrin, including from bovine milk, may reduce the risk of sepsis and NEC due to its immunomodulatory and antibacterial properties.

Nine studies were identified that looked at the effect of bLf supplementation on risk of infection in preterm and LBW infants. The first study by Manzoni *et al.* (2009) was a randomised, controlled, double-blind study in 472 VLBW infants (500-1500g) based in Italy and New Zealand. Infants were orally administered 100mg bLf per day alone or with probiotics, or received a placebo, from birth for 30 days. Incidence of LOS was significantly lower in both intervention groups compared to the placebo group, with 5.9% of infants in the bLf group affected (RR 0.34 vs. placebo; 95%CI, 0.17-0.70; p=0.002), 4.6% in the bLf plus probiotic group (RR 0.27 vs. placebo; 95%CI, 0.12-0.60; p<0.001) and 17.3% in placebo group. Decrease in incidence was

observed for both bacterial and fungal sepsis. NEC stage 2 or greater was less frequent in the bLf plus probiotic group versus placebo, while no significant difference between bLf alone and placebo was seen. In an extension of the trial in order to further investigate NEC, Manzoni *et al.* (2014) included 743 VLBW infants from Italy and New Zealand, receiving the same treatment. In this larger study group bLf supplementation led to significantly lower incidence of NEC (2%, RR 0.37 vs. placebo, 95% CI, 0.136-1.005, p=0.055) and also in the bLf plus probiotic group (0%, RR 0.00 vs placebo, p<0.001), compared to the control group (5.4%).

Akin *et al.* (2014) investigated whether oral bLf at a dose of 200mg/day reduces nosocomial sepsis episodes and NEC in premature infants in a randomised, controlled, double-blind trial in 50 VLBW (<1500g BW) or premature (<32 weeks) infants. bLf or placebo (control) was given throughout hospitalisation. bLf administration resulted in fewer nosocomial sepsis episodes in bLf treated infants vs. control group (4/22 vs 8/25, but this did not reach statistical significance) and a lower number of sepsis attacks (4 vs. 14), leading to a statistically significantly lower number of sepsis attacks per 1000 patient days (4.4 vs. 17.3/1000 patient days, p=0.007). Fewer infants in the bLf group developed NEC in bLf (4/22 vs 8/25 in control group), but this failed to reach statistical significance. Severe NEC was found in 5 out of 25 in the control group, while no severe NEC was observed in bLf group (p=0.05).

Ochoa *et al.* (2015) investigated the effect of oral bLf administration on occurrence of first episode of late-onset sepsis in 190 infants with low birth weight (<2500g) in another randomised, controlled, double-blind study. Infants received either 200mg bLf/kg BW/day or placebo for a duration of 4 weeks. In this group of LBW infants, risk of first episode of LOS was not significantly lower. Cumulative sepsis incidence was lower in the bLf group compared to placebo group (12.6% vs. 22.1%) but also failed to reach statistical significance; the authors noted that the difference became significant when taking into account timing of start of treatment as some children did not receive treatment from birth for medical reasons.

In a randomised, controlled, double-blind study in 139 neonates with a birth weight of <2000g, Kaur and Gathwala (2015) orally administered varying levels of bLf depending on weight of infants (BW 1000-1249g: 100 bLf mg/d; BW 1250-1499g: 150 bLf mg/d; BW 1500-1749g: 200 bLf mg/d; BW 1750-1999g: 250 bLf mg/d) or placebo for a period of 4 weeks starting with first day life. bLf administration led to a significantly lower incidence of first episode of culture-proven LOS (RR 0.21 vs. placebo; 95%CI 0.044-1.019; p = 0.036). Incidence of probable sepsis (no microorganisms isolated) was also significantly lower in the bLf vs. placebo group (RR0.26; 95%CI, 0.08-0.828; p = 0.016), as was the incidence of any LOS (RR 0.20 vs. placebo; 95%CI, 0.076-0.537; p=0.001) and sepsis-attributable mortality was also significantly lower (0% in bLf vs. 7.5% in placebo; p=0.027).

A small pilot study by Barrington *et al.* (2016) was set up primarily to investigate tolerability and acceptability of adding bLf to milk (mother's milk or preterm formula) in 79 infants born <31 weeks of gestation. The authors recorded clinical data, but noted that the study was not powered to identify significant differences in clinical outcomes. Relative risk of incidence of LOS and NEC was lower in the bLf group, but as expected this did not reach statistical significance.

The ELFIN trial, a large UK-based trial in 2182 very preterm infants (<32 weeks gestation), did not find a reduced risk of LOS when bLf was administered *enterally* (150mg/kg BW/day), with 29% of infants given bLf

developing LOS compared to 31% in the control group (ELFIN Trial Investigators Group, 2019). The study authors briefly discuss a possible reason for discrepancy between their findings and those by Manzoni *et al.* (2009) and Manzoni *et al.* (2014), proposing that exposure to other interventions might have contributed to the differences in findings. The authors noted the relatively high prevalence of invasive fungal infection in the Manzoni trials (7.7% of the control group), while in the ELFIN trial overall prevalence of LOS fungal infection was low, suggesting that practices were in place that significantly reduced the risk of fungal infection in the first place, in which case bLf administration would not be expected to additionally significantly reduce risk of fungal LOS, which may have impacted the overall LOS outcomes. The authors did not discuss mode of administration (oral versus enteral), as this may also have influenced the outcomes, noting that an enteral feed likely undergoes harsh heat treatments, which may have denatured lactoferrin present in the product.

Two further more recent trials failed to find a significant benefit of bLf administration in preterm infants, both noting the low number of overall incidence of infections. Ochoa *et al.* (2020) investigated the effect of adminstering 200mg/kg BW/day on prevention of LOS in 414 LBW infants (500-2000g). LOS or sepsis-associated death occurred in 22 (10.5%) infants in the bLf group vs. 30 (14.6%) in the placebo group; there was no statistically significant difference after adjusting for hospital and birth weight, with a hazard ratio of 0.73 (95% CI 0.42–1.26). For infants with birth weights <1500g the hazard ratio was 0.69 (95%CI, 0.39–1.25), once again failing to reach statistical significance. Interestingly, re-hospitalization rates during the 2-year follow-up were similar in both groups, except for significantly less bronchiolitis in the bLf group, with a rate ratio of 0.34 (95% CI 0.14–0.86). The study authors noted that their trial was underpowered to detect a statistically significant difference in LOS as the overall number of sepsis in both study arms was lower than expected. The authors also noted that human milk and colostrum inake in the study group was higher than in previous studies, which means all infants received a meaningful amount of human Lf, which may have limited the impact of additional bLf (Ochoa *et al.*, 2020).

A high prevalence of human milk feeding (~95% across both study arms), may have also contributed to a lack of statistically significant benefit of bLf treatment on prevalence of death and major morbidity, including LOS, in a randomised, controlled, double-blind trial in Australia and New Zealand, as noted by the study authors. The authors suggested that their study was likely underpowered due to lower than expected prevalence in the control group (Tarnow-Mordi *et al.*, 2020), which may have been due to the high level of human milk feeding. The LIFT trial included 1542 VLBW (<1500g) infants who received milk (mother's milk or other) either with bLf (200mg/kg BW/day) or without bLf up to 34 weeks' post menstrual age. There was no effect of bLf supplementation on in-hospital death or major morbidity, which occurred in 162 (21%) of 770 infants in the bLf group and in 170 (22%) of 771 infants in the control group (relative risk [RR] 0·95, 95% CI 0·79–1·14; p=0·60). There was also no significant difference in incidence of LOS, with 89 (12%) in bLf group and 108 (13%) in the control group, resulting in a relative risk of 0·83 (95%CI, 0·64–1·08) in the bLf vs. control group. The study authors also carried out a meta-analysis of trials investigating the effect of bLf on LOS and NEC, including in 5609 preterm infants. Meta-analysis resulted in a significant reduction of LOS with bLf supplementation (RR 0·79, 95% CI 0·71–0·88; p<0·0001; I^2=58%), but not NEC or all-cause mortality. In a sensitivity analysis, excluding three trials considered of lower quality because of risk of bias, a significant

overall effect of bLf supplementation on LOS persisted (RR 0·81; 95%C 0·73–0·90; p<0·0001) (Tarnow-Mordi *et al.*, 2020).

Overall, evidence from trials in preterm infants provides further support for a beneficial effect of bLf on reduced risk of infection.

3.3. Information related to the dietary intake or dietary exposure to bLf

3.3.1. Data to enable dietary intake or exposure of the target population to be estimated

Infant formula, including infant formula products for special dietary use, are likely to be the sole source of nutrition in formula-fed infants from birth to 6 months, with the infant consuming a progressively more diverse diet from 6 months of age onwards. Based on infant formula products available in countries where the use of bLf is permitted (e.g. USA, China), the percentage of infant formula products likely to contain bovine lactoferrin may, optimistically, be up to 50%.

3.3.1.1. Estimated exposure to bLf

Based on the maximum proposed levels of bLf in Infant formula products of 40mg/100kJ (which equals approximately 1100mg/L) (Table 2-16) and assumed formula intakes being similar to human milk intakes in Australian and New Zealand infants, the maximum dietary exposure of infants (birth to 12 months) is shown in Table 3-10. These levels are somewhat lower than the estimated lactoferrin intake of breastfed birth to 6-month-old and 6 to 12-month-old infants (Table 3-2).

Table 3-10: Estimated intakes of bLf in formula-fed infants at proposed maximum levels and comparison with mean intakes of breastfed infants

	Mean bLf intakes in infants consuming formula fortified with bLf at maximum permitted levels[a] (mg/day)	**Mean bLf intakes in breast-fed infants [b] (mg/day)**
Infants Birth to ≤ 6 months	880	984-1136
Infants 6 to ≤12months	660	738-852

[a] Based on maximum permitted levels of 40mg/100kJ (~1100mg/L) and typical human milk intakes of 0.8L/day (birth to 6 months) and 0.6L/day (6 to 12 months) (Food Standards Australia New Zealand (FSANZ), 2016)
[b] Based on typical levels in human milk from Australian women postnatal day >15 (1230-1420mg/L) (Houghton *et al.*, 1985) and typical human milk intakes (Food Standards Australia New Zealand (FSANZ), 2016)

3.3.1.2.Estimated exposure to iron from bLf

Bovine lactoferrin contains small amounts of iron. Synlait bLf contains iron at levels <15mg/100g bLf powder. Adding levels at the maximum proposed level of 40mg/100kJ, which equals ~1100mg/L, bLf contributes up to 0.006mg iron per 100kJ or ~0.017mg per 100mL of made-up infant formula, meaning the contribution of bLf to iron intakes in infants is negligible (see Table 3-11). When formulating infant formula products, the contribution from bLf counts towards total iron content of the formula. Iron bound to bLf is available to the infant, as discussed in more detail in Section 3.2.1.2. See Table 3-13 for calculations on contribution of bLf to daily iron intake of infants and comparison to Nutrient Reference Values for Australia and New Zealand.

Table 3-11: Estimated contribution of bLf to iron levels in infant and follow-on formula at proposed maximum permitted levels

	Min iron levels in IF and FoF[a]	**Max iron levels in IF and FoF[a]**	**Max iron contributed from bLf[c]**	**Max contribution from bLf (%)**
Iron (mg/100kJ)	0.2	0.5	0.006	1.2-3%
Iron (mg/100mL)	0.57[b]	1.41[b]	0.017	

[a] Minimum and maximum requirements in Standard 2.9.1; [b]Based on energy range mid-point (2825kJ/L); [c]Based on maximum permitted levels of 40mg/100kJ and maximum iron levels of 15mg/100g bLf

3.3.2. Data on the recommended level of formula consumption for the target population

Daily maximum intake levels based on a typical feed guide for infant formula, using a Synlait manufactured product sold in Australia and New Zealand, is presented in Table 3-12. Applying the proposed maximum addition to infant formula of 40mg/100kJ, exposure gradually increases over the first months of life, and peaks at 1011-1264mg/day in exclusively formula-fed infants if feeding guide is followed. As older infants increasingly start consuming complementary food, their exposure to bLf will be decreasing due to exposure from other foods being limited.

Table 3-12 Daily maximum intake of bLf based on the feeding guide of a Synlait manufactured infant formula

Age of infant	Water (mL)	Level scoops of powder[a]	Total volume per feed (mL)	Energy per serve (kJ)	Number of feeds/ day	Total energy/ day (kJ)	Daily maximum intake of bLf (mg)[b]
0-4 days	50	1	56	158	5-6	790-948	316-379
5 days - 4 weeks	100	2	112	316	6-8	1896-2528	758-1011
1-4 months	150	3	168	474	5-6	2370-2844	948-1138
4-6 months	200	4	224	632	4-5	2528-3160	1011-1264
>6 months	200	4	224	632	3-4	1896-2528	758-1011

[a]1 level scoop ~ 7.5g of powder; 1 scoop of powder provides ~158kJ; 1 scoop of powder added to 50mL water yields approximately 56mL of formula.
[b]Based on maximum allowable level of 40mg/100kJ.

Table 3-13: Daily maximum intake of iron from bLf based on the feeding guide of a Synlait manufactured infant formula

Age of infant	Energy per serve (kJ)	Number of feeds/ day	Total energy/ day (kJ)	Daily max. iron contribution from bLf addition (mg)	% of Recommended Dietary Intake[c]	% of Upper Limit[c]
0-4 days	158	5-6	790-948	0.047-0.057	N/A	0.24-0.29%
5 days - 4 weeks	316	6-8	1896-2528	0.114-0.152	N/A	0.57-0.76%
1-4 months	474	5-6	2370-2844	0.142-0.171	N/A	0.71-0.86%
4-6 months	632	4-5	2528-3160	0.152-0.19	N/A	0.76-0.95%
>6 months	632	3-4	1896-2528	0.114-0.152	1.03-1.38%	0.57-0.76%

[a]1 level scoop ~ 7.5g of powder; 1 scoop of powder provides ~158kJ; 1 scoop of powder added to 50mL water yields approximately 56mL of formula.
[b]Based on maximum allowable level of 40mg bLf/100kJ and maximum iron level of 15mg/100g bLf.
[c]Nutrient reference values for Australia and New Zealand

3.3.3. Information relating to exposure to the substance from other sources

The dietary exposure of infants to lactoferrin from non-human milk sources is predominantly from milk-based formulas currently available (see Table 3-2 in Section 3.1.1 for estimated intakes from non-bLf-fortified formula), and bovine milk-based products such as weaning yoghurts (noting that cow's milk is not recommended before age 1 year). The lactoferrin concentration of bovine milk is approximately 100mg/L (Barth & Behnke, 1997; Cheng *et al.*, 2008; Rainard *et al.*, 1982), meaning that exposure of infants to bLf from dairy products such as yoghurt would be relatively low (around 15mg from 150mL yoghurt).

3.4. Information related to labelling requirements under Part 2.9 of the Code

3.4.1. Information related to safety or nutritional impact of the proposed labelling change

There is no significant safety or nutrition impact of the proposed labelling requirements. Clarity of the source (milk) and mandatory allergen labelling of the presence of milk ingredients in infant formula products address safety issues. As a protein component, any additional contribution to the total protein content of the food from the relatively small level of added bLf, will be accounted for in the stated protein value.

In addition, as noted earlier, bLf will be listed in the ingredient list and will also be included in the Nutrient Information Statement. Parents who choose to formula-feed and are aware of lactoferrin may choose a formula containing bLf and thereby replace a similar formula not containing bLf. Synlait does not anticipate any nutritional concerns with this replacement seeing that any Infant formula products sold in Australia and New Zealand must meet strict regulatory standards. Synlait also does not anticipate that mothers who are breastfeeding choose to switch to formula because of addition of bLf to formula, as further discussed in Section 2.6.2.

It is important to note that Standard 1.2.7-4 prohibits health and nutrition claims on Infant formula products. Furthermore, attention cannot be drawn to the addition of nutritive substances on pack, nor can the benefits be communicated, and specifically formula cannot be labelled with the word “humanised” or “maternalised” or any word or words having the same or similar effect (Standard 2.9.1-24). Hence the inclusion of bLf in Infant formula products is only likely to be noted by those caregivers who pay attention to product composition when making a choice in formula selection, not a driver to initiate formula feeding.

3.4.2. Information to demonstrate that proposed labelling will be understood by consumers

Globally bLf is increasingly being used as a nutritive ingredient in foods, particularly infant formula products and dairy-based foods. The ability to include bLf in Infant formula products enables our own diverse ANZ intended populations to receive these nutritional benefits that they seek from foods manufactured overseas. Consumers that have heard of lactoferrin and understand the benefits are searching the web for foods containing lactoferrin. Sections 2.6.1 and 2.6.2 discuss consumer understanding of bLf in more detail. Consumers are generally familiar with the nutrition information statement on pack and therefore will be able to identify lactoferrin as a component and make an informed decision. The inclusion of lactoferrin in the ingredients listing as an ingredient of a compound ingredient or as an individual ingredient, combined with an allergen statement, allows the consumers to understand lactoferrin is a product derived from milk, and therefore will help consumers understand that the product is not suitable for infants with cow’s milk allergy. Inclusion of lactoferrin in the statement of nutrition information enables consumers to understand nutrition content for each 100mL consumed.

3.5. Information related to internationally recognised standards, codes of practice, recommendations and guidelines

Please refer to Section 1.8 for relevant information.

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