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Supporting document 1

Review of new toxicology, clinical safety, growth and development studies

Review of Application A1155 - 2'-FL and LNnT in infant formula and other products

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Executive summary

FSANZ has reviewed additional studies of the safety and effects on infant and toddler growth of 2'-O-fucosyllactose (2'-FL) and lacto-N-neotetraose (LNnT), which have been published since the 2019 assessment of A1155 by FSANZ.

The additional pharmacokinetic, toxicological and clinical studies on these oligosaccharides do not indicate a need to amend the conclusions of the previous assessment.

Additional information on the pharmacokinetics of HMOs confirms that small amounts of these substances are absorbed following oral intake and excreted intact in the urine. The majority are not absorbed in the small intestine but pass to the large intestine, where they are metabolised by intestinal bacterial enzymes or excreted unchanged in the faeces.

Recently published studies detected human milk oligosaccharides (HMOs) including 2'-FL in amniotic fluid and umbilical cord blood collected at birth, indicating that infants are likely to already be exposed to HMOs during development *in utero*.

New genotoxicity studies with 2'-FL in combination with other oligosaccharides confirmed the absence of mutagenicity, clastogenicity and aneugenicity. A 90 day oral toxicity study in neonatal rats with an 8:1 mixture of 2'-FL and difucosyllactose (DFL) found no adverse effects at doses up to 5000 mg/kg bw/day, or 4444 mg/kg bw/day 2'-FL. A 90 day dietary toxicity study in rats with 2'-FL in combination with four other oligosaccharides at 10% in the diet also found no adverse effects.

Newly available information from three clinical studies with healthy infants found that formula containing 2'-FL is well tolerated with no indications of adverse effects, consistent with the findings of previously reviewed studies. A real-world evidence study of infants fed formula containing 2'-FL (1.0 g/L) and LNnT (0.5 g/L) also found the formula was well tolerated.

$2'\text{-FL}_{micro}$ and LNnT_{micro} do not contain detectable proteins and are therefore 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.

Three relevant studies on infant growth were published since the initial assessment of evidence. One cohort study found an association between the concentration of several HMOs including 2'-FL and LNnT and weight-Z-score of infants aged 3 -12 months. The second cohort study found that the concentration of 4 HMOs in human milk samples from high weight gain infants of secretor mothers were significantly different to those in normal weight infants of secretors at 5 months of age, and 2 were significantly different at 9 months of age. Associations were also found between HMO content, anthropometry and weight velocity from birth to 5 months. However in addition to the study design limitations described by both authors, observational cohort studies are a weak source of evidence for the effect of individual HMOs on growth and development due to the difficulty in controlling for other factors. One randomised controlled trial found that infants who consumed formula that only differed from control formula by the addition of 0.25 g/L 2'-FL had similar weight-for-age and length-for-age percentiles as control groups at enrolment and after 6 weeks was similar for both groups. Based on the available evidence and limited absorption of HMOs, FSANZ maintains the conclusion that the addition of 2'-FL and LNnT to formula at levels normally found in human milk is unlikely to affect growth.

Since the proposed maximum concentrations of 2'-FL and LNnT (2.4 and 0.6 g/L, respectively) are within the range of naturally occurring levels in human milk (1.0 – 7.8 g/L and 0.04 – 1.08 g/L, respectively), there are no safety concerns associated with the addition of 2′-FL, alone or in combination with LNnT, to infant formula products and formulated supplementary foods for young children (FSFYC).

FSANZ concludes there are no public health and safety concerns associated with the addition of 2′-FL alone or in combination with LNnT to infant formula products and FSFYC at the proposed levels.

Table of contents

Abbreviations

2′-FL	2′-O-fucosyllactose
2′-FL_{chem}	2′-O-fucosyllactose produced by chemical synthesis
2′-FL_{micro}	2′-O-fucosyllactose produced by microbial fermentation
2′-FL_{human}	2′-O-fucosyllactose naturally occurring in human milk
3-FL	3-fucosyllactose
3’-SL	3’-siallylactose
6’-SL	6’-siallylactose
BW	Body weight
DFL or DFLac	Difucosyllactose
DFLNH	Difucosyllacto-N-hexaose
FDSLNH	Fucodisialyllacto-N-hexaose
FOS	Fructo-oligosaccharide
FMI	Fat mass index
FSFYC	Formulated supplementary foods for young children (or ‘toddler milk’)
GOS	Galacto-oligosaccharide
HMO	Human milk oligosaccharide
ITT	Intention to treat
LNFP I	Lacto-N-fucopentaose
LNT	Lacto-N-tetraose
LNnT	Lacto-N-neotetraose
$LNnT_{micro}$	Lacto-N-neotetraose produced by microbial fermentation
$LNnT_{chem}$	Lacto-N-neotetraose produced by chemical synthesis
$LNnT_{human}$	Lacto-N-neotetraose naturally occurring in human milk
NOAEL	No observed adverse effect level
scFOS	Short-chain fructo-oligosaccharide

1. Introduction

A number of new toxicity and clinical studies of 2'-FL and/or LNnT have become available since the time FSANZ's previous safety assessment was published (FSANZ 2019). These studies have been reviewed by FSANZ and are discussed below, together with a summary of the outcomes of the previous assessment.

2. Toxicology assessment

2.1 Previous FSANZ evaluation

The toxicological database previously evaluated by FSANZ included:

- information on the absorption, distribution, metabolism and excretion of HMOs including 2′-FL and LNnT
- *in vitro* genotoxicity studies of 2'-FL and LNnT
- an *in vivo* micronucleus assay with 2'-FL
- subchronic toxicity studies of 2'-FL and LNnT in rats, including studies in neonatal rats
- a subchronic toxicity study of 2'-FL in neonatal piglets
- human clinical studies of 2'-FL and/or LNnT in infants, children and adults

Studies were conducted with Glycom's 2'-FL_{micro} and $LNnT_{micro}$, as well as 2'-FL and LNnT produced from other sources. Evidence was provided demonstrating that the substances produced by Glycom are identical to 2'-FL and LNnT naturally occurring in human milk, and the substances produced by other manufacturers are also reported to identical to those in human milk. Studies with all these forms of 2'-FL and LNnT are therefore considered to be relevant for the evaluation.

FSANZ's assessment concluded there were no public health and safety concerns associated with the addition of 2'-FL to IFP and FSFYC at concentrations up to 2.4 g/L, alone or in combination with LNnT at 0.6 g/L.

HMOs were found to be resistant to hydrolysis by digestive enzymes in *in vitro* studies using human or porcine enzyme preparations or intestinal brush border membranes. Small amounts of ingested HMOs including 2'-FL and LNnT are absorbed, while a large proportion pass to the large intestine where they are fermented by the intestinal microbiota or excreted intact in the faeces. A study comparing breastfed infants with those consuming infant formula containing 2′-FL_{chem} found no evidence to suggest that absorption or urinary elimination of 2′-FL_{chem} is significantly different to that of 2′-FL_{human}.

Both 2'-FL and LNnT were not genotoxic *in vitro* or *in vivo*. No treatment-related adverse effects were observed in subchronic oral toxicity studies in neonatal rats at doses up to 5000 mg/kg bw/day 2'-FL or LNnT, or in older rats at doses greater than 7000 mg/kg bw/day 2'-FL. A 3-week study with neonatal piglets administered milk replacer formula containing 2'-FL at concentrations up to 2 g/L also found no adverse effects.

In human studies, infant formula supplemented with 2'-FL and LNnT was well tolerated with age-appropriate increases in body weight and other growth measures, with no significant increases in adverse events. 2'-FL and LNnT were also well tolerated in studies with children and adults.

The proposed maximum concentration of 2'-FL (2.4 g/L) is similar to the mean concentration

in mature human milk, and the proposed maximum concentration of LNnT (0.6 g/L) is also within the range of LNnT concentrations in human milk.

The overall no observed adverse effect level (NOAEL) of 5000 mg/kg bw/day 2'-FL in neonatal animals is more than 7-fold greater than the highest estimated P90 dietary intake for 3 month old infants (660 mg/kg bw/day), 12-fold higher than the highest estimated P90 intake for 12 month old infants (400 mg/kg bw/day), and 16-fold greater than the highest estimated P90 intake for children aged 2-3 years (310 mg/kg bw/day).

For LNnT, the NOAEL of 5000 mg/kg bw/day is more than 30-fold greater than the highest estimated P90 dietary intake for 3 month old infants (160 mg/kg bw/day), 50-fold greater than the highest estimated P90 intake for 12 month old infants (100 mg/kg bw/day), and more than 60-fold greater than the highest estimated P90 intake for children aged 2-3 years (77 mg/kg bw/day.

2.2 Additional toxicological and clinical safety studies

The applicant provided details of several new studies available in the scientific literature, and FSANZ also conducted a literature search in PubMed on 7th April 2020. Newly available data include investigations of the presence of HMOs in amniotic fluid and umbilical cord serum, in vitro genotoxicity studies with a mixture of 2'-FL and difucosyllactose (DFL) produced by fermentation from a single source, and a 90 day oral toxicity study with 2'-FL/DFL in neonatal rats. In addition, genotoxicity studies and a 13 week oral toxicity study with a mixture of 2'-FL and four other human milk oligosaccharides are available.

Several new human clinical studies of tolerance and hypoallergenicity are also available. In addition, the applicant for another source of 2'-FL (Jennewein, A1190) submitted several previously published pharmacokinetic studies of 2'-FL and other oligosaccharides that were not reviewed as part of A1155.

2.3 Toxicological studies

2.3.1 Absorption, distribution, metabolism and excretion

A study of preterm mother-infant dyads demonstrated the presence of intact HMOs including 2'-FL and LNnT in human milk and in the faeces of breastfed infants. They have also been found in the urine of breastfed infants, supporting the limited metabolism of these substances following absorption (De Leoz et al 2013). 2'-FL, LNnT and other HMOs have also been detected in the urine of pregnant and lactating secretor women (Hallgren and Lundblad 1977).

When HMOs reach the colon they may be metabolised by intestinal bacteria such as certain *Bifidobacterium* and *Bacterioides* species through the action of glycoside hydrolases and other enzymes, resulting in cleavage to disaccharides and monosaccharides (Marcobal and Sonnenburg 2012). Induction of α-L-fucosidase, an enzyme that metabolises 2'-FL, and production of lactate and short-chain fatty acids has been observed following culture of various *Bifidobacterium* and *Bacterioides* species in the presence of 2'-FL (Yu et al. 2013a). Faeces from healthy adults and patients with Crohn's disease or ulcerative colitis have been found to contain glycosidases which degrade mucin oligosaccharides, including α-L-fucosidase (Hoskins and Boulding 1981. Rhodes et al 1985). Several strains of faecal bacteria obtained from healthy adults have been shown to produce extracellular glycosidases capable of degrading mucin oligosaccharides (Hoskins et al 1985).

A recent study found several HMOs including 2'-FL, 3-fucosyllactose, difucosyllactose and 6'-sialyllactose were present in amniotic fluid collected from pregnant women at birth. These HMOs were also detected in maternal urine collected at birth and maternal milk obtained four days postpartum. The most dominant HMO in all three samples was 2'-FL (Wise et al 2018).

Up to 18 HMOs have also been detected in the serum of the umbilical vein at birth. Concentrations of the majority of HMOs, particularly secretor-associated fucosylated HMOs including 2'-FL, were correlated with levels in maternal serum. Using an *ex vivo* human placenta perfusion model, 2'-FL was shown to transfer from maternal to fetal circulation, suggesting that HMOs in cord blood may be of maternal origin (Hirschmugl et al 2019).

2.3.2 Toxicological studies with 2'-FL

Subchronic toxicity studies

90-day oral toxicity study with a mixture of Glycom's 2'-FL and difucosyllactose in neonatal rats (Phipps et al. 2018) Regulatory status: GLP; conducted in compliance with OECD TG 408 (1998) and the EMEA guideline on repeated dose toxicity (2010)

The test item in this study was an 8:1 mixture of 2'-FL and difucosyllactose (DFL) produced together from microbial fermentation of a single modified strain of *E. coli* K-12 (92.2% 2'-FL/DFL w/w; Batch No. CPN6317 1000517FD). The concentration of 2'-FL, DFL and other carbohydrates was 82.5%, 9.7% and 4.32% w/w, respectively. Water was used as the vehicle control. Juvenile Crl:CD(SD) rats (10/sex/group) were administered 0, 1000, 3000 or 5000 mg/kg bw/day 2'-FL/DFL by oral gavage from postnatal day (PND) 7 for 90 days. An additional reference group of rats received 5000 mg/kg bw/day fructooligosaccharides (FOS). Total carbohydrate doses were 0, 1092, 3276, 5460 or 5320 mg/kg bw/day, respectively. An additional 5 males and 5 females were included in the vehicle control, high dose 2'-FL/DFL and FOS groups and retained with no test article administration for 4 weeks after the dosing period to assess the reversibility of any observed effects. Animals were observed for morbidity and mortality at least twice daily. Detailed physical examinations were performed on all neonates from PND 7 to PND 20 (Days 1 – 14 of dosing) and once weekly from PND 21 onwards. Ophthalmoscopy was performed on all animals in the vehicle control, FOS and high dose 2'-FL/DFL groups during the last week of dosing. Body weights were recorded daily for the first two weeks of dosing and twice weekly thereafter. Food consumption was recorded twice weekly from weaning on PND 21. Animals were monitored for timing of sexual maturation (balano-preputial separation for males and vaginal opening for females), eye opening and air righting reflex. Auditory and visual function (startle response and pupil closure response) were examined on PND 20 (Day 16 of dosing) and length of the left ulna was monitored from PND 14 (Day 8 of dosing). Neurobehavioural evaluations comprised a functional observational battery test on all animals during Week 11 of dosing and assessment of spatial learning and memory (using the Morris water maze) during Week 12. Blood and urine samples were collected from fasted animals for haematology, coagulation, clinical chemistry and urinalysis parameters just prior to scheduled necropsy. For some haematology parameters in the vehicle control (male and female) and the low and high dose groups (female) 9 samples were analysed rather than 10. An explanation for this was not provided. Animals were killed on the day after receiving the final dose (PND 98) or at the end of the recovery period (PND 126) and subjected to a full macroscopic necropsy including measurement of organ weights. Tissues were collected from all animals and a histopathological examination was performed on those from the vehicle control and 5000 mg/kg bw/day 2'-FL/DFL groups.

No deaths, treatment-related clinical signs or opthalmoscopic findings were observed during the study. Mean body weights and food consumption were reported to be similar in the 2'-FL/DFL groups and control animals throughout the dosing and recovery periods. No

treatment-related differences in the age or body weight at which animals attained sexual maturation, surface and air-righting reflexes were reported. The mean age for balano-preputial separation was significantly higher in males given 5000 mg/kg bw/day 2'-FL/DFL compared with vehicle controls (45.4 days versus 43.7 days, $p < 0.05$), but this minor difference was considered to be due to abnormally low vehicle control values compared with historical control data (historical control values not reported). Mean ulna growth during the dosing and recovery periods was similar in all groups. No treatment-related effects on neurobehavioural observations in the functional observational battery and Morris maze tests were observed. Significantly lower activity counts in high dose females compared with controls ($p < 0.05$, control value not reported) were considered unrelated to treatment as there was no dose-response relationship and similar findings were not seen in males. No treatment-related changes in haematology, coagulation, clinical chemistry and urinalysis parameters and organ weights (relative to body weight) were observed. A number of statistically significant changes were observed in these parameters (Tables 1 – 5), but these were not associated with a dose-response, were restricted to one sex, and/or were within historical control ranges for the test facility. No adverse macroscopic or microscopic changes in organs and tissues were observed. Findings for animals treated with FOS were similar to those for animals treated with 2'-FL/DFL.

Table 1 Haematology and coagulation values for male rats administered 2'-FL/DFL by oral gavage for 90 days

Parameter	**Dose (mg/kg bw/day)**					**Historical control data (5^{th} – 95^{th} %ile)**
	0	**1000**	**3000**	**5000**	**5000 FOS**	
Haematology						
WBC ($x10^9$/L)	11.53 ± 2.96	9.18 ± 1.55	9.85 ± 1.94	11.95 ± 2.90	15.89 ± 4.16**	8.77 – 22.71
RBC ($x10^{12}$/L)	7.46 ± 0.33	8.32 ± 0.31**	8.04 ± 0.46**	8.04 ± 0.67	7.36 ± 0.20	7.07 – 9.02
Hb (g/dL)	14.8 ± 0.4	15.4 ± 0.5*	15.2 ± 0.4*	15.3 ± 0.8*	14.8 ± 0.4	13.20 – 16.20
Haematocrit (L/L)	0.39 ± 0.01	0.45 ± 0.02**	0.45 ± 0.01**	0.43 ± 0.04**	0.39 ± 0.01	0.38 – 0.47
MCV (fL)	52.7 ± 1.5	54.5 ± 1.0*	56.5 ± 2.3**	53.3 ± 1.7	52.9 ± 0.9	49.30 – 57.50
MCH (pg)	19.8 ± 0.6	18.5 ± 0.6*	19.0 ± 0.9*	19.1 ± 1.2*	20.1 ± 0.5	17.20 – 20.20
MCHC (g/dL)	37.6 ± 0.6	33.9 ± 0.6**	33.6 ± 0.5**	35.9 ± 2.5**	37.9 ± 0.4	33.00 – 36.70
Platelets ($x10^9$/L)	891 ± 110[a]	814 ± 87	845 ± 72	859 ± 116	944 ± 64	Not reported
Retic ($x10^{12}$/L)	0.17 ± 0.03	0.15 ± 0.02	0.16 ± 0.02	0.17 ± 0.02	0.18 ± 0.02	Not reported
RDW (%)	12.4 ± 0.6	11.7 ± 0.3*	11.6 ± 0.5*	12.3 ± 0.8	12.7 ± 0.4	10.5 – 13.00
Neutrophils ($x10^9$/L)	1.57 ± 0.84	1.22 ± 0.23	1.41 ± 0.20	1.76 ± 0.52	2.14 ± 0.80	Not reported
Lymphocytes ($x10^9$/L)	9.47 ± 2.29	7.53 ± 1.35	7.93 ± 1.81	9.64 ± 2.38	13.08 ± 3.38**	6.66 – 17.12
Monocytes ($x10^9$/L)	0.26 ± 0.15	0.19 ± 0.04	0.28 ± 0.04	0.24 ± 0.11	0.38 ± 0.15	Not reported

Parameter	Dose (mg/kg bw/day)					Historical control data (5th – 95th %ile)
	0	1000	3000	5000	5000 FOS	
Eosinophils ($x10^9$/L)	0.16 ± 0.06	0.11 ± 0.04	0.09 ± 0.02*	0.18 ± 0.11	0.17 ± 0.06	0.06 – 0.37
Basophils ($x10^9$/L)	0.04 ± 0.01	0.08 ± 0.03**	0.08 ± 0.03**	0.07 ± 0.04**	0.06 ± 0.03*	0.02 – 0.19
LUC ($x10^9$/L)	0.03 ± 0.01	0.06 ± 0.01*	0.06 ± 0.02**	0.06 ± 0.03**	0.05 ± 0.02	0.04 – 0.23
Coagulation						
APTT (sec)	22.4 ± 2.1	19.5 ± 4.3	21.8 ± 5.0	23.0 ± 5.8	20.0 ± 4.4	Not reported
PT (sec)	20.6 ± 1.5	23.1 ± 2.3*	23.6 ± 3.2*	22.2 ± 2.8*	22.9 ± 2.2*	20.50 – 33.00

* Significantly different from vehicle control ($p < 0.05$)
** Significantly different from vehicle control ($p < 0.01$)
APTT, activated partial thromboplastin time; FOS, fructooligosaccharide; Hb, hemoglobin; LUC, large unstained cells; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; n, number of animals; PT, prothrombin time; RBC, red blood cell count; Retic, absolute reticulocyte count; RDW, red cell distribution width; WBC, total white blood cell count
[a] = n=9 rather than 10

Table 2 Haematology and coagulation values for female rats administered 2'-FL/DFL by oral gavage for 90 days

Parameter	Dose (mg/kg bw/day)					Historical control data
	0	1000	3000	5000	5000 FOS	
Haematology						
WBC ($x10^9$/L)	10.29 ± 3.86	6.92 ± 0.93*	6.34 ± 1.37**	10.82 ± 5.44	10.64 ± 2.08	5.91 – 20.18
RBC ($x10^{12}$/L)	7.06 ± 0.34	7.80 ± 0.33**	7.83 ± 0.36**	7.08 ± 0.71	6.95 ± 0.27	6.79 – 8.52
Hb (g/dL)	14.7 ± 0.4	14.9 ± 0.7	14.9 ± 0.7	14.3 ± 0.7	14.8 ± 0.5	13.20 – 15.70
Haematocrit (L/L)	0.38 ± 0.02	0.44 ± 0.02**	0.44 ± 0.02**	0.39 ± 0.04	0.38 ± 0.01	0.37 – 0.45
MCV (fL)	53.9 ± 1.2	56.9 ± 1.38**	56.6 ± 1.41**	55.2 ± 0.90	54.1 ± 1.1	50.30 – 57.00
MCH (pg)	20.8 ± 0.68	19.2 ± 0.78**	19.0 ± 0.70**	20.3 ± 1.34	21.3 ± 0.62	17.60 – 20.20
MCHC (g/dL)	38.6 ± 0.85	33.7 ± 0.86**	33.6 ± 0.95**	36.8 ± 2.85	39.3 ± 0.70	33.70 – 36.90
Platelets ($x10^9$/L)	976 ± 105	907 ± 70	813 ± 117*	881 ± 138*	872 ± 114*	Not reported
Retic ($x10^{12}$/L)	0.17 ± 0.01	0.189 ± 0.02	0.16 ± 0.04	0.15 ± 0.04	0.16 ± 0.03	Not reported
RDW (%)	11.7 ± 0.6	11.5 ± 0.4	11.3 ± 0.3	11.7 ± 0.6	11.7 ± 0.5	9.80 – 11.70
Neutrophils ($x10^9$/L)	1.05 ± 0.23	0.84 ± 0.16	0.88 ± 0.24	1.13 ± 0.21[a]	1.41 ± 0.43**	Not reported

Parameter	Dose (mg/kg bw/day)					Historical control data
	0	1000	3000	5000	5000 FOS	
Lymphocytes ($x10^9$/L)	8.80 ± 3.65	5.75 ± 0.87**	5.15 ± 1.14**	7.67 ± 1.23[a]	8.80 ± 2.03	4.78 – 13.58
Monocytes ($x10^9$/L)	0.22 ± 0.05	0.15 ± 0.03**	0.12 ± 0.04**	0.17 ± 0.08**,[a]	0.24 ± 0.08	Not reported
Eosinophils ($x10^9$/L)	0.14 ± 0.04	0.08 ± 0.03*	0.09 ± 0.04*	0.09 ± 0.03*,[a]	0.11 ± 0.04	0.05 - 0.50
Basophils ($x10^9$/L)	0.03 ± 0.02	0.04 ± 0.02**	0.05 ± 0.02**	0.04 ± 0.02*,[a]	0.03 ± 0.01	0.01 – 0.11
LUC ($x10^9$/L)	0.05 ± 0.03	0.05 ± 0.02	0.05 ± 0.02	0.05 ± 0.02[a]	0.04 ± 0.02	0.03 – 0.28
Coagulation						
APTT (sec)	16.8 ± 2.3[a]	16.8 ± 1.5[a]	17.8 ± 1.5	18.9 ± 2.9[a]	18.5 ± 2.3	Not reported
PT (sec)	24.0 ± 2.2	22.4 ± 2.2[a]	23.1 ± 1.9	23.4 ± 3.6[a]	23.6 ± 2.6	20.50 – 30.70

* Significantly different from vehicle control ($p < 0.05$)
** Significantly different from vehicle control ($p < 0.01$)
APTT, activated partial thromboplastin time; FOS, fructooligosaccharide; Hb, hemoglobin; LUC, large unstained cells; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; n, number of animals; PT, prothrombin time; RBC, red blood cell count; Retic, absolute reticulocyte count; RDW, red cell distribution width; WBC, total white blood cell count
[a] = n=9 rather than 10

Table 3 Clinical chemistry values for males and females administered 2'-FL/DFL for which statistically significant changes were observed

Parameter	Dose (mg/kg bw/day)				
	0	1000	3000	5000	5000 FOS
Males					
Sodium (mmol/L)	144 ± 1	142 ± 1	143 ± 1	143 ± 1	142 ± 1**
Chloride (mmol/L)	100 ± 1	99 ± 1	100 ± 1	99 ± 1*	98 ± 2**
Calcium (mmol/L)	2.39 ± 0.06	2.42 ± 0.07	2.40 ± 0.06	2.45 ± 0.06	2.44 ± 0.07*
Phosphorus (mmol/L)	2.04 ± 0.27	2.00 ± 0.17	2.25 ± 0.28	2.23 ± 0.20	2.15 ± 0.19
AST (U/L)	74.7 ± 7	85 ± 12*	84 ± 8*	75 ± 5	78 ± 10
Urea (mmol/L)	5.39 ± 0.75	6.16 ± 0.61	5.40 ± 1.10	4.70 ± 1.27	4.75 ± 0.78
Creatinine (µmol/L)	27 ± 3	25 ± 3	25 ± 2	23 ± 2**	25 ± 3
Albumin (g/L)	34 ± 1	33 ± 1	32 ± 2**	35 ± 2	35 ± 1
Females					
Sodium (mmol/L)	142 ± 1	142 ± 2	141 ± 1	141 ± 1**	141 ± 1*
Chloride (mmol/L)	99 ± 1	100 ± 2	100 ± 1	98 ± 2	98 ± 1

Parameter	Dose (mg/kg bw/day)				
	0	1000	3000	5000	5000 FOS
Calcium (mmol/L)	2.43 ± 0.08	2.51 ± 0.10	2.43 ± 0.05	2.54 ± 0.08**	2.41 ± 0.07
Phosphorus (mmol/L)	1.77 ± 0.23	1.83 ± 0.31	1.80 ± 0.21	2.03 ± 0.24*	1.77 ± 0.24
AST (U/L)	70 ± 12	86 ± 13*	82 ± 14	70 ± 12	69 ± 8
Urea (mmol/L)	5.69 ± 0.58	6.77 ± 0.76*	6.86 ± 1.16*	5.80 ± 1.09	5.94 ± 0.48
Creatinine (µmol/L)	27 ± 1	29 ± 3	29 ± 3	29 ± 3	27 ± 2
Albumin (g/L)	40 ± 2	37 ± 3*	37 ± 2*	39 ± 4*	38 ± 2

* Significantly different from vehicle control ($p < 0.05$)
** Significantly different from vehicle control ($p < 0.01$)

Table 4 Urinalysis values for males and females administered 2'-FL/DFL for which statistically significant changes were observed

Parameter	Dose (mg/kg bw/day)					Historical control data
	0	1000	3000	5000	5000 FOS	
Males						
pH	7.7 ± 0.6	7.5 ± 0.5	7.8 ± 0.8	7.7 ± 1.0	8.0 ± 0.7	6.50 – 8.70
Total creatinine (µmol)	82.49 ± 7.00	75.61 ± 12.51	83.82 ± 22.52	66.96 ± 22.54	78.33 ± 14.45	29.50 – 78.30
Females						
pH	6.7 ± 0.4	6.8 ± 0.3	7.1 ± 0.5	7.4 ± 0.9*	6.8 ± 0.4	6.00 – 8.40
Total creatinine (µmol)	45.35 ± 6.69	42.45 ± 5.63	41.25 ± 8.27	36.08 ± 9.78*	42.45 ± 8.19	19.10 – 41.70

* Significantly different from vehicle control ($p < 0.05$)

Table 5 Organ weights relative to body weight (g/100 g) for males and females administered 2'-FL/DFL for which statistically significant changes were observed

Parameter	Dose (mg/kg bw/day)				
	0	1000	3000	5000	5000 FOS
Males					
Kidneys	3.332	3.674**	3.387	3.393	3.153
Seminal vesicles	1.745	1.991*	1.893	1.660	1.673
Thymus	0.321	0.400*	0.387*	0.399*	0.331
Females					
Kidneys	2.071	2.041	1.997	2.077	1.959
Thymus	0.338	0.340	0.337	0.330	0.336

* Significantly different from vehicle control ($p < 0.05$)
** Significantly different from vehicle control ($p < 0.01$)

The NOAEL of 2'-FL/DFL in this study was 5000 mg/kg bw/day, the highest dose tested. Based on an 8:1 2'-FL:DFL ratio, this is equivalent to 4444 mg/kg bw/day 2'-FL.

7 day pilot oral toxicity study with 2'-FL$_{micro}$ (produced by Jennewein) in combination with four other HMOs in rats (Parschat et al 2020) Regulatory status: GLP; non-guideline

A pilot study was conducted with a mixture of five HMOs produced by fermentation by Jennewein, including 2'-FL, in preparation for a 90-day toxicity study. The test substance was a dry powder blend of a fixed combination of 2′-FL (47.1% dry weight), 3-fucosyllactose (3-FL; 16.0%), lacto-N-tetraose (LNT; 23.7%), 3'-sialyllactose (3′-SL; 4.1%), 6'-sialyllactose (6′-SL; 4.0%) and other carbohydrates (5.1%). The HMOs were produced individually by fermentation, followed by removal of the production strain and further purification and concentration, but their purity was not reported. The report states that nuclear magnetic resonance (NMR) analysis and mass spectrometry (MS) were performed to unambiguously prove chemical equivalence to the human milk HMOs, but these data were not shown. Female CD rats aged 60 days (5/group) were fed either a standard diet or the same diet supplemented with the test substance (10%) for seven days. Animals were monitored daily for viability, clinical signs, body weight, food and water consumption.

No mortalities occurred during the study. No treatment related changes in behaviour, appearance and consistency of the faeces, body weight, body weight gain, or food consumption were reported.

13 week oral toxicity study with 2'-FL$_{micro}$ (produced by Jennewein) in combination with four other HMOs in rats (Parschat et al 2020) Regulatory status: GLP; conducted in compliance with OECD TG 408 (1998)

The test item in this study was a dry powder blend of five HMOs, 2′-FL (47.1% dry weight), 3-FL (16.0%), LNT (23.7%), 3′-SL (4.1%), 6′-SL (4.0%) plus other carbohydrates (5.1%). CD rats (10/sex/group) aged 65 days were fed a standard control diet or the same diet containing the test item at 10% for 91 days. The mean intake of the test item ranged from 5010 – 6880 mg/kg bw/day for males and 6260 – 7910 mg/kg bw/day for females, equal to 2360 – 3240 mg/kg bw/day 2'-FL in males and 2950 – 3730 mg/kg bw/day 2'-FL in females. Animals were housed individually. Clinical signs were monitored daily with detailed clinical observations made weekly. In week 13, animals underwent neurological screening to evaluate sensory reactivity to different stimuli (auditory, visual and proprioceptive stimuli), grip strength and locomotor activity. Body weight was assessed weekly and food and water consumption were monitored daily. Ophthalmology and auditory examinations were performed on all animals before the start of the study and one week before the end of the treatment. Blood and urine samples were collected from fasted animals prior to necropsy to assess haematology, coagulation, clinical chemistry and urinalysis parameters. At necropsy animals were examined macroscopically, organ weights were recorded and tissue samples collected for histopathological evaluation.

No mortalities occurred during the study. One male animal in the control group gained weight at a lower rate than the other control males. No changes in behaviour or external appearance were noted in this animal, but at necropsy a number of lesions and changes in haematology and clinical chemistry parameter were observed. These changes were considered to be spontaneous but the animal was excluded from all analyses. No clinical signs of toxicity were observed and there were no changes in appearance and consistency of the faeces. No treatment-related changes in food and drinking water consumption, body weight and body weight gain were reported. No treatment-related changes were observed in the neurological screen, ophthalmology and auditory examinations, haematology, coagulation, clinical chemistry and urinalysis parameters. Absolute and relative organ weights were not affected by treatment and no macroscopic or microscopic changes in organs and tissues were

reported.

The NOAEL for the test item in this study was 10% in the diet (equal to 5670 mg/kg bw/day for males (2670 mg/kg 2′-FL) and 6970 mg/kg bw/day for females (3280 mg/kg bw/day 2′-FL), the highest concentration tested.

Genotoxicity studies

Recently published papers include details of genotoxicity studies with a mixture of Glycom's 2'-FL and DFL at an 8:1 ratio (Phipps et al. 2018) and with a mixture of five oligosaccharides produced by fermentation by Jennewein, including 2'-FL at 47.1% dry weight (Parschat et al. 2020). These studies were GLP compliant and conducted according to appropriate test guidelines.

2′-FL showed no evidence of mutagenic, clastogenic or aneugenic activity in these assays (Table 6).

Table 6 Summary of new genotoxicity studies with 2′-FL preparations

Test	Test system	Test article	Concentration or dose range	Result	Reference
8:1 mixture of 2'-FL$_{micro}$ and DFL$_{micro}$ produced by Glycom					
Bacterial reverse mutation test (Ames test, OECD TG 471 [1997])	*S. typhimurium* strains TA98, TA100, TA1535 & TA1537; *Eschericia coli* strain WP2 uvrA (pKM101)	2'-FL/DFL$_{micro}$ (82.5% (w/w) 2'-FL; 9.7% DFL; Batch No. CPN6317 1000517 FD) Vehicle: water	Plate incorporation test: 5 – 5000 µg/plate Pre-incubation test: 50 – 5000 µg/plate	Negative ± S9	(Phipps et al. 2018)
In vitro mammalian cell micronucleus assay (OECD TG 487 (2016))	Cultured human peripheral blood lymphocytes	2'-FL/DFL$_{micro}$ (82.5% (w/w) 2'-FL; 9.7% DFL; Batch No. CPN6317 1000517 FD) Vehicle: water	500 – 2000 µg/mL	Negative ± S9	(Phipps et al. 2018)
Mixture of 5 HMOs produced by fermentation by Jennewein					
Bacterial reverse mutation test (Ames test, OECD TG 471 [1997])	*S. typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537	2'-FL (47.1% dry weight); 3-fucosyllactose (16%); lacto-N-tetraose (23.7%); 3'-sialyllactose (4.1%); 6'-sialyllactose (4%), other carbohydrate (5.1%) Vehicle: water	5 – 600 mg/plate (2.05 – 246 mg /plate 2'-FL)	Negative ± S9	Parschat et al. 2020

Test	Test system	Test article	Concentration or dose range	Result	Reference
In vitro mammalian cell micronucleus assay (OECD TG 487 (2016))	Cultured human lymphocytes	As above	7.5 – 60 mg/mL (3.1 – 24.6 mg/mL 2'-FL)	Negative ± S9	Parschat et al. 2020

2.3.3 Human studies

Clinical studies with infants

Infant study with formula containing 2'-FL and LNnT (Román et al. 2020)

An open label, non-randomised, prospective real-world evidence[1] study evaluated growth and tolerance of an infant formula containing 2'-FL 1.0 g/L and LNnT (0.5 g/L). The formula was a partially hydrolyzed, 100 % whey term infant formula providing 67 kcal/100 mL, containing 1.9 g protein, 11.5 g carbohydrates, and 5.1 g lipids per 100 kcal powder, as well as 2'-FL, LNnT and *Lactobacillus reuteri* (DSM 17938). The study was conducted at six centres in Spain. Healthy, term infants were enrolled at age 7 days – 2 months. The study groups were infants exclusively formula-fed (FF), infants fed a mixture of formula and human milk (MF) and infants who were exclusively breastfed (BF). Formula-fed infants were eligible to participate if their parent(s) had decided to formula feed prior to enrolment. Breastfed infants were eligible if they had been exclusively breastfed since birth, and their parent(s) had decided to continue exclusively breastfeeding until at least four months of age. Exclusion criteria included any known intolerance/allergy to cow's milk (formula-fed groups only), conditions requiring infant feedings other than those specified in the protocol, evidence of significant systemic disorders or parental refusal to participate. FF and MF groups were fed the study formula for approximately 8 weeks (56 days). Anthropometric measures including weight, length and head circumference were taken at baseline and again at the end of the study. The infant's gastrointestinal (GI) symptom burden was assessed via the Infant Gastrointestinal Symptom Questionnaire (IGSQ), a validated 13-item questionnaire that assesses GI-related signs and symptoms as observed by parents over the previous week in five domains: stooling, spitting up/vomiting, gassiness, crying, and fussing. A composite IGSQ score is derived by summing the scores for each domain with a possible range of 13 to 65, where higher values indicate greater GI distress and values ≤ 23 indicate no digestive distress. The IGSQ was administered at baseline, week 4, and week 8. Parents of MF and FF infants also completed a formula satisfaction questionnaire at weeks 4 and 8. Adverse Events (AE) were recorded from the time of enrolment for the duration of the study. All AEs were assessed by the site investigator for duration, intensity, frequency, and relationship to study formula.

At the start of the study 82, 62 and 63 infants were enrolled in the FF, MF and BF groups, respectively. The number who remained in the study and had tolerance and growth measures at 8 weeks were 66, 48 and 45 in the FF, MF and BF groups, respectively. The analysis was restricted to infants who completed the study. Baseline characteristics were similar in all three groups, with the exception of lower body weights in infants in the FF group compared with the MF and BF groups. Composite IGSQ scores indicated low levels of GI distress in all three groups at all time points, with no significant differences between groups.

[1] Real-world evidence refers to evidence on risks or benefits of a substance based on observational data collected outside of a clinical research setting.

Analysis of scores for the individual IGSQ domains found no significant differences between groups for four of the five domains (gassiness, fussiness, crying, and spitting-up/vomiting). For stooling, FF infants had scores significantly higher than those in the BF group at baseline (3.47 ± 1.8 versus 2.32 ± 0.82; $p < 0.001$), however the scores improved over the course of the study and no significant differences between FF and BF stooling scores were observed at the end of the study (2.71 ± 1.26 versus 2.18 ± 0.39; $p = 0.07$). Stooling scores of MF infants were significantly higher than BF infants at baseline (2.93 ± 1.21 versus 2.32 ± 0.82; $p = 0.045$) and at week 8 (2.85 ± 1.52 versus 2.18 ± 0.39; $p = 0.025$). In total 58 AEs were experienced by 49 subjects, with a similar incidence in all three groups (18, 21 and 19 AEs in the BF MF and FF groups, respectively). Three AEs were considered potentially related to the study formula: two cases of cow's milk intolerance (one each in the MF and FF groups) and one instance of irritability in the FF group. Six serious AEs occurred (4 in the FF group and 2 in the MF group), all of which were cases of bronchiolitis not considered to be related to the study formula. No significant differences in anthropometric measures were found at the end of the study. Nearly all parents reported that they were satisfied with the infant formula. It was concluded that the infant formula was well tolerated.

Hypoallergenicity trial of infant formula containing 2'-FL and LNnT (Nowak-Wegrzyn et al. 2019) Regulatory status: Non-GLP

A whey-based, extensively hydrolysed infant formula (EHF) containing 2'-FL (1.0 g/L) and LNnT (0.5 g/L) was assessed for clinical hypoallergenicity in a group of children with cow's milk protein allergy (CMPA). The 2'-FL and LNnT supplements were confirmed as being free of residual milk proteins. The test formula had a protein/peptide content of 2.2 g/100 kcal while the control formula contained 2.47 g/100 kcal protein/peptides, with the macronutrient and micronutrient profiles of both formulas otherwise almost identical. Taste and appearance of the two formulas were reported to be indistinguishable. The source of 2'-FL and LNnT used in this study was not specified. Infants and children aged between 2 months and 4 years with documented CMPA were recruited from 12 study sites in the USA, and 67 children (mean age 24.5 ± 13.6 months; 67.2% [45] male) were included in the intention to treat (ITT) population. Most subjects identified as Caucasian/White (56.3%), with 45.3%, 3.1% and 1.6% identifying as Black/African American, Asian and native Hawaiian/Pacific Islander, respectively. CMPA diagnosis was confirmed by a reported convincing allergic reaction to cow's milk or milk-containing product and presence of milk-specific serum IgE (>0.7 kU_A/L) or a positive skin prick test with a wheal > 5 mm (90.6% of participants), a milk-specific serum IgE level > 15 kU_A/L or a skin prick test wheal ≥ 10 mm.

Children underwent double-blind, placebo-controlled food challenges (DBPCFC) with the test and control formulas in a blinded, cross-over trial. The first challenge occurred 3 – 28 days after enrolment and the second was conducted 2 – 7 days after the first challenge. Participants were asked to fast for 1 hour prior to each DBPCFC session. Participants were not permitted to use antihistamine (except eye drops) during the 7 days prior to the first challenge, or oral steroids within 14 days before enrolment. The initial dose in the food challenge was a lip smear, followed by increasing oral doses of formula at 10 – 15 minute intervals, up to a total volume of 180 mL for subjects ≤ 1 year of age and 240 mL for subjects > 1 year. Children were observed for a minimum of 1 hour after the final dose, and any allergic signs or symptoms (cutaneous, gastrointestinal, respiratory or cardiovascular) were documented on a standardised DBPCFC data collection form. The challenge was considered evaluable if subjects had consumed a minimum of 100 mL of formula, and the challenge outcome was assessed according to pre-defined pass/fail criteria for each symptom, in line with recommendations of the American Academy of Allergy, Asthma & Immunology and European Academy of Allergy & Clinical Immunology (Sampson et al. 2012). If a subject successfully passed both DBPCFC sessions, a 1-week open home challenge with the test formula was conducted to confirm the absence of any delayed allergic reactions. Participants were asked to drink a minimum of 240 mL test formula daily for a period of 7 – 9 days, with

daily recording of clinical parameters including potential allergic symptoms and other adverse events. Based on guidance from the American Academy of Pediatrics, hypoallergenicity was confirmed if the 95% lower bound confidence interval (CI) for the proportion of subjects without allergic reactions in the DBPCFC was greater than 90%.

Test formula was administered first to 36 children (Test → Control group) and 31 received the Control formula first (Control → Test group). Two children in the Test → Control group were unable to consume the required minimum amount of formula (100 mL) during the DBPCFC with test formula and one subject was outside the required age range. Statistical analysis was performed with the remaining 64 subjects (modified ITT [mITT] cohort). One patient was erroneously adminstered both challenges with Test formula. This patient was included in the mITT cohort but excluded from the per protocol (PP) cohort. A further two children in the Test → Control group withdrew before completing the challenge with control formula. The remaining 61 patients completed both DBPCFC and were included in the PP cohort (n=61).

A 12-month old girl reacted during both DBPCFC with widespread urticaria and an erythematous rash, after ingesting a total of 165 mL of test formula and 85 mL control formula. No allergic reactions were observed in any of the other children. Analysis of the mITT cohort found that 63/64 subjects (98.4%; 95% lower bound CI 92.8%) tolerated the test formula and 61/62 subjects who completed the challenge with control formula (98.4%, 95% lower bound CI 92.6%) tolerated it. The test formula therefore met the defined criteria for hypoallergenicity. The per-protocol analysis also indicated that the test formula was hypoallergenic: 60/61 (98.4%, lower bound CI 92.5%) subjects tolerated both the test formula and the control formula. Of the 62 children who completed both DBPCFC, 61 completed the open challenge with test formula following exclusion of the subject who failed both DBPCFC. Fifty-five subjects consumed the required minimum 240 mL test formula/day. Two patients reported gastrointestinal symptoms: one vomited on Day 1 but completed the home challenge without further issues, while another developed diarrhoea attributed to gastroenteritis on the final day of the challenge. No other significant gastrointestinal symptoms were reported and no serious adverse events occurred.

It was concluded that the formula supplemented with 2'-FL and LNnT met the clinical criteria for hypoallergenicity in children with CMPA.

Infant study with 2'-FL in combination with short-chain fructo-oligosaccharides – follow-up report (Reverri et al. 2019) Regulatory status: Conducted in accordance with Good Clinical Practice

In a review of clinical studies with infant formula containing 2'-FL (Reverri et al. 2019), additional details were provided on a study with infant formula containing 2'-FL in combination with short-chain FOS (scFOS), previously reported as an abstract and reviewed in the previous SD1 for A1155 (Kajzer et al. 2016). The new review by Reverri et al. provides additional experimental details and results to those previously reported.

The study was a prospective, randomised, multi-centre, double-blind controlled tolerance study in healthy term infants aged 0-8 days. Gastrointestinal tolerance of infants fed an infant formula containing 0.2 g/L 2′-FL and 2.0 g/L scFOS (Abbott Nutrition, Columbus, OH, USA; n=46) was compared to a control formula without oligosaccharides (n=42) and a human milk fed reference group (n=43). Both formulas were standard intact milk protein-based infant formulas with the same nutrient composition except for oligosaccharide content. Infants were exclusively fed formula or human milk from enrolment until 35 days of age. Daily intake records were maintained by parents from enrolment to 14 days of age, and for three days before the visit at 35 days of age. Daily stool records were kept by parents throughout the course of the study. The primary variable was the average mean rank stool consistency

(MRSC) score, and secondary measures included stool number, formula intake details (volume, number of feedings and percent of feedings with spit-up/vomit within one hour of feeding), anthropometric measures (including weight and length) and adverse events.

In total 41 infants in the 2'-FL group completed the study duration, 36 in the control formula group and 42 in the human milk-fed group. No differences in average MRSC scores between groups from enrolment to 35 days of age were observed. The control formula group had a significantly higher MRSC score compared with human milk-fed infants from enrolment to 14 days of age (2.41 ± 0.09 versus 2.07 ± 0.08; p = 0.0409), but there were no significant differences between the control formula and 2'-FL (2.31 ± 0.10) groups or the 2'-FL and human milk-fed groups. The average number of stools per day was significantly higher in the human milk-fed group (5.5 ± 0.4) than the formula with 2'-FL (1.9 ± 0.2; p < 0.0001) and the control formula (2.1 ± 0.2; p < 0.0001) groups. No clinically significant differences in formula intake or the percentage of feedings associated with spit-up/vomit were observed among the three groups from enrolment to 35 days of age. There were no differences in anthropometric measures between groups at enrolment or at 35 days of age. No safety concerns were identified with the study formulas and adverse events were reported to be similar in all three groups, although the data are not presented in the report.

It was concluded that formula containing 2'-FL (0.2 g/L) and scFOS (2.0 g/L) was well tolerated in infants.

Infant study with 2'-FL in combination with Bifidobacterium lactis (Storm et al. 2019)
Regulatory status: Conducted in accordance with Good Clinical Practice

In a randomised, controlled, double-blind, multi-centre study, feeding tolerance of 2'-FL (source unspecified) in a 100% whey, partially hydrolysed infant formula supplemented with *Bifidobacterium animalis* spp *lactis* strain Bb12 (*B. lactis*) was assessed. Healthy full-term infants (n=79) aged 14 ± 5 days who had been exclusively formula fed for at least 3 days prior to enrolment were randomised to receive the test formula or a control formula *ad libitum* for 42 days. The only difference between the formulas was that the test formula contained 0.25 g/L 2'-FL. An Infant Gastrointestinal Symptom Questionnaire (IGSQ) was administered and anthropometric measurements were taken on visits at enrolment and again after approximately 42 days of feeding. For two days before the second visit, caregivers completed a diary of formula intake, stool characteristics, spit-up, vomiting and durations of crying and fussing. Adverse events were reported throughout the study and assessed for duration, intensity, frequency and relationship to test product. The primary objective of the trial was to compare IGSQ scores between groups following consumption of the formula. The intention-to-treat (ITT) population comprised all infants who took any amount of study formula (38 test; 40 control), while the per-protocol (PP) population (30 test; 33 control) excluded subjects who were unlikely to have had full exposure to the study formulas.

Average formula consumption was not significantly different between groups. Mean IGSQ scores for the test and control groups were similar for both the ITT and PP analyses at baseline and at the end of the study. At the end of the study mean IGSQ scores in the PP analysis were 20.9 ± 4.8 in the test group and 20.7 ± 4.3 in the control group (p = 0.82). Stool frequency and consistency were similar between groups. More stools were reported to be difficult to pass in the control group than the test group (3% test and 21% control; p = 0.04), but the number of infants reported as having difficult to pass stools was not significantly different (13% in test versus 29% in control; p = 0.14). No significant differences in duration of crying and fussing or vomiting frequency were observed. The proportion of babies reported to have any spit-up was not significantly different between groups. Among babies with reported spit-up, significantly more in the test group had > 5 spit-ups per day compared with controls. Spitting up was not considered to be an issue for either formula, however, as only one subject in each group had spit-up reported as an adverse event and in both cases it

was classed as ‘mild’. No serious adverse events were reported, and the overall incidence of adverse events was similar between groups. Subjects in the control group had a significantly higher incidence of reported infections and infestations than the test group (23% versus 8%; p = 0.05), but the authors suggested that this finding should be interpreted with caution due to the small number of cases reported (9 versus 3, respectively).

The authors concluded that the partially hydrolysed formula supplemented with 0.25 g/L 2’-FL was well tolerated.

Clinical feeding experience of infants fed a partially hydrolysed whey-based formula with 2’-FL (Reverri et al. 2019) Regulatory status: Non-GLP

The review of clinical studies with infant formula containing 2’-FL by Reverri et al. (2019) also includes details of an unpublished clinical feeding experience study conducted to assess the effects of switching to a partially hydrolysed whey-based formula (PHF) supplemented with 2’-FL on symptoms of formula intolerance.

The prospective, multi-centre, single arm study investigated healthy term formula-fed infants given a low lactose (<2 g/L) PHF containing 0.2 g/L 2’-FL and 1.8 g/L scFOS (Abbott Nutrition, Columbus, OH, USA). At enrolment, parents completed a baseline tolerance assessment questionnaire that assessed infants’ symptoms of formula intolerance over the previous three days. Infants were eligible for inclusion in the study if they were identified by parents as ‘very fussy’ or ‘extremely fussy’. Infants (aged 7 – 42 days at study entry; mean age ± SEM 25.7 ± 1.4 days) consumed the PHF with 2’-FL as their sole source of nutrition *ad libitum* for 28 days. Symptoms of formula intolerance were assessed by parents in a daily diary that included the severity of fussiness and gassiness, number of spit-ups associated with feeding and hours of crying. Study visits took place at enrolment and on study days 7 and 28. The primary variable was change in fussiness severity from baseline compared to the first full day of study formula feeding. Secondary variables were change in gassiness severity, number of spit-ups and hours of crying; anthropometric measurements and adverse events were also assessed.

In total 59 infants were enrolled in the study, 47 of whom were evaluable on study day 1 and 32 on day 28. After one day of consuming the PHF containing 2’-FL, 63.8% of infants showed an improvement in fussiness symptoms. The median reduction in the severity of fussiness was statistically significant after one day ($p < 0.0001$) and severity of fussiness continued to decrease throughout the duration of the study. Significant improvements in the severity of gassiness, number of spit-ups and hours of crying were also observed after one day, and these improvements were maintained over the course of the study. Infants had significantly fewer colicky symptoms (the combination of fussiness, gassiness and crying) in one day after initiation of feeding (median 22% fewer; $p < 0.0001$), and 30%, 33% and 40% fewer colicky symptoms after two, seven and 28 days, respectively. The authors stated that growth (weight and length) was normal, although specific values were not reported. Adverse event reports were noted to show no safety concerns, although further details were not provided.

It was concluded that the PHF containing 0.2 g/L 2’-FL and 1.8 g/L scFOS was safe and well tolerated by healthy term, fussy infants.

Clinical studies with adults

Study of 2’-FL and LNnT in adults with irritable bowel syndrome (Palsson et al. 2020)

A conference abstract provides limited details of a multi-centre, open label trial in adult patients with irritable bowel syndrome (IBS) designed to determine whether supplementation

with a combination of 2'-FL and LNnT may improve bowel symptoms. A total of 317 subjects (70.7% females; mean age 44.0 years, range 18-93 years) recruited from 17 sites around the US were given 5 g of a 4:1 mixture of 2'-FL and LNnT daily by mouth for 12 weeks. Bowel habits, IBS symptoms and quality of life were assessed at baseline and every 4 weeks during the intervention.

The full 12 week intervention was completed by 245 patients. Intention-to-Treat analysis found a significant reduction in abnormal bowel movements and IBS symptom severity compared with baseline during the intervention period. A significant increase in health-related quality of life scores was also observed. The study product was reported to be well tolerated in most subjects, and the only common side effects were mild gastrointestinal symptoms such as abdominal discomfort, distention and flatulence.

3. Effects on infant and toddler growth

3.1 Previous FSANZ evaluation

As part of the *safety, technical and health effects assessment* for Application A1155, FSANZ considered the evidence provided by the Applicant and undertook a literature search in Pubmed to identify additional relevant studies. One cohort study in breastfed infants and four clinical trials of infant formula were reviewed (FSANZ 2019).

Cohort study

An exploratory study by Sprenger et al. (2019) investigated the effect of human milk HMO concentration including 2'-FL, LNnT, and three other HMOs on the growth of fifty breastfed Singaporean infants. Anthropometric data were collected at birth, 30, 60, and 120 days postpartum and milk samples collected at 30, 60, and 120 days after birth. 2'-FL secretor status was determined from the 30 day milk samples. Mean length, weight, head circumference and body mass index by maternal secretor status were plotted on the sex-specific WHO growth charts and were not significantly different from chart median values at birth or any other time points.

Clinical studies

Puccio et al. (2017) compared the effect of formula containing 2'-FL (1.0 -1.2 g/L) and LNnT (0.5 - 0.6 g/L) to control formula without oligosaccharides on infant weight gain (g/day) in a randomised controlled trial in 175 infants over 4 months. The study was powered to detect a 3 g/week difference in weight between groups. Weight gain was not significantly different between groups.

Marriage et al. (2015) examined the growth of infants that consumed formula containing two oligosaccharides (test formula 1: 0.2 g/L 2′-FL and 2.2 g/L GOS; test formula 2: 1.0 g/L 2′-FL and 1.4 g/L GOS) compared to formula containing 2.4 g/L GOS in a randomised controlled study that was also powered to detect a 3 g/day or greater difference in body weight. No significant differences between groups was observed for weight, length, or head circumference over the four month study period.

Two additional studies that were assessed by FSANZ were not used in the body of evidence. A clinical trial undertaken by Kajzer et al. (2016) studied the effect of formula containing 2'-FL (0.2 g/L) and FOS (2 g/L) to control formula without oligosachharides on infant growth until 35 days postpartum. The authors reported that no significant difference in anthropometric data between groups was observed however further details were not provided. FSANZ considered that the study was not of adequate duration to determine changes in body weight

or other anthropometric measurements. Additional details of the study were described in a recent publication (Reverri et al. 2018) however information relating to weight gain were not provided. A study by Prieto et al. (2005) investigated the effects of formula containing LNnT on babies age 6 - 24 months however insufficient details were provided in the publication to include in the body of evidence.

As discussed in Section 2.3.1 HMOs are not hydrolysed by digestive enzymes *in vitro* and data indicate that a large proportion of HMOs remain intact until they reach the large intestine where they are fermented by intestinal microbiota or excreted.

FSANZ concluded from the available evidence that no adverse effects on infant growth are expected at concentrations requested by the Applicant.

3.2 Additional studies on infant and toddler growth

As part of the review, a literature search was conducted in PubMed on 16 March 2020 to identify additional studies using search terms described in Appendix 1. Two relevant cohort studies and one clinical trial were identified (Larsson et al. 2019; Storm et al. 2019; Lagström et al. 2020).

Larsson et al. (2019) undertook an exploratory study that compared the HMO composition of human milk samples from 13 exclusively breastfed infants with high weight (HW) gain with 17 exclusively breastfed infants with normal weight (NW) gain. The HW cohort included infants with a weight-for-age-Z-score (WAZ) >2 and at least 1 standard deviation (SD) increment in WAZ during the first 5 months post-partum and the NW group had a WAZ of -1 to +1. HMO analysis was undertaken at 5 - 6½ and 9 months and maternal secretor status was determined based on the presence or near absence of 2'-FL and lacto-N-fucopentaose (LNFP I).

Of the 21 HMOs analysed, the concentration of 4 HMOs were significantly different in the HW secretor compared to the NW secretor groups at 5 months and two were significantly different at 9 months. LNnT concentration was significantly lower in the HW secretor group than the NW secretor group at 5 and 9 months (median 592 nmol/mL vs 817 nmol/mL, p = 0.012 and 424 nmol/mL vs 736 nmol/mL, p = 0.049 respectively); difucosyl-lacto-N-hexaose (DFLNH) concentration was significantly lower in the HW group at 5 months (15 vs 25 nmol/mL, p = 0.045) and difucosyllactose (DFLac) was higher in the HW group at 5 months (888 vs 781 nmol/mL, p = 0.045). HMO-bound fucose was significantly higher at 5 and 9 months (16580 vs 14981, nmol/mL, p = 0.033, and 18115 vs 14994 nmol/mL, p = 0.049 respectively).

Associations between HMO content, anthropometry at 5 months and weight velocity from birth to 5 months were analysed by Spearman's correlation. 2'-FL was positively associated with weight velocity (g/week) from 0 - 5 months (Rho = 0.5, p = 0.015) and fat mass index (FMI, kg/m^2) (Rho = 0.468, p = 0.024) at 5 months in secretor mothers. LNnT was negatively associated with weight velocity (Rho = -0.531, p = 0.009) and FMI (Rho = -0.447, p = 0.033) at 5 months. In addition, DFLNH was negatively associated with weight velocity from 0 - 5 months (Rho = -0.502, p = 0.015) and FMI at 5 months (Rho = -0.434 p = 0.039). DFLac was positively associated with weight velocity (Rho = 0.417, p = 0.048) and fucosyl-disalyl-lacto-N-hexaose (FDSLNH) was negatively associated with fat free mass index (Rho = -0.424, p = 0.044). HMO-bound fucose was positively associated with weight velocity (Rho = 0.565, p = 0.005) and FMI (Rho = 0.489, p = 0.018). Total HMO was positively associated with weight velocity (Rho = 0.496, p = 0.016) and FMI (Rho = 0.466, p = 0.02).

The study authors noted several limitations in the study including small sample size that limited the power to detect differences between groups, and a lack of adjustment for

confounding factors due to the small sample size. In addition, analysis did not commence until 5 - 6 ½ months by which time growth velocity had decreased and some infants had commenced complementary feeding. The mean duration of exclusive breastfeeding was reported as 5.14 months and 5.54 months for the HW and NW groups respectively. Adjustment could not be made for other milk components including fat, lactose and protein due to the small sample size.

Lagström et al. (2020) investigated the association between human milk oligosaccharide composition and child growth up to age five. Eight hundred and two mother and baby pairs from a Southwest Finland longitudinal cohort were included in this study. Anthropometric data of infants were recorded at 3, 6, and 8 months and at 1, 2, 3, 4, and 5 years of age and human milk samples obtained at 3 months were analysed for HMO content and maternal 2'-FL secretor status. Birth weight-Z-scores were calculated using reference values for the Finnish population. Statistical analysis was undertaken using a hierarchical linear mixed model for repeated measurements of height and weight-Z-scores to model their associations with HMO concentrations and were adjusted for mode of delivery, sex, birth weight-Z-score, maternal pre-pregnancy BMI, HMO, and HMO * time interaction as explanatory factors. The derived variable of the logarithm of 2'-FL to the logarithm of LNnT ratio was examined using a hierarchical linear mixed model for repeated measurements.

Of the study population, 699 mothers (87.2%) were secretors and 103 were non-secretors (12.8%). After adjusting for other explanatory factors, statistically significant associations were found between the concentration of several HMOs and weight-Z-scores in children age 3 -12 months with secretor mothers; with partial regression coefficient β for 2'-FL: +0.21, [95% confidence interval (CI): 0.03, 0.39], p = 0.02; LNnT: β = -0.225, [95% CI: -0.39, 0.06], p = 0.007; HMO-bound fucose: β = +0.837, [95% CI: 0.28, 1.39], p = 0.003; 3-FL: β = +0.182, [95% CI: 0.04, 0.32], p = 0.012; 3'-SL: β = +0.161, [95% CI: 0.03, 0.29], p = 0.017; DFLac: β = +0.154, [95% CI: 0.02, 0.29], p = 0.028; and LSTb: β = -0.149, [95% CI: -0.27, -0.03], p = 0.017.

6'SL was negatively associated with weight-Z-scores in this age group in non-secretor mothers (β = -0.375, [95% CI: -0.61, -0.14], p = 0.002). LNnT was negatively associated with weight-Z-scores (β = -0.213, [95% CI: -0.38, -0.04], p = 0.014) and three HMOs were positively associated with weight-Z-scores in children age 1 - 5 years in secretor mothers: HMO-bound fucose (β = +0.610, [95% CI: 0.04,1.18] p = 0.037), 3-FL (β = +0.162, [95% CI: 0.01, 0.31], p = 0.032) and 3'-SL (β = +0.153, [95% CI: 0.02, 0.29], p = 0.028).

Total HMO concentration was positively associated with weight-Z-scores in secretor mothers in children age 3-12 months (β = +0.901, [95% CI: 0.03, 1.77], p = 0.042) but negatively associated in non-secretor mothers (β = -3.086, [95% CI: -5.82, -0.35], p = 0.027). HMO diversity was negatively associated with weight-Z-scores in children age 3 - 12 months with secretor mothers (β = 0.048, [95% CI: -0.09, -0.01], p = 0.017). 2'FL was positively associated with height-Z-score in secretor mothers age 3 -12 months (β = 0.229, [95% CI: -0.042, 0.416], p = 0.016) and LNnT was negatively associated with height-Z-score in secretor mothers age 3 -12 months (β = -0.255, [95% CI: -0.428, -0.082], p = 0.004). and 1- 5 years (β = -0.247, [95% CI: -0.432, -0.062], p = 0.009). HMO-bound fucose was positively associated with height-Z-score in secretor mothers age 3-12 months (β = 0.788, [95% CI: 0.199, 1.376], p = 0.009). and 1- 5 years (β = 0.707, [95% CI: 0.078, 1.337], p = 0.028).The 2'-FL/LNnT ratio was associated with weight and height-Z-scores in children of secretor mothers age 3 month to 5 years.

The authors noted several limitations of the study, including the use of a single human milk sample at 3 months (compositional changes occur during the course of lactation). Not all infants were exclusively breastfed at the time of sampling and the duration of breastfeeding varied between participants, with a median breastfeeding time of 10 months. Analysis of the

macronutrient composition of the human milk samples was not undertaken. Most statistically significant results were in secretor mothers, but this may be due to the larger size of this cohort. The authors noted that no conclusions on causation of HMO profiles and growth patterns can be drawn from the observational study and would require testing as part of an intervention study.

Clinical trials

A study by Storm et al. 2019 compared the tolerance of partially hydrolysed whey protein infant formula containing 2'-FL (0.25 g/L) and *Bacillus lactis* with a control formula that did not contain 2'-FL. The study had a randomised, double blinded, parallel, multicentre design and was conducted at 7 sites in the United States. Subjects were enrolled at 14 ± 5 days of age, weighed and randomised to receive either the test or control formula. Infants were formula fed *ad libitum* until day 42. Further details of the study are provided in Section 2.1.3 Human studies - *Clinical studies with infants.*

The primary objective of the study was to assess the tolerance of the formula containing 2'-FL using a validated tool of feeding tolerance measured by stool patterns, frequency of spitting up, degree of flatulence and general demeanour. Secondary outcomes that included stool frequency, consistency and ease of passing stools and weight gain were analysed using the intention to treat (ITT) cohort that included 38 subjects in the test group and 40 participants in the control group. Weight-for-age and length-for-age percentiles were calculated for the ITT population using the World Health Organisation growth charts.

The average volume of formula consumed was similar between groups, 0.7 kg (± 0.39 kg) in the ITT test formula group and 0.68 kg (± 0.34 kg) in the control formula group. A significant difference was not observed between either the ITT or PP groups for infant gastrointestinal symptoms score on Day 42 (ITT: 22.5 ± 6.4 vs 20.8 ± 4.5, p = 0.19; PP 20.9 ± 4.8 vs 20.7 ± 4.3, p = 0.82). Weight-for-age percentile was similar for test and control groups: 43.5 ± 26.2 at enrolment and 45.9 ± 26.3 after 6 weeks for the test group compared to 43.3 ± 21.0 at enrolment and 42.8 ± 25.4 after 6 weeks for the control group, however statistical analysis was not undertaken. Length-for-age percentile was similar for test and control groups: 44.7 ± 28.5 at enrolment and 53.9 ± 33.9 after 6 weeks for the test group compared to 45.0 ± 29.8 at enrolment and 53.4 ± 33.2 after 6 weeks

Some limitations of the study were noted including the short duration, the concentration of 2'-FL being lower than the amount requested in the application (0.25 g/L vs 1.2 g/L) and the lack of statistical analysis of the effect of test formula on weight. Analysis of the effect on weight gain in the PP population would be useful for comparison as 21% of participants did not adhere to the study protocol.

FSANZ become aware of an additional clinical trial in infants that was published after the updated literature search was conducted. This study, summarised in section 2.1.3, reported no differences in infant growth between infants exclusively fed test formula containing added 2'-FL and LNnT, infants fed a mixture of test formula and human milk, and infants who were exclusively breastfed. However, this was a non-randomised, open label study, and the test formula also contained *Lactobacillus reuteri* (Román et al. 2020).

4. Discussion and conclusions

Pharmacokinetic, toxicological and clinical studies on 2'-FL and LNnT submitted by the applicant or published since FSANZ's earlier assessment did not indicate a need to amend the conclusions of the previous evaluation.

Additional information on the pharmacokinetics of HMOs confirms that small amounts of these substances are absorbed following oral intake and excreted intact in the urine. The majority of HMOs are not absorbed in the small intestine but pass to the large intestine, where they are metabolised by intestinal bacterial enzymes or excreted unchanged in the faeces.

Recently published studies found the presence of HMOs including 2'-FL in amniotic fluid and umbilical cord blood collected at birth, indicating that infants are likely to already be exposed to HMOs during development *in utero*.

New genotoxicity studies with 2'-FL in combination with other oligosaccharides identical to those in human milk confirmed the absence of mutagenicity, clastogenicity and aneugenicity. A 90 day oral toxicity study in neonatal rats with an 8:1 mixture of 2'-FL and DFL found no adverse effects at doses up to 5000 mg/kg bw/day, or 4444 mg/kg bw/day 2'-FL_{micro}. A 90 day dietary toxicity study in rats with 2'-FL_{micro} in combination with four other HMOs at 10% in the diet (equal to 2670 and 3280 mg/kg bw/day 2′-FL in males and females, respectively) also found no adverse effects.

Newly available information from three human clinical studies with healthy infants found that formula containing 2'-FL is well tolerated with no indications of adverse effects, consistent with the findings of previously reviewed studies. A real-world evidence study of infants fed formula containing 2'-FL (1.0 g/L) and LNnT (0.5 g/L) also found the formula was well tolerated.

2'-FL_{micro} and $LNnT_{micro}$ do not contain detectable proteins and are therefore 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.

Since the proposed maximum concentrations of 2'-FL and LNnT (2.4 and 0.6 g/L, respectively) are within the range of naturally occurring levels in human milk (1.0 – 7.8 g/L and 0.04 – 1.08 g/L, respectively [FSANZ 2019]), there are no safety concerns associated with the addition of 2′-FL, alone or in combination with LNnT, to infant formula products and formulated supplementary foods for young children (FSFYC).

Three relevant studies on infant growth were published since the initial assessment of evidence. One cohort study found an association between HMO concentration and weight Z score of infants aged 3 - 12 months, however in addition to the design limitations described by the author, observational cohort studies are a weak source of evidence for the effect of individual HMOs on growth and development due to the difficulty in controlling for other factors. One clinical study found that weight-for-age percentile was similar for both groups.

Following the assessment of recent evidence FSANZ maintains the conclusion that based on the available evidence and limited absorption of HMOs the addition of 2'-FL and LNnT to formula at levels normally found in human milk should not affect growth.

FSANZ concludes there are no public health and safety concerns associated with the addition of 2′-FL alone or in combination with LNnT to infant formula products and FSFYC at the proposed levels.

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Appendix 1: Search strategy for infant and toddler growth assessment

Human milk oligosaccharide or carbohydrate or 2’-FL or 2’-fucosyllactose or LNnT or lacto-N-neo-tetraose
AND
Milk or breast or formula
AND
Anthropometric or weight or growth or development
AND
Child or infant or baby or maternal
AND
Year 2018 to 2020

Best matches for (((((((child[Title/Abstract]) OR infant[Title/Abstract]) OR baby[Title/Abstract]) OR maternal[Title/Abstract]) AND ("2017/12/01"[PDat] : "2020/12/31"[PDat]))) AND (((((Anthropometric[Title/Abstract]) OR weight[Title/Abstract]) OR growth[Title/Abstract]) OR development[Title/Abstract]) AND ("2017/12/01"[PDat] : "2020/12/31"[PDat]))) AND (((((milk[Title/Abstract]) OR breast*[Title/Abstract]) OR formula[Title/Abstract]) AND ("2017/12/01"[PDat] : "2020/12/31"[PDat]))) AND (((((((human milk oligosaccharide[Title/Abstract]) OR carbohydrate[Title/Abstract]) OR 2’-FL[Title/Abstract]) OR 2’-fucosyllactose[Title/Abstract]) OR LNnT[Title/Abstract]) OR lacto-N-neo-tetraose[Title/Abstract]) AND ("2017/12/01"[PDat] : "2020/12/31"[PDat])):

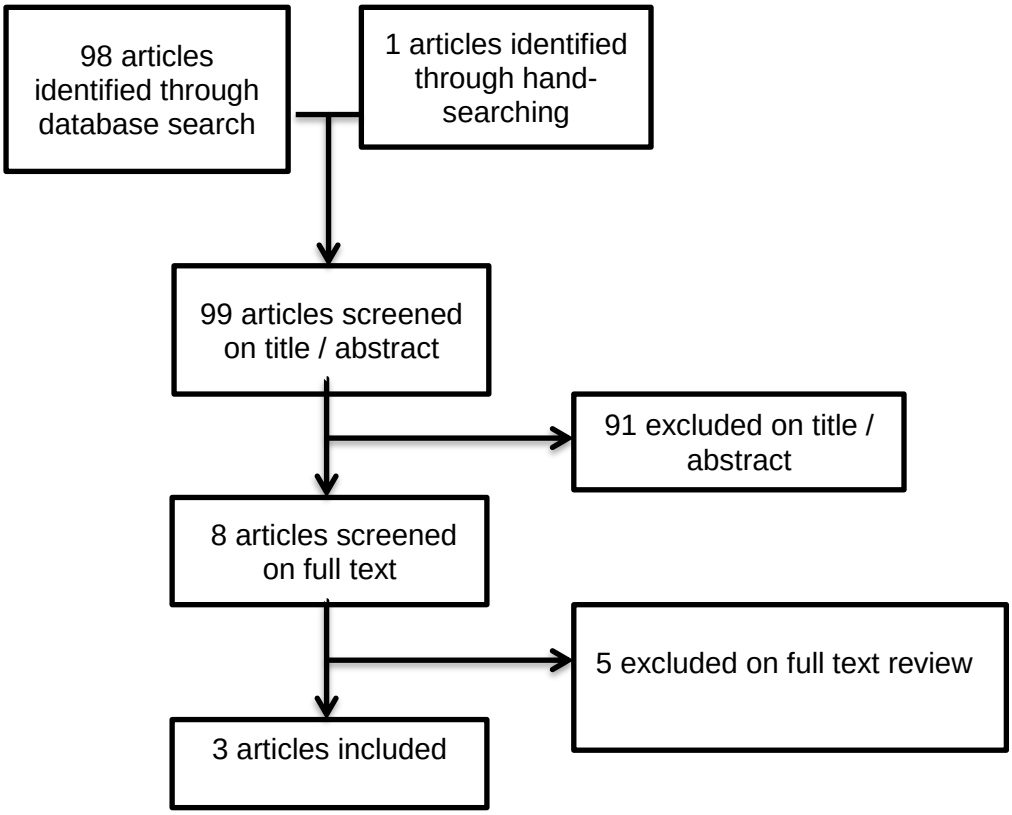

Figure A1 PRISMA diagram of study identification for review

Table A1: Clinical trials in infants and toddlers using formula containing 2'-FL and/or LNnT

Reference Country where study conducted	Age at recruitment ; duration of study	N randomised (control, intervention) N followed-up	Added oligo-saccharide content of control formula (g/L)	Concentration in tested infant formula		Power/ sample size	Difference in weight vs control
				2′-FL (g/L)	LNnT (g/L)		
Kajzer et al. (2016) (Abstract only) US	0-8 days; Followed until 35 days of age	42, 46 N followed up: 36,41 Also 43 (42 followed-up) in a non-randomised breastfed comparison group	0	0.2 plus 2 g/L scFOS	0	Not reported	Abstract stated "there were no differences between groups for anthropometric data"
Marriage et al. (2015) US	0-5 days; Followed until 119 days of age	101, 104 (Formula 1), 109 (Formula 2) N followed up: 84, 81, 83 Also 106 in a non-randomised breastfed comparison group (90 followed-up)	2.4 g/L GOS	Test formula 1: 0.2 plus 2.2 g/L GOS	0	To detect a 3 g/day difference or greater using a 2-sided test	0.2 g/day less in boys; 1 g/day less in girls (not an intention-to-treat analysis)
				Test formula 2: 1.0 plus 1.4 g/L GOS	0		1 g/day more in boys, 0.3 g/day less in girls (not an intention-to-treat analysis)
Puccio et al. 2017 Belgium and Italy	0-14 days; Followed until 4 months of age	87, 88 N followed-up: 58, 64 although an intention-to-treat (ITT) analysis was done	0	1.0-1.2	0.5-0.6	To detect -3 g/day difference or greater using a 1-sided test	Intervention group gained 0.13 g/day less weight (95% CI: -1.63 to 1.37) i.e. 13 g over 100 days (ITT analysis)

Reference Country where study conducted	Age at recruitment ; duration of study	N randomised (control, intervention) N followed-up	Added oligo-saccharide content of control formula (g/L)	Concentration in tested infant formula		Power/ sample size	Difference in weight vs control
				2′-FL (g/L)	LNnT (g/L)		
Prieto 2005 Chile	6-24 months; Duration 16 weeks	113, 115 N followed-up: 101, 102	0	0	0.2	Not reported	LNnT group gained 0.07 g less in weight and 0.37 cm less in length than control group over the 16 weeks
Storm et al. 2019 US	14 ± 5 days; Followed until day 42	40, 38 N followed up: 33, 30	0	0.25	0	Powered to investigate formula tolerance, not weight difference	“Weights and lengths were similar between groups after 6 weeks” Weight for age percentile: Control: 42.8 ± 25.4 Test: 45.9 ± 26.3 (p-value not reported)