
PEER REVIEW OF A1155 SAFETY, TECHNICAL AND HEALTH ASSESSMENT

Prof (adj) Andrew Bartholomaeus

Prof (adj) Diamantina Institute University of Queensland

Prof (adj) Faculty of Health, University of Canberra

Principal BartCrofts Scientific Services Pty Ltd

Bartcrofts@gmail.com

0428691234

Contents

| | |
|---|-----------|
| PEER REVIEW OF A1155 SAFETY, TECHNICAL AND HEALTH ASSESSMENT | 3 |
| Background | 3 |
| General Considerations | 3 |
| Context 3 | |
| Safety Studies on Nutrients | 4 |
| Neonatal Rat models | 5 |
| Chemistry 6 | |
| History of Human Exposure | 7 |
| GM safety assessment | 7 |
| Available toxicological and clinical evidence | 7 |
| Assessment of the effect on infant growth..... | 8 |
| Potential for allergenicity | 8 |
| Other aspects | 9 |
| Risk Benefit Considerations | 9 |
| Overall Conclusions | 9 |
| REFERENCES | 11 |

Peer Review of A1155 Safety, Technical and Health Assessment

Background

FSANZ has requested an expert review of the Safety, technical and health assessment of application A1155 from Glycom seeking permission to add two human milk identical oligosaccharides to infant formula. Following completion by FSANZ of the approval report the Australia and New Zealand Ministerial Forum on Food Regulation requested that FSANZ review its approval of the application. FSANZ has defined the scope for this review as a consideration of;

- History of human exposure
- GM safety assessment
- Available toxicological and clinical evidence
- Assessment of the effect on infant growth
- Potential for allergenicity
- Other aspects as identified

The review does not extend to assessment of the efficacy of 2'-FL or LNnT with regard to any claimed benefit.

Application A1155 requests permission to add 2'-*O*-fucosyllactose (2'-FL) and lacto-*N*-neotetraose (LNnT) derived by microbial fermentation from genetically modified (GM) *Escherichia coli* K-12, to infant formula. These compounds are human milk identical oligosaccharides (HiMO).

General Considerations

Context

Infant formula is the sole or a substantive portion of the food intake of infants and toddlers where breastfeeding is either not possible or for other reasons not desirable or not optimal. For risk and health assessment purposes the appropriate context for consideration of whether a proposed composition of an infant formula is appropriate for the target population is of course human breast milk. While this observation is superficially rather facile, breast milk is a food with a highly variable composition and any comparison must accommodate this natural variability. Sources of variation include the genetics of the mother, the duration of lactation, the mother's diet and fluid intake, the feeding demands of the child and the health of the mother. Breast milk composition therefore varies between mothers and from day to day for any individual mother. In the absence of convincing evidence of adversely altered health outcomes in children related to variations in specific components of breast milk at levels across the normal range, a presumption that inclusion of individual components of human breast milk is safe and appropriate at levels within the normal range is reasonable. Indeed,

preventing the addition of normal human breast milk components to infant formula at levels consistent with the normal range is arguably, at least potentially, risk generating. In this respect I note that the FSANZ health Assessment indicates that;

“2’-FL and LNnT are also naturally present in human milk, providing a history of human exposure to these substances for breastfed infants. The requested maximum concentrations of 2’-FL_{micro} (1.2 g/L) and LNnT_{micro} (0.6 g/L) in infant formula products, and the 2’-FL concentration used in the dietary intake assessment (based on 2.4 g/L), are within the range of concentrations reported in mature human milk (1.0 – 7.8 g/L for 2’-FL secretors and 0.09 – 1.08 g/L for LNnT)”

Indeed, the proposed levels are considerably below the upper range of normal human breast milk levels.

Safety Studies on Nutrients

Toxicology and safety studies are essentially hypothesis free in that no specific effect of treatment is anticipated and multiple parameters are included to enable identification of any effects that may occur. Consequently, in animal studies such as those described in the FSANZ health assessment of A1155 numerous clinical, hematological, histological, and health-related parameters are typically measured. If statistical significance is set at the standard default value of $p < 0.05$, then on average 1 parameter in 20 (i.e. 5%) is expected to be statistically significantly different between treatment groups purely by chance, and a few of these might even be expected to show evidence of a dose relationship, again purely by chance (McDonald, 2009). So common and predictable are these random statistically significant differences that every guideline toxicology study can be expected to report these. Consequently, interpretation of statistically significant findings relies on a consideration of biological consistency and plausibility which is based on the identification of appropriate changes in correlating parameters and consistency across studies. The FSANZ assessment has appropriately therefore dismissed as incidental a number of isolated statistically significant effects in a number of the studies provided by the applicant. A more detailed discussion of this issue can be found in Bartholomaeus et al. (2013).

Although standard toxicology study designs are usually appropriate as a component of the safety assessment of low intake nutrients the classical approach to utilizing the results of such studies in the risk assessment is not appropriate. With a few exceptions related to food availability and preservation, there are generally no adverse population health consequences that arise from the establishment of very conservative (ie low) human health reference values (HRVs) for chemicals in food. Consequently, the risk assessment process for chemical additives and contaminants in food incorporates multiple layers of precaution which act to reduce permitted intakes and therefore levels in food, and thereby provide a high degree of protection. Because nutrients are a normal, and generally essential or beneficial, component of the diet this aspect of the toxicological paradigm for risk assessment is generally inappropriate for nutrients. The FSANZ health assessment for Application A1155 has

appropriately used a margin of safety approach (MOE) which focuses on the degree to which doses in animal studies which produce no identifiable adverse effects exceed those proposed for the target human population. Here too, however, the principal, most robust and precautionary guide to safety of normal human breast milk components added to formula is the range of such substances in normal human breast milk.

For non-digestible sugars such as 2'-FL and LNnT, very high doses such as those used in the animal studies provided by the applicants may disturb gastrointestinal function through physico-chemical effects, such as establishment of a high osmotic load in the gut resulting in soft and loose stools, or the generation of gas and discomfort due to fermentation in the lower bowel. These types of effects are concentration (in the gut contents) dependent rather than dose (on a per kg bw basis) dependent and tend to dissipate rapidly as intakes, and therefore GIT concentrations, are reduced. Alterations in faecal consistency in some of the animal studies may be of this nature and would not indicate a risk to the target population at levels within the normal human breast milk range for these oligosaccharides.

Neonatal Rat models

The length of gestation for mice and rats is very short (19 – 22 days) and the pups are born very immature. The blood brain barrier and gut are considerably less developed than in the human neonate for example. In the neonatal pup the gut matures slowly during lactation (post-natal days day 0 – 21) with a rapid period of maturation occurring during the short transition from milk to solid food. As a result, macromolecule absorption in human and rat neonates is quite different. Although some absorption of macromolecules such as oligosaccharides results from intestinal M cell immunological sampling of gut contents in human neonates this is quite limited. The neonatal rat resembles a human infant born prematurely and in particular the gut is more permeable to large molecules such as oligosaccharides (Sanglid, 2006; Tiechberg, et al., 1990). As a consequence, where dosing of rat pups commences prior to weaning (day 21) as occurred for a number of studies submitted for A1155, they will be considerably *more sensitive* to systemic toxicity, local irritation and osmotic related effects than human term neonates due to comparative gut immaturity. Therefore, an absence of apparent toxicity in such studies provides quite robust evidence of an absence of hazard but the presence of effects, particularly if mild and confined to high doses, does not in isolation necessarily constitute evidence of hazard to term human neonates.

Gastrointestinal maturation in relation to birth and weaning

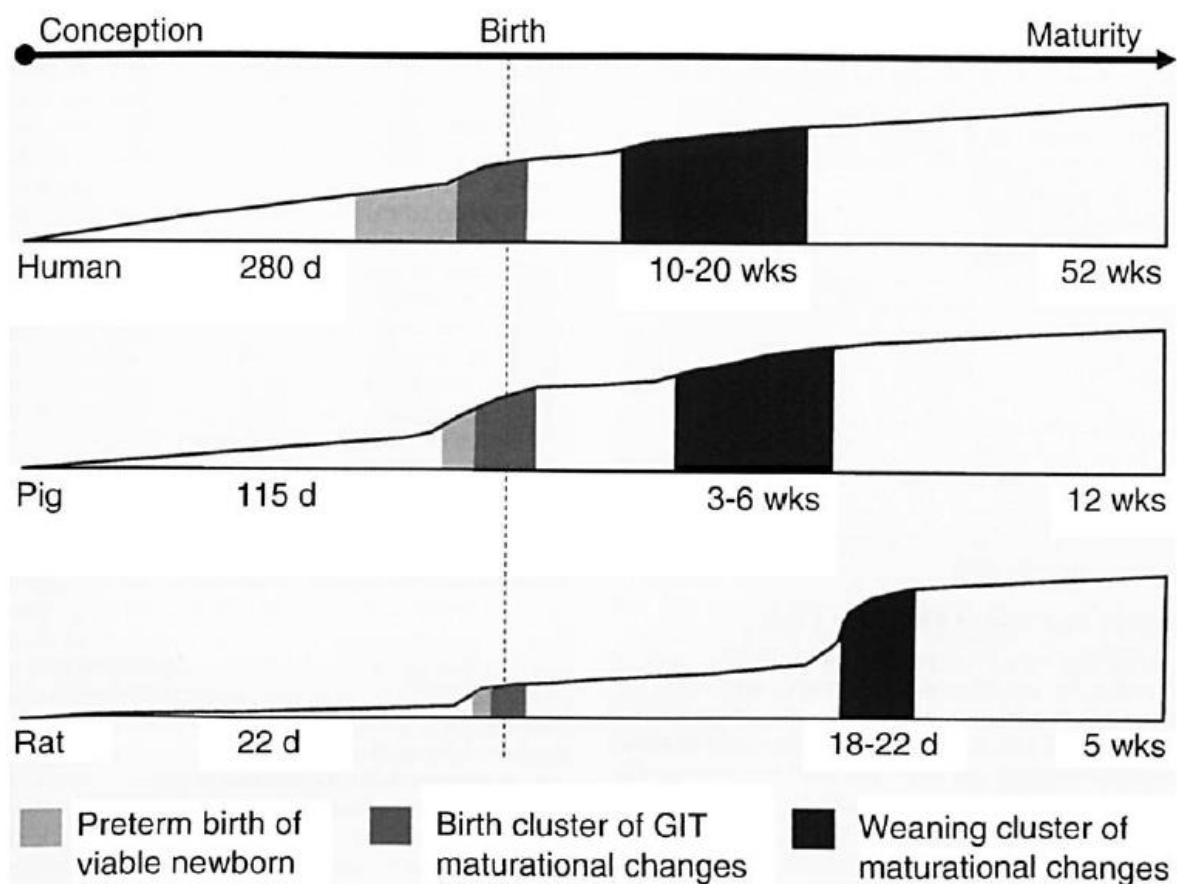


Figure 1. The timing of GIT maturation in three different groups of mammalian species. In humans and other primates, GIT development is slow and maturation starts early (in fetal life). In most small rodents and carnivorous species, the developmental changes occur relatively quickly and late (postnatally around weaning). GIT maturation in large domestic animals (pigs, sheep, cattle) is intermediate (i.e., maturation is rapid during the period from shortly before birth to shortly after weaning). Around weaning (black areas) and birth (dark grey areas), maturation is particularly rapid, resulting in a weaning cluster and a birth cluster of maturational changes. Birth of viable preterm neonates occurs over a wider range of gestational ages in humans compared with domestic animals like the pig (light grey areas).

Chemistry

2'FL and LNnT are comparatively simple molecules consisting of 3 and 4 monosaccharide moieties respectively, in a chain. A variety of analytical methods confirmed that these compounds synthesized by either microbial culture or chemical means are identical to those produced by human breast tissue and present in human milk, as would be expected given their simplicity. All methods of synthesis, whether biological or chemical, generate impurities (intermediates, byproducts etc), including normal human breast tissue. The impurities identified in material produced microbially were predominantly carbohydrates consisting of residual starting materials naturally present in human breast milk at higher levels or formed during heat treatment of milk (such as pasteurisation). The nature of the impurities does not raise a plausible health or safety concern at the levels that will be present in infant formula (or that for older children).

History of Human Exposure

Both 2'FL and LNnT are normal human milk constituents and the range of normal values extends well above the levels requested by the applicant and that proposed by FSANZ to be approved in infant and follow on formula. The microbially produced oligosaccharides are chemically identical to the normal human milk constituents. On this basis there is a clear history of human consumption of both 2'-FL and LNnT by neonates and older children at levels that substantially exceed that requested by the applicant and approved by FSANZ.

GM safety assessment

The GM safety assessment has considered the host organism, E.coli K12, the source or donor organisms for the genes required for the production of the oligosaccharides, the mechanism of the genetic modifications, the nature of the genes transferred, the safety of novel proteins, the history of safe use of the enzymes expressed by the modifications, and bioinformatic assessment of the potential allergenicity or toxicity of the expressed proteins.. No public health or safety concerns were identified by the GM safety assessment. Genetic modification of food organisms and food additive producing organisms such as E.coli K12 has been performed for about 30 years. During this period there has not been a single instance of the process of genetic engineering in and of itself introducing, generating or otherwise leading to the production of toxic or allergenic proteins or secondary metabolites not associated with either the modified organism or the gene donor.

I concur with the conclusions of the GM safety Assessment that no identifiable public health or safety concerns arise from the genetic modification of the production organism E.coli K12.

Available toxicological and clinical evidence

Given that both 2'FL and LNnT are identical to that found in normal human breast milk and are added at levels well within the normal range found in human milk, the conduct of animal and genotoxicity studies for the assessment of safety are arguably unnecessary. The applicant has however provided an extensive range of animal, human and in vitro studies which support the safety of both 2'-FT and LNnT when used as proposed. A number of studies commenced dosing prior to weaning, when rat pups have an immature gut more permeable to macromolecules than human neonates and are therefore *more sensitive* to potential adverse effects than human neonates. The doses administered were quite high and more than adequate to demonstrate safety, particularly given the increased sensitivity of neonatal rats. Doses of 5000 mg/kg bw equate to approximately 50,000 ppm in the diet or approximately 5% of the composition of the diet. Higher levels would risk invalidating the studies due to dietary imbalance, feed avoidance due to taste issues or localized gut concentration related effects not relevant for the proposed levels of use in infant and follow on formula. A number of the studies dosed the rats for 90 days which is approximately 12% of the animal's life expectancy or roughly equivalent to 9-10 years in human terms. The duration of dosing is therefore more than adequate to model the proposed pattern of use. The range of parameters

assessed in the animal studies was extensive and included behavioral and developmental assessment in addition to the normal battery of parameters included in OECD test guideline compliant studies. As is normal and expected from studies involving a large number of comparisons a number of random but statistically significant results arose which were appropriately dismissed as incidental by the FSANZ evaluators. Some minor variations at high doses that may have been treatment related remained within normal background ranges (historical control ranges) and were appropriately concluded to be non adverse.

Genotoxicity studies were predictably negative.

A number of clinical trials have been conducted in healthy infants. These studies involved relatively small numbers of subjects and many included a range of combinations of oligosaccharides in addition to 2'-FL and/or LNnT. As would be expected however formula containing 2'-FL and or LNnT is well tolerated in the target population.

Assessment of the effect on infant growth

As the FSANZ assessment has concluded:

Trials in infants have tested 2'-FL in combination with either scFOS, GOS, or LNnT. The highest tested concentrations of 2'-FL and LNnT in infant formula were 1.2 and 0.6 g/L, respectively. None of these studies found a difference in growth compared to a control formula. Based on this, and the limited oral absorption of 2'-FL and LNnT, FSANZ concludes that no adverse effects on growth are expected at concentrations corresponding to those typically observed in human milk.

This conclusion is reasonable, consistent with the available data and logically consistent with the addition of human breast milk identical oligosaccharides to formula at levels well within the normal range found in normal human breast milk.

Potential for allergenicity

For multiple reasons the use of genetic engineering to modify *Escherichia coli* K-12 to produce these oligosaccharides does not introduce a risk of production of protein allergens as an impurity.

2'FL and LNnT are human milk identical oligosaccharides, are not themselves allergenic, and are highly purified prior to addition to formula. Due to the degree of purification of the final ingredient it is highly unlikely that novel protein or DNA will actually be present.

The structural requirements for protein allergenicity are very specific and extensive. The incidental modification of endogenous non allergenic proteins of *Escherichia coli* K-12 to be allergenic through the process of gene insertion (or in this case plasmid insertion) is highly implausible. For a protein to be allergenic it must first be resistant to digestion which requires very specific and generally extensive structural modification of the protein. In addition, the protein must be recognized by the immune system to initiate a response, which also requires very specific modification. There are no known plausible additions, deletions or substitutions

of amino acids in endogenous proteins that would randomly convert an otherwise nontoxic or nonallergenic protein into a protein with toxic or allergenic properties (Steiner, et al., 2013).

Escherichia coli K-12 has a long history in the production of food enzymes and biopharmaceuticals and has consequently been thoroughly characterized. No potential for the production of allergenic proteins has been reported.

As Concluded by the FSANZ assessment

2'-FL_{micro} and LNnT_{micro} does not contain detectable proteins and is therefore considered unlikely to pose an allergenicity concern. A recently published double blind, placebo-controlled food challenge study demonstrated that infant formula containing 1.0 g/L 2'-FL and 0.5 g/L LNnT was hypoallergenic in children with cow's milk protein allergy, consistent with this conclusion.

Other aspects

Risk Benefit Considerations

A review of the clinical evidence in support of claimed health benefits has been sought from clinical experts in the field. In terms of the risk component of that consideration however there is an absence of identifiable plausible risk from the addition of human milk identical oligosaccharides to infant and follow on formula at levels that are within the normal range and well below the upper bound of the normal range for human breast milk. In these circumstances the most conservative (or precautionary), risk mitigating, approach would seem to be to permit and encourage adjustment of infant formula composition to more closely approximate human breast milk to the greatest extent possible whilst acknowledging the wide natural variability of the composition of human breast milk. Given that variability in composition, there cannot be a point estimate for a "best" or "ideal" level of components such as 2'-FL or LNnT.

Overall Conclusions

Application A1155 requests permission to add 2'-O-fucosyllactose and lacto-N-neotetraose derived by microbial fermentation from genetically modified (GM) *Escherichia coli* K-12 to infant formula. These oligosaccharides are chemically identical to that occurring naturally in human breast milk and the proposed level of addition is well below the upper range found in normal human breast milk. On this basis, in the absence of evidence of adverse health outcomes in exclusively breast-fed infants ingesting these oligosaccharides at levels near the upper range of normal there is no *prima facie* basis for concern regarding the safety of their addition to infant and follow on formula. Indeed, preventing the addition of normal human breast milk components to infant formula at levels consistent with the normal range is arguably, at least potentially, risk generating.

Animal studies employing high doses and using the neonatal rat model, which is likely to be substantially *more sensitive* to any adverse health effects due to their relative gut immaturity compared to human neonates, yielded no findings of toxicological or developmental concern.

Clinical studies in the target population also found no evidence of adverse developmental outcomes or intolerance to 2'-FL or LNnT as would be expected. Limitations in the numbers of subjects in the trials do not raise concerns given the absence of effects at high doses in a highly sensitive animal model, that these oligosaccharides are chemically identical to those in human milk and are to be added at levels within the normal range.

The data provided in support of the safety of the proposed addition of the two oligosaccharides is adequate and sufficient given the nature of the substances, the context of the proposal, and specifically given the addition will result in infant formula more closely approximating the normal range of human breast milk composition. In my view there is nothing either present or absent in the data that would provide a scientifically justifiable basis for rejecting this application on the grounds of safety. In my view, the balance of precaution favors addition of normal human breast milk substances to formula to more closely reflect the range of breast milk composition rather prevention of addition, unless data is available that demonstrates that the normal variation of the levels of these substances present in human breast milk is robustly associated with variation in neonatal and infant health and developmental outcomes in a direction that would justify prohibition of their addition.

The FSANZ Safety, Technical and Health Effects Assessment is of high quality, reflects careful and competent evaluation of the available data and the conclusions are consistent with and proportionate to the data available. Having considered the data and arguments presented by FSANZ in their assessment I agree with their overall conclusion that no plausible hazards, and therefore risks, are identifiable for these oligosaccharides at the levels proposed to be permitted by FSANZ.

References

- Bartholomaeus, A., Parrott, W., Bondy, G. & Walker, K., 2013. The use of whole food animal studies in the safety assessment of genetically modified crops: limitations and recommendations.. *Critical Reviews in Toxicology*, 43(S2), pp. 1-24.
- McDonald, J., 2009. *Handbook of biological statistics. 2nd Ed.* Baltimore: Sparky House Publishing.
- Sanglid, P., 2006. Gut Responses to Enteral Nutrition in Preterm Infants and Animals. *Experimental Biology and Medicine*, pp. 1695-1711.
- Steiner, H. et al., 2013. Evaluating the potential for adverse interactions within genetically engineered breeding stacks. *Plant Physiology*, Volume 161, pp. 1587-1594.
- Tiechberg, S. et al., 1990. Development of the Neonatal Rat Small Intestinal Barrier to Nonspecific Macromolecular Absorption: Effect of Early Weaning to Artificial Diets. *Paediatric Research*, 32(1), pp. 31-57.