FOOD STANDARDS Australia New Zealand
Te Mana Kounga Kai – Ahitereiria me Aotearoa

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Supporting document 1

Nutritional safety assessment report – Application A1173

Minimum protein in follow-on formula

Executive summary

The nutritional safety assessment aimed to determine whether a reduction in the minimum protein requirement for follow-on formula from 0.45 g/100 kJ to 0.38 g/100 kJ is supported by protein levels in human milk, and whether the lower minimum protein requirement will support adequate dietary protein intake and normal growth in infants consuming follow-on formula in addition to complementary foods.

To determine if the requested lower minimum amount of protein in follow-on formula is supported by protein levels in human milk 5 to 12 months post-partum, 11 original research studies were reviewed. There are different methods to determine the protein content in human milk and follow-on formula. The Code prescribes the protein content in follow-on formula to be calculated from nitrogen, using specified conversion factors. Nitrogen-based methods (Kjeldahl or Dumas) are used most often for determining protein in infant formula. These determine crude protein by measuring the total nitrogen content of the sample, then converting to protein using conversion factors. Crude protein overestimates protein content in human milk, because human milk contains a high proportion of non-protein nitrogen (NPN), around 20 to 27% of total nitrogen. The proportion of NPN in infant formula has been reported to range from 5 to 16% of total nitrogen, depending on protein source and manufacturing processes. The nitrogen-based methods may be adapted to measure the nitrogen in only the protein content of the sample (i.e. excluding NPN), which is referred to as true protein. Research studies often use colorimetric methods to determine protein in human milk. These methods, however, have not been well validated for measuring protein in human milk and infant formula, and comparisons with crude or true protein values are variable.

Therefore, priority was given to studies reporting crude (n=4) or true (n=1) protein. Reported protein concentrations (g/L) were converted to protein per 100 kJ using the established average energy density value for human milk beyond 6 months post-partum, of 288 kJ/100 mL. Mean values of crude protein in human milk ranged from 0.34 to 0.40 g/100 kJ, and true protein concentration in the single study measuring this was 0.29 g/100 kJ. These values are consistent with human milk protein levels determined by other reviews examining human milk protein levels. The requested minimum protein requirement (0.38 g/100 kJ) falls within the range of human milk protein values established in this assessment, and is consistent with protein concentrations reported for Australian women (albeit determined by colorimetric methods), of 0.29 g/100 kJ to 0.38 g/100 kJ.

The potential effect of a lower protein follow-on formula on infant growth was assessed. Two randomised controlled trials were examined, which compared weight gain from 3 months of age to 12 months, in infants fed a lower protein formula (0.39 g crude protein/100 kJ) compared with higher protein formula (study 1: 0.65 g/100 kJ; study 2: 0.51 g/100 kJ), and

with breastfed infants. Both studies indicated slower weight gain between 3 and 12 months for infants fed the lower protein formula compared with the higher protein group.

However, the differences only reached statistical significance between 3 and 6 months in study 1. Compared with breastfed infants, study 2 reported higher weight gain in infants fed either formula, whereas study 1 reported greater weight gain in the higher protein group and no difference in the lower protein group.

The dietary intake assessment showed no risk of inadequate protein intakes for Australian and New Zealand infants if the minimum protein in follow-on formula was be lowered to 0.38 g/100 kJ, assuming the infants were also consuming appropriate complementary foods. Australian and New Zealand infants consume adequate amounts of dietary protein from complementary foods, and are therefore not reliant on follow-on formula to meet all of their protein requirements.

Based on the nutritional safety assessment, it is concluded the requested lowering of the minimum protein requirement in infant follow-on formula from 0.45 g/100 kJ to 0.38 g/100 kJ is appropriate and safe. Published research studies have shown the crude protein value of 0.38 g/100 kJ falls within the mean levels found in human milk from 5 to 12 months post-partum. In addition, randomised, controlled trials reported no adverse effects on growth in infants consuming the lower protein formula from 3 months to 12 months of age. Finally, the dietary intake assessment indicates that protein intakes of Australian and New Zealand infants would remain adequate if the minimum protein level for follow-on formula was lowered to 0.38 g/100 kJ.

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1. Introduction

1.1 Objective of the assessment

Standard 2.9.1 – Infant Formula Products of the *Australia New Zealand Food Standards Code* (the Code) prescribes the minimum amount of protein in follow-on formula as 0.45 g/100 kJ. The applicant has requested that this value be reduced to 0.38 g/100 kJ, given that this level more closely aligns with protein levels in human milk 6 to 12 months post-partum, and that lower protein levels would help infants fed follow-on formula as their main milk source achieve growth trajectories similar to breastfed infants. The objectives of this nutritional safety assessment, therefore, are to confirm whether the proposed lower minimum protein level is consistent with levels found in human milk 6 to 12 months post-partum, and that the lower minimum level will support adequate dietary protein intake and normal growth in infants consuming follow-on formula in addition to a progressively diversified diet.

1.2 Approach of the assessment

The Ministerial Policy Guideline on the *Regulation of Infant Formula Products* (the Policy Guideline) states that the nutritional composition of human milk should be used as a primary reference for determining the composition of follow-on formula. In addition, the Policy Guideline states that the composition of follow-on formula must strive to achieve as closely as possible the normal growth and development of healthy full-term breastfed infants at the appropriate age when follow-on formula is used as the principal source of liquid nourishment in a progressively diversified diet.

This assessment aims to determine whether a reduction in the minimum amount of protein in follow-on formula from 0.45 g/100 kJ to 0.38 g/100 kJ is supported by: (1) currently available studies on human milk composition; and (2) available safety studies on infant growth and development. It also aims to establish whether the minimum protein level of 0.38 g/100 kJ for follow-on formula poses a risk of inadequate dietary protein intake.

The approach undertaken for this assessment was to:

- Compare the analytical methods used to measure protein levels in human milk and infant formula/follow-on formula products.
- Consider previous assessments of human milk protein levels by scientific advisory groups and key systematic reviews.
- Conduct a literature review to identify new research conducted since previous reviews.
- Determine whether the lower minimum protein in follow-on formula is consistent with average levels in human milk 6 to 12 months post-partum.
- Review the literature to confirm that a lower minimum protein level in follow-on formula poses no nutritional risk as assessed by infant growth and adequacy of dietary protein intake.
- Consider whether the lower minimum protein level will support adequate dietary protein intake and normal growth in infants consuming follow-on formula in addition to a progressively diversified diet.

1.3 Definitions and terminology

Adequate Intake[1] means the average daily nutrient intake level based on observed or experimentally-determined approximations or estimates of nutrient intake by a group (or groups) of apparently healthy people that are assumed to be adequate.

Crude protein is based on all nitrogen-containing substances in human milk is calculated from the total nitrogen content of a food multiplied by a conversion factor (usually 6.25 based on the nitrogen content of mixed proteins – see report text).

Follow-on formula[2] means an infant formula product represented as either a human milk substitute or replacement for infant formula and which constitutes the principal liquid source of nourishment in a progressively diversified diet for infants aged from six months.

Infant[2] means a person under the age of 12 months.

Non-protein nitrogen (NPN) consists mainly of free amino acids, peptides, and urea. In human milk between 20 to 27% of total nitrogen is present as NPN.

Soy-based formula[2] means an infant formula product in which soy protein isolate is the sole source of protein.

True protein is based on the all nitrogen-containing substances minus NPN multiplied by an appropriate conversion factor (e.g. 6.38 for milk proteins). Therefore, the calculation excludes nitrogen that may be metabolically available, e.g. amino acids, small peptides, urea, aminosugars, nucleotides, carnitine, and choline.

2. Nutrition Assessment

2.1 Measuring protein in milk

2.1.1 Variability of protein levels in human milk

Human milk is considered to contain the ideal levels of nutrients for infants, thus it is used as the reference for the nutrient content of infant formula. Protein levels in human milk can vary considerably between women (Gidrewicz and Fenton 2014). Maternal health, BMI and some infant characteristics (e.g. infant size and volume of milk intake) may influence protein levels after 6 months post-partum (Nommsen et al. 1991). However, maternal diet has no effect on human milk protein levels, there appear to be no diurnal variances or differences between fore- and hind-milk (Mitoulas et al. 2002), and reported differences in protein levels between preterm and term milk during early lactation are no longer evident after 12 weeks (Saarela et al. 2005; Gidrewicz and Fenton 2014). A recently published systematic review of 26 studies showed protein concentrations in human milk were stable between 4 to 6 months through to 12 months (Lönnerdal et al. 2017).

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[1] Source: NHMRC (2006).

[2] Source: Standard 11.2 – Definitions used through the Australia New Zealand Food Standards Code http://www.comlaw.gov.au/Series/F2015L00385

Differences in sample processing and analytical methods are major sources of variability in human milk protein concentrations reported in the literature (Gidrewicz and Fenton 2014). There are several protein determination methods available, but as each method is based on different analytical principles the results are not directly comparable. Furthermore, the lack of certified reference materials to standardize results and limited reporting of quality control measures limits comparisons between laboratories even when the same analytical method is used. These limitations are discussed further in the next section.

2.1.2 Measurement of protein in human milk and infant formula products

The methods for determining protein in infant formula and human milk have previously been reviewed by FSANZ and are summarised in Table 1 (FSANZ 2013). For this assessment, only the analytical methods used in the studies reviewed will be discussed. The Code does not prescribe a protein determination method but the minimum protein level of follow-on formula is based on crude protein values (ANZFA 1999). The Code requires protein content to be calculated using the formula: *Protein content=NC x F*, where *NC* is the nitrogen content of the formula, and *F* is a specified nitrogen conversion (NCF) (refer to discussion in section 2.1.3).

Table 1:   Methods to determine protein content in human milk[a]

Method	Measure of	Procedure	Limitations
Kjeldahl	Nitrogen	Digestion then conversion of organic nitrogen to ammonia which is detected by titration Crude protein determined from total nitrogen in the sample; true protein determined after removing non-protein nitrogen from total nitrogen	Crude protein includes nitrogen from non-protein constituents, whereas true protein excludes metabolically important nitrogen from non-protein sources; different proteins require different conversion factors depending on the amino acid composition of the sample protein
Dumas	Nitrogen	Measures nitrogen released by combustion of milk sample	Requires expensive instrumentation; different proteins require different conversion factors depending on the amino acid composition of the sample protein
Spectroscopic	Protein	Direct detection by UV spectroscopy at 280 nm	Interference by other milk constituents
Colorimetric (e.g. Bradford, Lowry, BCA methods)	Protein	Interaction of proteins with reagents to form a complex which is measured spectrophotometrically	Requires a reference standard that reproduces reagent-binding properties of breast milk proteins
Total amino acids (sum weight of amino acids)	Amino acids	Amino acid analysis	Analytically demanding; requires determination of recovery coefficients for each amino acid determination

[a] Adapted from FSANZ (2013)

Methods that measure nitrogen

Crude protein is determined by measuring the total nitrogen concentration in a sample, then applying a nitrogen to protein conversion factor (NCF; see below) to estimate protein concentration. This estimate is termed 'crude protein' because it includes nitrogen from non-protein sources referred to as non-protein nitrogen (NPN) and includes: free amino acids, small peptides, and urea (Elgar et al. 2016; Moore et al. 2010).

The Kjeldahl method for the determination of crude protein is the most commonly used method, and is accredited by the Association of Official Analytical Chemists International (AOAC) (Koletzko et al. 2005; Elgar et al. 2016). This method is often used to validate other protein determination methods.

Crude protein methods overestimate protein content if there is a high amount of NPN in the sample (Lönnerdal 2003). Human milk has a high proportion of NPN, around 20 to 27% of total nitrogen, with urea providing approximately 50% of the NPN (FSANZ 2016; EFSA 2017). The proportion of NPN in milk-based formulas has been reported to range from 5 to 16% of total nitrogen, depending on protein source and manufacturing processes (Rudolff and Kunz 1997). The Dumas combustion method is another nitrogen-based method to determine crude protein and results from this method compare well with the Kjeldahl method (Wiles et al. 1988).

The Kjeldahl method can be adapted to allow measurement of nitrogen in only the protein fraction of a sample to give an estimate of 'true protein'. After precipitation and filtration of protein from the sample, true protein is determined by either directly measuring the nitrogen in the protein, or by measuring the NPN in the remaining solution then deducting the non-protein content from total nitrogen (Moore et al. 2010). Both methods require the conversion of nitrogen to protein using appropriate NCF.

True protein values are comparable with crude protein values if there is very little NPN in the sample. However, the true protein methods have not been well standardized to human milk or infant formula products (Moore et al. 2010). As infants use some nitrogen from non-protein sources for protein synthesis and other biological functions, excluding NPN will underestimate the utilizable amount of nitrogen in human milk or formula (EFSA 2017).

Methods that directly measure protein

Studies sometimes directly measure protein in human milk using colorimetric methods, such as the Bradford, bicinchoninic acid assay (BCA), and Lowry methods (Mitoulas et al. 2002; Elgar et al. 2016; Dewey et al. 1984). Generally these methods are easier and quicker to perform than the Kjeldahl method. The colorimetric methods, however, have not been well validated to the Kjeldahl method for human milk or infant formula (Moore et al. 2010). The assays tend to be sensitive to structural differences in milk constituents, and to interfering substances that may give falsely elevated true protein levels (Keller and Neville 1986). Furthermore, the colour responses are dependent on the amino acid composition of the protein, and therefore require standard reference materials with the same protein composition as the sample of interest (Moore et al. 2010). Purified human milk protein standard reference materials are recommended when measuring protein in human milk (Donovan and Lönnerdal 1989). Nevertheless, a comparative study has shown that when using a human milk standard reference material the Bradford and BCA methods gave higher results than both Kjeldahl true protein (by 34%) and crude protein (by 9%) (Donovan and Lönnerdal 1989). The Lowry method gave similar results to true protein, and underestimated crude protein.

Other standard reference materials, such as human or bovine serum albumin (BSA) gave variable results when comparing colorimetric assays to the Kjeldahl methods (Keller and Neville 1986; Donovan and Lönnerdal 1989).

Overall these studies highlight the difficulties in comparing results between studies unless the same analytical assay and standardized reference materials are used.

2.1.3 Nitrogen to protein conversion factors

The NCF is the factor that is used to multiply the nitrogen content of a food or food ingredient (measured by nitrogen-based methods) to determine the protein content.

The Code specifies two conversion factors: 6.38 for cow milk-based formula, and 6.25 for all other protein sources.

The 6.25 NCF was derived from the average nitrogen content in mixed foods, which is approximately 16% (Maubois and Lorient 2016). Thus, 1 g of nitrogen is equivalent to 6.25 g of protein. The 6.38 conversion factor is an average of conversion factors for casein (6.36) and whey (6.41) proteins in milk. While the relative proportions of whey and casein differ between early lactation (80/20) and late lactation (50/50), the average conversion factor remains the same, at 6.38 (Maubois and Lorient 2016). Using 6.25 when 6.38 may be more appropriate will underestimate crude protein by 2%. This underestimation is considered minimal given the large variation in NPN (5 to 16% of total nitrogen) in cow milk-based infant formula (Rudolff and Kunz 1997).

2.1.4 Summary

While the Code does not require protein in human milk or follow-on formula to be measured using any specific method, it does require protein content in an infant formula product to be estimated from nitrogen using specified nitrogen to protein conversion factors (6.38 for cow milk-based formula, and 6.25 for other formula). Therefore, when setting human milk protein levels as a benchmark for formula, the values used must be comparable to nitrogen-based methods. Studies have often used colorimetric methods to measure protein in human milk, given these methods are inexpensive and quicker to perform than the nitrogen-based Kjeldahl method. These methods, however, have not been well validated for measuring protein in human milk and infant formula and do not use NCF to convert nitrogen to protein. Given the lack of standardized reference materials with which to compare results between studies, comparisons of results obtained from colorimetric assays with nitrogen-based methods should be made with caution.

2.2   Protein content in human milk

2.2.1 Approach - determining protein content in human milk

To establish a range of protein concentrations in human milk, original research studies were identified from the scientific literature (see Section 2.2.2). From these studies, the range of protein concentrations per 100 mL was determined. Many studies did not report energy density components or use sampling protocols that reduce variations in fat content to allow comparisons between studies.

Therefore, protein concentrations were converted to protein-energy density values using the average human milk energy density value of 288 kJ/100 mL for human milk beyond 6 months post-partum (Butte and King 2005).[3]

Studies that reported using internationally accredited analytical methods (by AOAC or the International Dairy Federation), with no further details given, were assumed to have measured crude protein by the Kjeldahl method, using a nitrogen to protein conversion factor of 6.38, given this method is the most commonly used method for determining protein in milk samples.

2.2.2 Identification of literature

The applicant provided 3 studies reporting human milk concentrations from 6 to 12 months post-partum (Mitoulas et al. 2002; Dewey et al. 1984; Nommsen et al. 1991), and a

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[3] Reported as 2.8 kJ/g in the original study (Butte and King 2005), and converted to kJ/mL for this assessment using the relative density factor of 1.03 g/mL for human milk.

systematic review reporting temporal trends in protein concentrations in human milk over the first 12 months of lactation (Lönnerdal et al. 2017). Additional studies were identified for this assessment from examining the references listed in key reviews (Gidrewicz and Fenton 2014; Lönnerdal et al. 2017; Hester et al. 2012; EFSA 2017; NHMRC 2006). A search on the PubMed electronic database was also undertaken using the search terms: human milk, proteins/analysis, breast feeding. Studies were included in this assessment irrespective of the protein determination method used, given that the Code does not specify the method that must be used when establishing human milk protein concentrations.

Studies were included if they:
1. Included healthy women with term or preterm infants[4]
2. Reported human milk protein content at any time between 6 and 12 months, inclusive[5]
3. Reported results for protein concentration (g/L) in a format that allows the extraction of data.

Table A2.1 in Appendix 2 lists the studies that were identified through this search and the reasons for exclusion. From this list, 11 studies were identified as fulfilling the inclusion criteria above. Two studies were identified for the Australian population, but none were identified for the New Zealand population.

2.2.3 Results from primary research studies

Results from the studies are presented in Table 2, expressed as absolute concentrations (g/100 mL) by month of lactation. Crude protein ranged from 0.99 to 1.14 g/100 mL (Feng et al. 2016; Saarela et al. 2005; Yamawaki et al. 2005; Nagra 1989). One study reported true protein, of 0.83 g/100 mL (Stuff and Nichols 1989). This was similar to protein determined by the total amino acid method (0.82 g/100 mL (Feng et al. 2016).

The results from the colorimetric assays varied considerably (0.80 to 1.24 g/100 mL). Some of the variability is likely to be explained by differing assays used and the type of standard reference material used.

Specifically, the two studies using the Lowry method with BSA or unspecified protein standards (likely to be BSA) gave higher results (1.14 to 1.24 g/100 mL) than studies using other colorimetric assays with human milk standard reference materials (0.83 to 1.07 g/100 mL) (Allen et al. 1991; Mitoulas et al. 2002; Gridneva et al. 2018).

The results from the studies for Australian women differed (Mitoulas et al. 2002; Gridneva et al. 2018). Using the same colorimetric method, average protein concentration reported by Mitoulas et al (2002) of 0.83 g/100 mL was lower than the concentration reported in a more recent study (Gridneva et al, 2018), of 1.02 g/100 mL. The results from Mitoulas et al (2002) are similar to true protein and protein determined from total amino acids (Feng et al. 2016; Stuff and Nichols 1989). The results by Gridneva et al. (2018) are similar to the crude protein concentrations (1.097 g/100 mL) reported for women from 9 countries including Australia (Feng et al. 2016). Feng et al (2016) found that there were generally no differences in protein concentrations of human milk between the countries. Therefore it is reasonable to assume that protein content of human milk of Australian and New Zealand women is comparable to international values.

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4 Protein concentration in human milk does not appear to differ between women with term and preterm infants (Saarela et al. 2005; Gidrewicz and Fenton 2014).

5 Although the age range for infant follow-on formula specified in the Code is from 6 to <12 months, human milk values for 12 months were included in this analysis because milk protein concentrations appear to be stable from 6 to 12 months (Lönnerdal et al. 2017).

Table 2:   Reported human milk protein content from 5 to 12 months post-partum

Reference (Country)	Method for determining protein	Months post-partum	N	Protein (g/100 mL)
Feng et al. 2016 (9 countries)	Crude protein (Dumas)	5 to 6	23	1.097
	Protein (Total amino acid)	5 to 6	23	0.821
Saarela et al. 2005 (Finland)	Crude protein (Kjeldahl)	6	20	1.14
Yamawaki et al. 2005 (Japan)	Crude protein (Kjeldahl)	6 to 12	39	0.99
Nagra et al. 1989 (Pakistan)	Crude protein (Kjeldahl)	6	20	1.01
		9	18	0.99
		12	17	1.01
Stuff & Nichols 1989 (US)	True protein (Kjeldahl)	6	45	0.82
		9	8	0.84
Gridneva et al. 2018 (Australia)	Colorimetric (Bradford; human milk standard)	6	19	0.97
		9	15	1.07
Mitoulas et al. 2002 (Australia)	Colorimetric (Bradford; human milk standard)	6	15	0.80
		9	6	0.83
		12	5	0.83
Dewey et al. 1984 (US)	Colorimetric (Lowry; unspecified standard)	7 to 11	27	1.24
Nommsen et al. 1991 (US)	Colorimetric (Lowry; Bovine Serum Albumin standard)	6	45	1.14
		9	28	1.16
		12	32	1.24
Allen et al. 1991 (US)	Colorimetric (BCA; Human milk standard)	6	13	1.00
Michaelas et al 1994 (Denmark)	Protein (Spectrophotometric)	6	27	0.77
		8	14	0.77

2.2.4 Results from systematic reviews

To-date only one systematic review and meta-analysis has reported human milk protein content from the 6th month of lactation (Lönnerdal et al. 2017). This review examined studies published up to March 2015, to describe the temporal trends in human milk protein concentration over the first year of lactation. Studies were included that reported true protein determined by the Kjeldahl true protein method, and studies using various colorimetric assays. The key finding relevant to the present assessment is that human milk protein concentration declines rapidly during the first few weeks of lactation, then remained stable from 4 to 12 months, at around 1.1 g/100 mL. The authors did not examine energy density, and therefore the study does not provide information on the temporal trends in protein by energy density over the course of lactation. Studies have shown that the energy-providing macronutrients in human milk remain constant from 6 to 12 months (Dewey et al. 1984; Mitoulas et al. 2002; Nommsen et al. 1991); therefore, protein per 100 kJ should also remain stable.

2.2.5 Human milk protein content in terms of energy density

The Code prescribes the protein content in infant formula to be determined from nitrogen-based methods. Therefore, to facilitate comparisons between protein levels in human milk and follow-on formula, the range of crude and true protein concentrations were determined for this assessment. After conversion to g/100 kJ using the average energy density of

288 kJ/100 mL (as discussed in Section 2.2.1), mean crude protein in human milk 6 to 12 months post-partum ranged from 0.34 to 0.40 g/100 kJ, and true protein was 0.29 g/100 kJ. The requested minimum protein content for follow-on formula, of 0.38 g/100 kJ, falls within the ranges of crude and true protein levels reported for human milk. Moreover, the requested minimum is consistent with results from studies involving Australian women, using colorimetric methods, of 0.29 and 0.38 g/100 kJ (Gridneva et al. 2018; Mitoulas et al. 2002).

2.2.6 Conclusions from other authoritative bodies

When setting protein intake recommendations for Australian and New Zealand infants, the National Health and Medical Research Council (NHMRC) based the Adequate Intake (AI) for infants aged 0 to 6 months (AI 10 g/day) and 7 to 12 months (AI 14 g/day) on the average intake of protein from human milk[6], with additional protein from complementary foods added for infants 7 to 12 months (NHMRC 2006). Human milk protein concentration up to 6 months of lactation was estimated as 1.27 g/100 mL, based on studies measuring protein with colorimetric assays (Mitoulas et al. 2002; Nommsen et al. 1991; Dewey et al. 1984, 1984; Dewey and Lonnerdal 1983) and one study reporting Kjeldahl crude protein up to 12 weeks (Butte et al. 1984). Protein concentrations for 7 to 12 months post-partum was estimated as 1.1 g/100 mL based on colorimetric results reported by Dewey et al (1984), Mitoulas et al (2002), and Nommsen et al (1991).

The IOM used similar data as the NHMRC when setting the Dietary Reference Intakes for infants, except they did not use the results from the study by Mitoulas and colleagues (2002) when setting the Recommended Dietary Allowance for infants 7 to 12 months (of 11 grams per day) (Institute of Medicine 2005). Thus, they estimated average protein levels in human milk 7 to 12 months post-partum at a slightly higher value than the NHMRC, of 1.21 g/100 mL.

In response to a request to reduce the minimum allowed protein content in follow-on formula in the EU, the ESFA examined systematic reviews assessing human milk protein concentrations up to 12-weeks (Gidrewicz and Fenton 2014; Lönnerdal et al. 2017; Hester et al. 2012) and 12-months (Lönnerdal et al. 2017) post-partum. They also highlighted the studies by Michaelsen and colleagues, who measured true protein with an infrared analyser (Michaelsen et al. 1990), and the study by Nommsen et al (1991). From this evidence base, the panel concluded that the mean crude protein was 1.1 g/100 mL at 6 months, corresponding to about 0.38 g/100 kJ (EFSA 2017). These values are consistent with the range of protein concentrations in human milk determined for this assessment.

2.2.7 Summary

Based on studies reporting protein content measured using nitrogen-based methods, crude protein in human milk 5 to 12 months post-partum ranged from 0.99 to 1.14 g/100 mL, corresponding to 0.34 to 0.40 g/100 kJ. True protein was 0.83 g/100 mL, corresponding to 0.29 g/100 kJ. These values align with the reports by other governmental agencies and the EFSA. The requested minimum protein in follow-on formula, of 0.38 g/100 kJ, falls within the upper end of the range for crude protein in human milk and therefore is consistent with human milk protein concentrations reported for Australian women.

2.3 Higher protein content for soy-based follow-on formula

Internationally higher protein levels have been set for follow-on formulas based on soy protein isolate. For example, the European regulations set a minimum protein of 0.56 g/100 kJ for follow-on formula based on soy protein compared to 0.43 g/100 kJ for follow-on

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[6] Determined from an average volume of milk intake (0.78 L and 0.6 L for infants aged 0-6 and 7-12 months, respectively), plus protein concentration in human milk (NHMRC 2006).

formula based on cows' and goats' milk proteins. The rationale for the higher minimum for soy protein is due to (1) the NCF for soy protein and (2) the digestibility and amino acid availability compared to dairy protein sources.

Compared to cows' and goats' milk proteins (comprised mainly of whey and casein proteins), soy protein has a different amino acid composition and is structurally different due to side chain glycosylation. Glycosylation increases protein molecular weight but does not increase nitrogen content. Thus for soy protein, 1 g of nitrogen has been determined to be equivalent to 5.71 g of protein. The lower NCF for soy proteins is consistent with the current scientific literature on this topic (Evers et al. 2016; Krul 2019). Therefore scientifically the most appropriate NCF is 5.71 for follow-on formula based on soy protein.

However there is no consistency in the application of the 5.71 NCF for soy protein in regulations. As shown in Figure 1 below, use of the 6.25 conversion factor for soy protein instead of 5.71 would lead to a calculated protein content that is overestimated by about 10%. A higher minimum protein content for soy-based follow-on formula allows for overestimation of the calculated protein content and potential amino acid insufficiency. The higher value also allows for concerns that soy and other plant-based proteins may have lower digestibility and amino acid availability compared to dairy sources (Koletzko et al. 2013).

On this basis the EFSA (2014) supported the conclusions of the EC SCF (2003), recommending a higher minimum protein content for follow-on formula based on soy protein of 0.54 g/100 kJ.

The Code does not set a specific NCF for soy-based follow-on formula. Therefore a higher minimum protein amount is scientifically justified to ensure follow-on formula consumers are obtaining adequate protein and amino acids.

$$\text{Minimum Protein in FOF (per 100 kJ)} \qquad \text{NCF} \qquad \text{Amount of nitrogen measured by Kjeldahl needed to meet minimum protein amount}$$

$$0.38 \text{ g Protein} \times \frac{1 \text{ g Nitrogen(Kjeldahl)}}{5.71 \text{ g Protein}} = 0.0665 \text{ Nitrogen (Kjeldahl)}$$

$$0.38 \text{ g Protein} \times \frac{1 \text{ g Nitrogen(Kjeldahl)}}{6.25 \text{ g Protein}} = 0.0608 \text{ Nitrogen (Kjeldahl)}$$
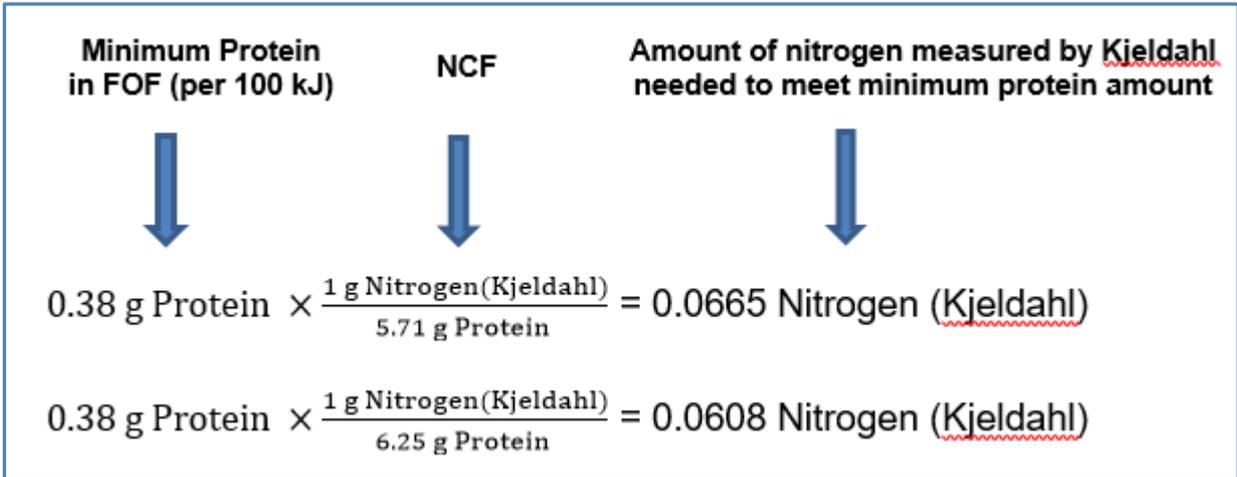
Figure 1: Calculation showing the amount of nitrogen measured by Kjeldahl required to meet the proposed minimum protein level of 0.38 g/100 kJ. Top equation uses the soy NCF of 5.71, bottom equation uses 6.25 as the NCF for other proteins (see section 2.1.3)

2.4 Safety studies lower protein follow-on formula (0.38 mg/100 kJ)

2.4.1 Impact of lower protein follow-on formula on infant growth and development

Formula-fed infants demonstrate faster weight gain compared with breastfed infants (Koletzko et al. 2009). As breastfed infants are the benchmark, some reduction in growth rate compared with current formula is not an adverse effect providing it is not less than breastfed infants.

Two studies were provided by the applicant to show that formula with a protein content of 0.39 g/100 kJ, with adequate complementary food, supports normal weight gain and growth rates in infants aged 3 to 12 months (Inostroza et al. 2014; Ziegler et al. 2015). No additional studies specifically examining the effect of lower protein follow-on formula on growth or other developmental outcomes were identified through a PubMed search using the search terms such as infant formula, protein, energy. Instead, the research to date has focussed on 'lower protein' formulas with protein concentrations at the current minimum, of 0.45 g/100 kJ, or higher.

The two studies included in the application were randomised, controlled trials examining growth trajectories of infants fed lower protein formula (1.61 or 1.65 g/100 kcal, equivalent to 0.39 and 0.40 g/100 kJ, respectively) compared with those fed higher protein formula (2.2 or 2.7 g/100 kcal, equivalent to 0.51 or 0.65 g/100 kJ) (Ziegler et al. 2015; Inostroza et al. 2014) (summarised in Appendix 2 Table A2.2)[7].

The amino acid profiles the of the study formulas generally met the amino acid specifications of the Code; with minor exceptions for each of the study formula. Both studies included a non-randomized breastfeeding reference group. The primary objectives were to compare differences in weight gain between the formula groups between the period 3 to 6 months of age, with power calculations based on this objective.

Secondary objectives included examining weight gain for the periods 3 to 12 months of age and 6 to 12 months of age, comparing weight gain between the formula and breastfed groups, and examining differences in other anthropometric measurements (e.g. weight–for–length z-scores). Inostroza et al (2014) specifically recruited infants with overweight mothers - a high-risk group for rapid infant growth – whereas while Ziegler et al (2015) did not specifically target women with high BMI, over 50% of the women in the formula groups had a BMI >25 kg/m^2.

Both studies indicated slower weight gain between 3 and 12 months for infants fed the lower protein formula compared with the higher protein group. However, the differences only reached statistical significance between 3 and 6 months in the study by Inostroza et al (2014). Compared with breastfed infants, Ziegler et al (2015) reported higher weight gain in infants fed either formula, whereas Inostroza et al (2014) reported greater weight gain in the higher protein group and no difference in the lower protein group.

Both studies had some limitations that should be considered when interpreting the results, as summarised in Table A2.2. Nevertheless, it can be concluded that there is a consistent direction of the association, with both studies showing slightly lower growth rates in infants fed the lower protein formula.

2.4.2 Conclusions from other authoritative bodies

The EFSA reviewed the studies by Inostroza et al (2014) and Ziegler et al (2015) as part of an assessment of the safety and suitability of reducing the minimum amount of protein to 0.38 g/100 kJ (EFSA 2017). EFSA noted that the studies were not specifically designed to meet EU regulatory definitions for infant formula or follow-on formula and that not enough information was provided to determine total energy intakes or contribution of the formula to protein intakes. Therefore, the panel noted that the two trials alone did not provide sufficient evidence to conclude on the safety and suitability of the lower protein levels in follow-on formula. However, they considered that the requested minimum protein level fell within the levels found in human milk 6 to 12 months post-partum (estimated as 1.6 g/100 kcal (or 0.38

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[7] The reported protein content in the lower protein formulas were converted from g/100 kcal using the conversion factor of 4.18, giving values of 0.3852 and 0.3947 g/100 kJ. With rounding, these values were 0.39 and 0.40 g/100 kJ, respectively.

g/100 kJ), and that their dietary modelling showed no adverse effect on protein intakes with the lower protein content in follow-on formula.

Thus, the panel concluded that the use of follow-on formula with a protein content of at least 1.6 g/100 kcal (0.38 g/100 kJ) from intact cow or goat milk protein, otherwise complying with all other EU regulations, is safe and suitable for EU infants consuming suitable complementary foods.

3.   Dietary Intake Assessment

3.1 Method

3.1.1 Scope of the dietary intake assessment

Follow-on formulas are suitable for infants aged from 6-<12 months of age, therefore only this population group was included in the dietary intake assessment. The assessment included an evaluation of protein intakes using different intake scenarios which were compared to the AIs for protein for infants for risk characterisation.

Two intake scenarios were considered:

1. follow-on formula is the single source of protein
2. model diet for 9-month-old infants: a mixed diet of follow-on formula and other solid foods and beverages

The amounts of protein per 100 kJ were converted to grams of protein per 100 g of follow-on formula in order to undertake the dietary intake calculations.

3.1.2 Intake Scenario 1: Follow on formula as the sole source of protein

For this scenario, it is assumed that follow-on-formula is the only source of protein in the diet (i.e. no protein from solid foods or other beverages). This provides the most protective estimate of protein intake when considering adequacy.

To calculate the theoretical dietary intake of protein the relevant estimated energy requirements specified in the Australian New Zealand Nutrient Reference Values (NRVs) for the age group considered[8] were used to estimate how much formula would have to be consumed to meet that requirement. Energy requirements for boys and girls from 6 to 12 months range between 2500 kJ/day and 3500 kJ/day depending on sex and month of age. The minimum permitted amount of protein contained in the follow-on formula (as ready to consume i.e. prepared with water) is then multiplied with the consumption amount to provide a theoretical intake. The minimum levels of protein assessed were:

•   the current minimum level in the Code (0.45 g/100 kJ equivalent to 1.19 g/100 g formula)
•   the proposed new minimum by the applicant of (0.38 g/100 kJ equivalent to 1.00 g/100 g formula).

Estimated protein intakes were calculated for 6, 9 and 12-month-old infants.

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[8]https://www.nrv.gov.au/nutrients

3.1.3 Intake Scenario 2: Mixed model diet for 9-month-old infants

By the age of 6-12 months, most infants consume a mixed diet and obtain nutrients from a range of foods in addition to human breast milk and/or infant/follow-on formula. On this basis, the impact of a lower protein level in follow-on formula was also assessed assuming that infants eat a mixed diet.

At the time of this assessment, FSANZ did not have any nationally representative data on the consumption of foods for infants aged 6-12 months. Therefore, a model diet was constructed for 9-months-old infants to represent consumption patterns at the mid-point of this age range. The methodology used to develop the infant model diet is described in Appendix 2.

For the model diet assessment, three concentrations of protein were used for follow-on formula (prepared with water):
- the protein content of standard follow-on-formula sourced from the food composition dataset AUSNUT 2011-13 of 1.80 g/100 g[9]
- the minimum level currently specified in the Code of 1.19 g/100 g
- the proposed lower level of 1.00 g/100 g.

3.2 Estimated dietary intakes

3.2.1 Intake Scenario 1: follow-on formula is the single source of protein

As shown in Table 4, the theoretical protein intake of 6-month-old males is 12 g/d using the current minimum protein level of follow-on formula as input and 10 g/day using the proposed reduced minimum protein content. For 6-month-old females, the protein intakes are very similar. As expected, with increasing age the theoretical protein intakes increase due to increased energy requirements being largest for 12-month-old males reaching 16 g/d and 13 g/d for current and proposed minimum protein contents respectively.

Table 4: Theoretical Dietary protein intake for differently aged infants based on using follow-on formula as the sole source of protein

Age (months)	Sex	Energy required (kJ/day)[1]	Formula required (kg/day)[2]	Theoretical Protein Intake (g/day)	
				Current minimum 1.19 g/100 g	Proposed minimum 1.00 g/100 g
6	M	2700	1.0	12	10
	F	2500	0.9	11	10
9	M	3100	1.2	14	12
	F	2800	1.1	13	11
12	M	3500	1.3	16	13
	F	3200	1.2	14	12

[1] Nutrient Reference Values for Australia and New Zealand https://www.nrv.gov.au/dietary-energy
[2] Based on a follow on formula energy content of 264 kJ/100 g (source: standard follow on formula AUSNUT 2011-13)

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[9]http://www.foodstandards.gov.au/science/monitoringnutrients/ausnut/ausnutdatafiles/Pages/foodnutrient.aspx

3.2.2 Intake Scenario 2: model diet for 9-month-old infants

Using the more complex assessment methodology based on a model diet that includes consumption of follow-on formula, solid foods and other beverages provides a more accurate estimate of protein intake using different protein contents of follow-on formula. Table 5 concentrations in the market place (AUSNUT data), the minimum protein content currently specified in the Code and the proposed lower minimum protein. Mean protein intakes were estimated at 24 g/day based on AUSNUT data, 21 g/day on the current minimum in the standard and 20 g with the proposed reduction in the protein minimum.

Estimated intakes of protein from non-nationally representative studies of infants were also reviewed by FSANZ. Nutrient intakes from these studies were estimated from dietary recall or record data collected from the infants. The estimated intakes determined by FSANZ above are similar to the mean protein intake of 29 g/day reported for 9-month-old infants of first-time mothers from the control arm of the Melbourne The Infant Feeding Activity and Nutrition Trial (InFANT) (Lioret et al. 2013). They were also similar to the estimated usual protein intakes of 24 g/day for 6-8-month-old infants from the NOURISH study for infants consuming a diet of formula and solid foods and beverages (Kavian et al, 2015).

Table 5 shows the contributors to protein intake based on the model diet for 9-month-old infants. As can be seen, the percentage contribution of follow-on formula to estimated protein intake decreases from 32 to 28% as the protein intake of the formula is reduced while the contribution of general foods increases accordingly.

Table 5: Contributors (%) to the protein intake of 9-month-old infants based on different protein concentrations in follow-on formula

Food Group	AUSNUT composition	Current minimum	Proposed minimum
Protein	1.80 g/100 g	1.19 g/100 g	1.00 g/100 g
Infant formulas	**42**	**32**	**28**
Other foods			
Poultry, game birds	10	11	12
Beef, veal, large game	6	7	7
Cheeses	5	5	6
White breads	<5	5	5
Multigrain, wholemeal, spelt, rye breads	<5	<5	5
Other foods (solid and fluid)	30	35	37
Total other foods	**58**	**68**	**72**

3.3 Risk Characterisation

3.3.1 Use of the Adequate Intake

The Adequate Intake (AI) is the average daily nutrient intake level based on observed or experimentally determined approximations or estimates of nutrient intake by group/s of apparently healthy people that are assumed to be adequate. AIs are set when there is insufficient evidence to set an EAR (NHMRC 2006).

The AIs for protein for infants up to 12 months of age are based on the average protein content and average consumption (0.78 L/day) of human milk relevant to infants of the same age. A different concentration of protein in human milk was used for the 0-6 month old AI (at

12.7 g/L) compared to the 7-12 month AI (at 11 g/L) based on changes in milk composition at different stages of lactation. There was also an additional allowance of protein from complementary foods for the 7 to 12 months AI of 7.1 g (based on nutrition survey data from the USA).

The AI for protein for infants 0-6 months is 10 g/day and for 7-12 months is 14 g/day.

3.3.2 Comparison of protein intakes from the different scenarios to the AI

For intake scenario 1, follow-on formula was assumed to be the sole source of protein for infants aged 6 to less than 12 months. As outlined in Table 4 above, infants aged 6 months have an estimated intake of protein from follow-on formula based on the lower proposed protein level that is equivalent to their AI and infants aged 9 and 12 months have estimated intakes that are between 1-3 g/day lower than the AI (depending on age and sex).

The proportion of infants not introduced to complementary foods by 7 months is low. The 2010 Australian National Infant Feeding Survey (AIHW, 2011) indicated that only 8.4% of Australian infants had not been introduced to solids by 6 months of age. The Infant Feeding in New Zealand report (NZ Ministry of Social Development, 2018) indicated that only 3.8% of New Zealand infants had not been introduced to solids by 7 months of age.

As mentioned above, AIs are based on the average protein content and average consumption of human milk relevant to infants at the same ages (NHMRC, 2006), with an additional allowance of protein from complementary foods for the AI for infants aged 7 to 12 months. Intakes of protein from follow-on formula as a sole source of protein that are slightly below the AI would be expected to be within the distribution of normal daily protein intakes of this population group. Therefore, FSANZ considered that older infants are not at risk of inadequate protein intake as a result of reducing the protein minimum in follow-on formula.

For intake scenario 2, which includes protein sourced from a mixed diet and follow-on formula, intakes based on the proposed minimum protein level in follow-on formula were higher than the AI (Table 6).

The mean protein intakes from Australian infant dietary studies (i.e. InFANT and NOURISH) are above the AIs for both NRV age groups.

Table 6: Dietary protein intakes of 9-month-old infants[1] consuming follow-on formula and mixed diets (intake scenario 2) compared to Adequate Intakes (AI)[2]

Source of protein concentration	Protein level in follow-on Formula (g Protein/ 100 g)	Mean protein intake (g/day)	AI for protein[2] 0-6 months (g/day)	AI for protein[2] 7-12 months (g/day)
AUSNUT	1.80	24		
Current protein minimum	1.19	21	10	14
Proposed protein minimum	1.00	20		

[1] Assumes same energy content (264 kJ/100 g) across all calculations using different protein concentrations in follow-on formula

[2] Nutrient Reference Values for Australia and New Zealand https://www.nrv.gov.au/nutrients/protein

3.4 Conclusions from Dietary Intake Assessment

If it is assumed that follow-on formula is the single source of protein, the theoretical protein intake of 6-month-old boys is 12 g/d and 11 g/d for girls using the current minimum protein in

the Code as input and 10 g/day for both genders using the proposed reduced minimum protein content. With increasing age, the theoretical protein intakes increase as a result of increasing energy requirements.

Estimated daily protein intake based on a minimum proposed follow-on formula protein content of 0.38 g/100 kJ and assuming the formula was the only source of dietary protein, was 10 g/day for boys and girls 6 months of age, which is equivalent to the AI for this age group, thus indicating sufficient protein intake. Protein intakes in this scenario, however, were 1-3 g/d lower than the AI of 14 grams of protein per day for infants 7-12 months based on the proposed lower minimum protein level.

It is unlikely that the lower protein follow-on formula would pose a risk of inadequate protein intake because older infants from 6 to less than 12 months of age would receive protein from complementary food sources. However, the data on age of introduction to solid food in Australia (NHMRC, 2011) and New Zealand (NZ Ministry of Social Development, 2018) indicate only a small declining proportion of infants are not introduced to solids at around 6 months of age. It is also relevant to note that the proposed minimum protein in follow-on formula aligns more closely to the protein content in human milk. As such, the same percentage of breastfed infants would likely not meet their protein AI in scenario 1.

Furthermore, the AI is likely to overestimate protein requirements because the AI is derived from population-based average dietary intakes, which tend to exceed requirements, rather than experimentally derived protein requirements (NHMRC 2006).

Using a more complex assessment methodology including other foods and beverages, the mean protein intake of 9-month old infants can be estimated at 24 g/day based on AUSNUT protein data, 21 g/day on the current standard and 20 g/day with the proposed reduction in minimum protein content. The percentage contribution of follow-on formula to estimated protein intake decreases as the protein intake of the formula is reduced while the contribution of all other foods increases. On this basis, it is considered unlikely that a lower minimum protein level for follow-on formula poses a risk of inadequate protein intake.

4. Nutritional safety assessment conclusion

Based on the evidence currently available, we have determined that crude protein content in human milk during the 6th to 12th months of lactation ranges from 0.34 to 0.40 g/100 kJ, and true protein is around 0.29 g/100 kJ. The requested minimum protein concentration for follow-on formula, of 0.38 g/100 kJ, falls within the levels of protein in human milk determined for this nutritional safety assessment.

Two randomised controlled trials showed no adverse effect on growth with a lower protein formula (0.39 and 0.40 g crude protein/100 kJ) compared with higher protein formula (0.51 to 0.65 g/100 kJ) when fed to infants between 3 months to 12 months of age. Furthermore, the dietary intake assessment showed no risk of inadequate protein intake for Australian or New Zealand infants if the protein in follow-on formula was lowered to 0.38 g/100 kJ as requested by the applicant.

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Appendix 1: Literature

Table A2.1: Identified studies reporting human protein milk levels from 6 to 12 months

Reference (country)	Participants	Human milk sampling	Method for determining protein and energy	Included/excluded
Gridneva et al. 2018 (Australia)	Mothers of healthy singleton, full-term, exclusively breastfed (at 2 and 5 months) infants.	Fore- and hind-milk samples from one feed.	Bradford Dye Binding assay (Bio-Rad commercial kit) – as per method by Mitoulas et al, below.	Included
Czosnykowska-Lukacka et al 2018 (Poland)	Lactating women up to >24 months post-partum (no inclusion criteria).	Full breastmilk sample collected from women 26 women up to 12 months post-partum.	Human milk analyser (Infrared method; gives crude and true protein measurement, NCF 6.38).	Excluded Not included in assessment because provided only combined protein values 1 to 12 months.
Feng et al. 2016 (9 countries)	18 to 40 year old mothers with healthy term, singleton infants 1 to 12 months post-partum.	Complete breast sample from 23 mothers collected between 1.00 pm and 5.00 pm.	Crude protein measured with FP-528 Nitrogen system, based on Dumas combustion method. Conversion factor 6.25. True protein measured by amino acid analysis. Energy not reported.	Included
Saarela et al 2005 (Finland)	Donor mothers who had delivered full-term infants, and mothers who had delivered preterm infants.	20 mothers collected foremilk and hind milk (single collection time), and 53 mothers collected full breast sample over 24-hours.	"IDF Standard 20A", which is Kjeldahl crude protein method (not sure of conversion factor, but is usually 6.38).	Included
Yamawaki et al 2005 (Japan)	Healthy, non-smoking, non-vitamin supplement using women aged under 40 yr.	One milk sample collected from 39 women at an intermediate time during suckling; no standardization of timing.	Kjeldahl crude protein, conversion factor 6.38.	Included

Reference (country)	Participants	Human milk sampling	Method for determining protein and energy	Included/excluded
Mitoulas et al 2002 (Australia)	Healthy mothers with term infants, having exclusively breast fed for at least 4 months.	Fore- and hind-samples collected over 24-hours from 15, 6 and 5 women during the 6th, 9th, and 12th months of lactation, respectively.	Bradford Dye Binding assay (Bio-Rad commercial kit). Human milk standard reference material.	Included
Vilalpando 1998 (Mexico and US)	Healthy women with term infants who were breastfed for 6 months.	Full breastmilk samples collected from 50 women over 24 hours.	Kjeldahl crude and true protein (no NCF reported).	Excluded Not included in assessment because provided only combined protein values 1 to 6 months.
Michaelsen 1994 (Denmark)	Women selling to milk bank.	Samples collected from 244 women, mostly hind-milk samples.	Infrared (IR) scanner (measures nitrogen-hydrogen bonds, therefore gives results for protein, free amino acids, oligopeptides).	Included
Allen et al 1991 (US)	Multiparous women who planned to exclusively breastfeeding for 6 months, and had successfully breast fed previously	One mid-feed sample collected during the morning.	BCA (Pierce commercial kit). Human milk standard material was developed by measuring Kjeldahl True Protein (conversion factor not stated).	Included
Nommsen et al. 1991 (US)	Healthy mothers and infants and no plan to introduce solid foods or other milk before 4 months.	Full breastmilk sample at each feed over 24 hours	Modified Lowry assay Bovine Serum Albumin as standard material.	Included
Nagra et al. 1989 (Pakistan)	Breastfeeding women, medium socio-economic status.	One fore- and hind-sample collected between 9 and 11, after woman had eaten breakfast.	No method given, other than a general "All nutrients were analysed using A.O.A.C methods".	Included
Stuff & Nichols 1989 (US)	Healthy women aged 18 to 36.	24-hour samples collected (further information given in prior report).	Kjeldahl true protein (reported only nitrogen values; for this assessment, nitrogen (N) was converted to protein using factor 6.38):	Included

Reference (country)	Participants	Human milk sampling	Method for determining protein and energy	Included/excluded
Dewey, Lonnerdal 1984 (US)	Breastfeeding women attending Lamaze classes and from general public.	One full breast sample at second feed of day	Modified Lowry. Standard material not described.	Included

Table A2.2: Randomised Controlled Trials

Study Country	Number of Subjects	Duration of study	Composition of study formula[a]	Outcome Summary	Study Quality and Limitations
Inostroza et al. (2014) Brazil	86 in each formula group 76 in breastfed group	3 to 24 months	Lower protein formula: Energy: 62.8 kcal/100 mL (262.5 kJ/100 mL) Protein: 1.65 g/100 kcal (0.39 g/100 kJ) Higher protein formula: Energy: 65.6 kcal/100 mL (274.2 kJ/100 mL) Protein: 2.70 g/100 kcal (0.65 g/100 kJ)	Primary objective Lower weight gain from 3 to 6 months in lower protein group compared with higher protein group ($p<0.05$). Secondary objectives (comparison of formula groups) No differences in weight gain from 6 to 12 months. Lower weight gain from 3 to 12 months in lower protein group compared with higher protein group ($p=0.015$). Secondary objectives (comparison with breastfed infants) No differences in weight gain between lower protein group and breastfed group. Trend for higher weight gain from 3 to 6 months ($p=0.071$), and higher weight gain from 6 to 12 months ($p=0.002$) in higher protein group compared with breastfed group.	Quality - Groups comparable at baseline; selection and allocation procedures described; disposition of subjects provided; appropriate outcomes, outcomes reliably measured. Limitations – high drop-out rate, particularly in the lower protein group (37%); limited follow-up after 9 months; sufficient statistical power not verified for detecting differences in growth between 6 and 12 months; generalisability uncertain (recruited only infants from overweight mothers); no information on protein intake from complementary foods. Overall – moderate quality

Study Country	Number of Subjects	Duration of study	Composition of study formula[a]	Outcome Summary	Study Quality and Limitations
Ziegler et al. (2015)	97 in each formula group 112 in breastfed group	3 to 12 months	Lower protein formula: Energy: 67.2 kcal/100 mL (280.9 kJ/100 mL) Protein: 1.61 g/100 kcal (0.39 g/100 kJ) Higher protein formula: Energy: 64.6 kcal/100 mL (270.0 kJ/100 mL) Protein: 2.15 g/100 kcal (0.51 g/100 kJ)	Primary objective No difference in weight gain from 3 to 6 months in lower protein group compared with higher protein group. Secondary objectives (comparison of formula groups) No differences in weight gain from 6 to 12 months, and trend for lower weight gain from 3 to 12 months (p=0.068) in lower protein group. Secondary objectives (comparison with breastfed infants) Higher weight gain in both protein groups compared with breastfed infants (p<0.05).	Quality - Groups comparable at baseline; selection and allocation procedures described; disposition of subjects provided; appropriate outcomes, outcomes reliably measured. Limitations –sufficient statistical power not verified for detecting differences in growth between 6 and 12 months; no information on protein intake from complementary foods. Overall – moderate quality

[a] *Values were reported in kcal, and were converted to kJ using the conversion factor 4.18.*

Appendix 2: Construction of a model diet for 9-month-old infants

By the age of 9 months, most infants will be consuming a mixed diet and will have intakes of nutrients from a range of foods in addition to human breast milk and/or infant formula. In the absence of nationally representative food consumption data for this population group, a model diet can be used to estimate intake for 9-month-old infants. The model diet is based on recommended energy intakes, mean body weight, the proportion of milk and solid foods in the diet and on food consumption data available for two-year-old children from the 2011-12 Australian National Nutrition and Physical Activity Survey.

The recommended energy intake for a 9-month-old boy (FAO 2004) at the 50th percentile weight (WHO 2006) is used as the basis for the model diet. Boys' weights are used because boys tend to be heavier than girls at the same age and therefore have higher energy and food requirements. The body weight of a 50th percentile 9-month-old boy is 8.9 kg.

The model diet assumes that 50% of energy intake comes from follow on formula and 50% from solids and other fluids (Hitchcock et al. 1986, Butte et al. 2004, PAHO 2003). The patterns of consumption of a two-year-old child from the 2011-12 National Nutrition and Physical Activity survey are scaled down and used to determine the solids and other fluids portion of the model diet.

Certain foods such as tree nuts, tea, coffee, alcohol and honey are excluded from the model diet for a number of reasons. Nuts are not recommended for infants because of the choking risk (NHMRC 2012), however, peanut butter was included in the model diet. Coffee (NHMRC 2012) and alcohol (ACT Government, 2013) are unsuitable for infants and therefore are excluded. Tea is not appropriate for infants to consume as it contains tannins and other compounds that bind to iron and other minerals which reduce their bioavailability (NHMRC 2012). Honey is not recommended for infants as it can contain the spores of *Clostridium botulinum* which is harmful to the immature infant gut (Brook 2007) and increases the risk of dental caries (NHMRC 2012).

Consumption of breakfast cereals is assumed to be in the form of infant cereal or rice-based breakfast cereals. Mixed grain breakfast cereals include bran-based cereals, and bran is not recommended in the diet of infants (ACT Government, 2013) due to the potential interference with the absorption of minerals (Murkoff, 2010). Consequently, mixed grain breakfast cereals, are excluded from the model diet.

Since cow's milk is not recommended as the main milk source for children aged less than 12-months of age (NHMRC 2012), all milk consumption was assumed to be in the form of follow on formula. Soy beverages (except soy infant formula) do not contain an appropriate balance of protein, fat and vitamins (ACT Government, 2013) and are inappropriate for infants (NHMRC 2012).

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