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

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## Supporting document 1

Risk and technical assessment – Application A1277

A1277 - 2′-FL from GM *Escherichia coli* K-12 (gene donor: *Helicobacter enhydrae*) in infant formula products

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# Executive summary

FSANZ has assessed an application from Inbiose N.V. to amend the Australia New Zealand Food Standards Code (the Code) to permit a new source organism for the production of 2′-fucosyllactose (2′-FL), a human milk oligosaccharide. The applicant's 2′-FL is produced by microbial fermentation using a genetically modified (GM) strain of *Escherichia coli* (*E. coli*) K-12.

The Code already permits 2′-FL from different source organisms for addition to infant formula products. The maximum permitted level is 96 mg/100 kJ, equivalent to 2.4 g/L. FSANZ has previously determined that there are no safety concerns associated with the addition of 2′-FL to infant formula products at concentrations up to 2.4 g/L. The primary purpose of the present assessment is therefore to assess the safety of 2′-FL produced by the new production strain.

The applicant's 2′-FL is chemically and structurally identical to the naturally occurring substance present in human milk. It is also chemically and structurally identical to 2′-FL previously assessed and permitted by FSANZ, therefore does not raise any safety concerns.

The *E. coli* K-12 host organism has a long history of use for the production of recombinant proteins and other products, and poses no risks to humans. No safety concerns arising from the gene donors were identified. Characterisation of the GM production strain confirmed that all introduced genes were both genetically stable and functional.

On the basis of the data provided, no potential safety concerns were identified in the assessment of the 2′-FL production strain *E. coli* K-12. Based on previous FSANZ assessments of 2′-FL and the toxicological assessment in the present application, it was concluded that there are no public health and safety concerns associated with 2′-FL produced from the new GM source organism that is the subject of this application.

The nutrition assessment concluded that, based on the available evidence, the addition of 2′-FL to infant formula products is unlikely to pose a risk to the normal growth of infants.

Based on these previous microbiological assessments, given the identical chemical structure and that the applicant has not requested any change in the maximum permitted level of 2′-FL added to infant formula products, the associated health benefits from the addition of 2′-FL to infant formula products for infants remain the same: (1) an anti-pathogenic effect; (2)

immunomodulation and (3) development of the gut microbiome through supporting growth of *Bifidobacteria* spp.

Overall the safety assessment concluded there are no public health and safety concerns associated with the addition of 2′-FL synthesised from a new source organism to infant formula products at the proposed use levels.

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# 1 Introduction

FSANZ received an application from Inbiose N.V to amend the Australia New Zealand Food Standards Code (the Code) to permit a new source organism for the production of 2′-fucosyllactose (2′-FL). The applicant's 2′-FL is produced by microbial fermentation using a genetically modified (GM) strain of *Escherichia coli* (*E. coli*) K-12.

Schedule 26 of the Code already permits 2′-FL from several source organisms for addition to infant formula products (*E. coli* K-12 containing the gene for alpha-1,2-fucosyltransferase from *Helicobacter pylori*; *E. coli* BL21 containing the gene for alpha-1,2-fucosyltransferase from *E. coli* O126; *E. coli* K-12 containing the gene for alpha-1,2-fucosyltransferase from *Bacteroides vulgatus*). The maximum permitted level of 2′-FL in infant formula products is 96 mg/100 kJ, equivalent to 2.4 g/L. The purpose of the present assessment is therefore to assess the safety of 2′-FL produced by the new production strain.

# 2 Food technology assessment

The food technology assessment provides information on chemical identification, physicochemical properties and specifications for the oligosaccharide proposed to be added to infant formula products. The assessment primarily aimed to address whether the microbiologically-synthesised 2′-FL proposed to be added to infant formula products is identical to that present in human milk. The assessment also considered the manufacturing process and the validity of analytical methods used to quantify and characterise 2′-FL during production and as a component of infant formula products.

FSANZ has assessed a number of recent applications requesting permissions for Human identical Milk Oligosaccharides (HiMO) in food. The information in this section has built on the reports written for the assessment of those applications. 2′-FL has been assessed in Applications A1155, A1190, A1233, A1251 and A1265 (FSANZ 2019b; FSANZ 2021; FSANZ 2022a; FSANZ 2022b; FSANZ 2023). Application A1155 assessed permitting both 2′-FL and lacto-N-neotetraose (LNnT, a constitutional isomer of LNT) in infant formulas and other products. Application A1265 assessed a blend of four HiMO products, being 2′-FL and difucosyllactose (DFL) referred as 2′-FL/DFL; lacto-*N*-tetraose (LNT); 6′-sialyllactose sodium salt (6′-SL) and 3′-siallyllactose sodium salt (3′-SL).

## 2.1 Chemical and physical properties

2′-FL is a component of the human milk oligosaccharide (HMO) fraction of human milk. The applicant produces its 2′-FL via microbial fermentation using a GM strain of *E. coli* K-12, which is detailed in section 3.1.

The chemical name and properties of 2′-FL that is requested to be permitted is provided in Table 1 with information as provided in the application and claimed by the applicant to be chemically and structurally identical to the 2′-FL of previously FSANZ approved and permitted applications.

2′-FL is an oligosaccharide that contains the sugar fucose (a hexose deoxy sugar with the chemical formula $C_6H_{12}O_5$) and so is called a 'fucosylated' HMO. 2′-FL is a trisaccharide consisting of the monosaccharides L-fucose, D-galactose and D-glucose. It can also be described as the monosaccharide L-fucose, and the disaccharide D-lactose, connected by an alpha (1→2) glycosidic linkage (Figure 1).

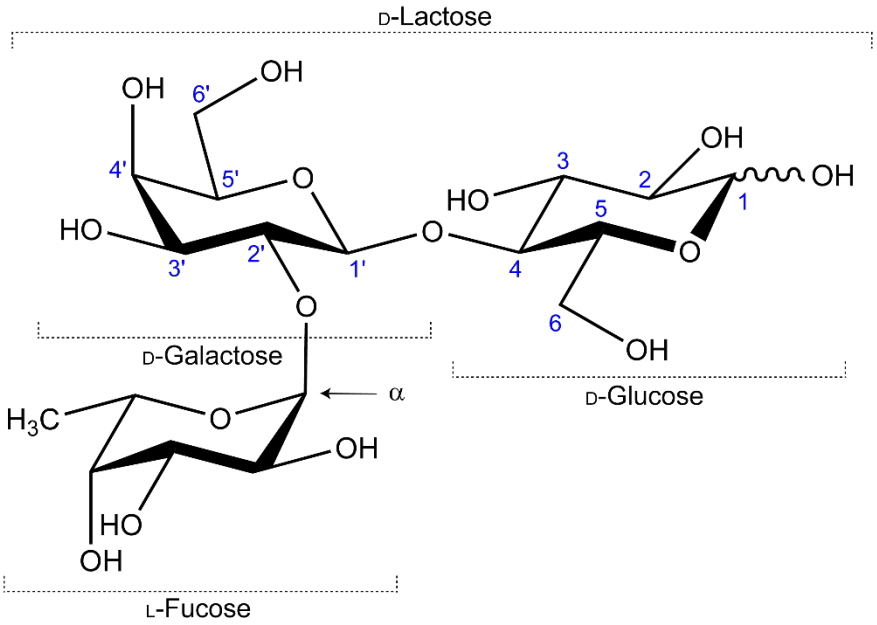


**Figure 1** *Molecular structure of 2'-FL.*

2′-FL is a white to off-white homogeneous powder that is readily soluble in aqueous solutions. It is poorly soluble in organic solvents.

**Table 1** *The nomenclature and chemical properties of 2'-FL.*

| Property | 2′-FL |
|---|---|
| Common name | 2′-fucosyllactose |
| IUBMB[1] Chemical name | α-L-fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-D-glucopyranose |
| Alternative common names | 2′-O-fucosyllactose<br>2′-O-L-fucosyl-D-lactose<br>2′-fucosyl-D-lactose<br>2′-FL |
| Alternative names[a] | fucosyl-α-1,2-galactosyl-β-1,4-glucose<br>α-L-Fuc-(1→2)-β-D-Gal-(1→4)-D-Glc |
| IUPAC[2] abbreviation[a] | Fuc-α-(1→2)-Gal-β-(1→4)-Glc |
| CAS[3] registry number | 41263-94-9 |
| Chemical formula | $C_{18}H_{32}O_{15}$ |
| Molecular weight | 488.44 g/mol |

[a] Fuc = fucose or fucosylpyranose; Gal = galactose or galactosylpyranose; Glc = glucose or glucosylpyranose

[1] The International Union of Biochemistry and Molecular Biology
[2] The International Union of Pure and Applied Chemistry
[3] Chemical Abstract Service

### 2.1.1 Chemical and structural equivalence of 2′-FL

The application included analytical data (Confidential Commercial Information) to support its claim that 2′-FL produced using its microbial fermentation process is chemically and structurally identical to the substance naturally present in human milk as the reference standard. The analytical methods provided used one dimensional $^{1}H$ and two dimensional $^{1}H$-$^{13}C$ HSQC (Heteronuclear Single Quantum Coherence) nuclear magnetic resonance (NMR) spectroscopy. FSANZ assessed the information provided and agreed with the applicant's conclusions that the spectral analysis confirms that the microbially produced substance has the same stereochemical configuration and three-dimensional structure as those naturally occurring in human milk.

Analyses were also provided on the applicant's 2′-FL compared to 2′-FL FSANZ had assessed and approved from earlier applications as well as the current specification for 2′-FL in S3—40 (specification for 2′-fucosyllactose sourced from *E. coli* K-12) within the Code. FSANZ assessed these summary results as well as the analytical results of three non-consequential batches of 2′-FL and agreed with the applicant's claim that their substance is chemically identical and at least as pure as such currently permitted forms and complies with S3—40. FSANZ also checked the applicant's analytical results compared to another specification for 2′-FL sourced from another microorganism being S3—45 (specification for 2′-fucosyllactose sourced from *E. coli* BL21) and confirmed it also meets the purity criteria.

In summary, FSANZ agrees with the applicant that its 2′-FL is chemically and structurally identical to 2′-FL already assessed and permitted by FSANZ from earlier applications.

### 2.1.2 Stability of 2′-FL under conditions of use

The applicant addressed the issue of stability of its 2′-FL by referring to stability results provided by other applications (A1155 and A1190, FSANZ 2019 and FSANZ 2021 respectively). The justification for this approach is that the chemical and structural results have confirmed that its product is identical to 2′-FL produced either chemically synthesised or by microbial fermentation and to those already assessed and permitted by FSANZ from earlier applications. FSANZ agrees with this conclusion as noted in the above section so this approach is accepted.

As summarised within the SD1 for the 2nd Call for Submissions (CFS) for A1155 the chemically synthesised 2′-FL powder by itself is stable for 60 months (5 years) when stored at 25°C and 60% relative humidity and 24 months at accelerated conditions of 40°C at 80% relative humidity (FSANZ 2019a).

Results provided also in A1155 indicated that the 2′-FL added to a powdered preparation to model a commercial infant formula powder product stored in gassed (nitrogen/carbon dioxide) cans stored at 4-37°C were stable up to 900 days.

FSANZ also assessed the stability of 2′-FL for another recent application, Application A1233 (FSANZ 2022a) which had very similar results.

The application also referred to various stability trials provided within various US Food and Drug Administration (FDA) Generally Recognized as Safe (GRAS) documents, which provided additional supporting evidence for the stability of 2′-FL. These are GRN 546 (FDA 2014), GRN 571 (FDA 2015), GRN 735 (FDA 2018a) and GRN 749 (FDA 2018b).

## 2.2 Manufacturing processes

The method of production for the applicant's 2′-FL is the same as that of earlier applications

so it is not reported in detail in this report. The production process for both 2′-FL and LNnT is summarised within SD1 of the 2nd CFS for A1155 (FSANZ 2019a).

2′-FL is produced by a microbial fermentation process using a modified strain of *E. coli* K-12. The production process is conducted in two stages: upstream processing (USP) and downstream processing (DSP). The USP can be considered the fermentation steps while the DSP captures the purification, isolation and concentrations steps.

## 2.3 Specifications

As noted in Section 2.1.1, the applicant's 2′-FL meets the requirements of the current specification for 2′-FL sourced from *E. coli* K-12, i.e. S3—40. It also meets the purity requirements for 2′-FL sourced from another microorganism, being S3—45.

### 2.3.1 Impurities

The levels of impurities of the applicant's 2′-FL comply with the S3—40 specification and are consistent with comparable approved 2′-FL.

The applicant's own specification and analytical results provided in the application indicate that its 2′-FL also meets the requirements of S3—4 related to contamination limits for lead, arsenic, cadmium and mercury.

The application contained information relating to possible impurities in the final purified 2′-FL including the residual starting materials, D-lactose and L-fucose as well as manufacturing by-products such as difucosyllactose and 2′-fucosyl-D-lactulose. Again, these are consistent with the current specification S3—40. Lactose, fucose and difucosyllactose are natural components of human milk.

The production microorganism is removed during the processing and purifications steps during production of 2′-FL. Qualitative polymerase chain reaction (PCR) methods were used to confirm that no residual DNA from the production microorganism remains in the final purified nutritive substance.

## 2.4 Analytical methods for detection

The applicant has in-house analytical methods for detecting and quantifying the presence of its 2′-FL. The analytical method uses Ultra-High Performance Liquid Chromatography coupled with Refractive Index detector (UHPLC-RI).

## 2.5 Food technology conclusion

FSANZ concludes from its assessment of the data provided in the application that the applicant's 2′-FL produced by a microbial fermentation method of production are chemically and structurally identical to naturally occurring substances in human milk. It is also chemically identical to 2′-FL already assessed and permitted by FSANZ.

The applicant's 2′-FL meets the requirements of the current specification for 2′-FL sourced from *E. coli* K-12, i.e. S3—40.

Stability studies of 2′-FL provided on identical products concluded that the nutritive substance as a powder is stable for 60 months (5 years) when stored at 25°C and 60% relative humidity and 24 months at accelerated conditions of 40°C at 80% relative humidity. When 2′-FL was added to a powdered preparation to model a commercial infant formula powder product

stored in gassed (nitrogen/carbon dioxide) cans stored at 4-37°C it was stable up to 900 days.

# 3 Safety assessment

## 3.1 GM production strain assessment

### 3.1.1 Host organism

*E. coli* K-12 is one of the most common bacterial laboratory strains. It was isolated from a patient stool sample in 1922 in California (Bachmann 1996). Comparative genome sequencing and proteomic analysis of the K-12 strain and its derivatives, to the well-characterised pathogenic strains, have identified differences in the K-12 cell wall structure associated with a reduced ability to colonise the human intestinal tract, as well as the absence of specific adhesive proteins and virulence factors associated with pathogenicity (Bachmann 1996; EPA 1997; Sahl et al. 2013). These studies have also shown reduced toxin production in K-12 strains and the absence of plasmids encoding antibiotic resistance. Under the U.S. National Institutes of Health (NIH) Research Involving Recombinant or Synthetic Nucleic Acid Molecules (NIH Guidelines, 2019), *E. coli* K-12 is classified as a Risk Group 1 agent which is reserved for organisms that are not human or animal pathogens.

*E. coli* K-12 has a long history of use in the human biopharmaceutical industry, with ~30% of currently approved recombinant therapeutic proteins in the United States (US) being produced in *E. coli* K-12, including biosynthetic human insulin in 1983 (Huang et al. 2012; Jozala et al. 2016), and the production of food enzymes which began in the 1980s (JECFA 1991).

*E. coli* K-12 is permitted as a source microorganism for the production of chymosin in the Code and has been approved for use in the production of lacto-*N*-neotetraose (LNnT) and 2′-FL. *E. coli* K-12 is considered a model organism and has been thoroughly characterised for use in research and industry, it is therefore considered a safe organism.

The host strain is *E. coli* K-12 MG1655 (ATCC700926; CGSC7740: genotype $F^-$lambda$^-$ *ilvG*$^-$ *rfb-50 rph-1;* serotype OR:H48:K-) was sequenced by Blattner et al. (1997) and published in GenBank (U00096.3). This strain was derived from W1485, a descendent of the original K-12 isolate, and has been maintained as a laboratory strain with minimal genetic manipulation. This strain has been used for the production of biopharmaceuticals and food additives.

### 3.1.2 Characterisation of the GM production organism

***Development of the GM production strain***

Several modifications were made to the *E. coli* K-12 host strain MG1655 to create the production strain INB-2FL_03. Collectively, these changes direct the biosynthesis of 2′-FL by the production strain.

A total of five genes were introduced into the host strain using standard molecular biology techniques. Four genes were introduced into the genome. These encode:

- lactose permease from *E. coli*;
- sucrose permease from *E. coli*;
- sucrose phosphorylase from *Bifidobacterium adolescentis*; and
- fructokinase from *Zymomonas mobilis*.

Together, these four genes facilitate the uptake of sucrose and lactose, as well as the conversion of sucrose into GDP-fucose. GDP-fucose is an important intermediate in the synthesis of 2′-FL. One additional gene encoding α-1,2-fucosyltransferase from *Helicobacter enhydrae* was introduced using an expression plasmid. α-1,2-fucosyltransferase is responsible for the synthesis of 2′-FL from GDP-fucose and lactose. Figure 2 provides an overview of the functions of the introduced genes in the biosynthesis of 2′-FL.

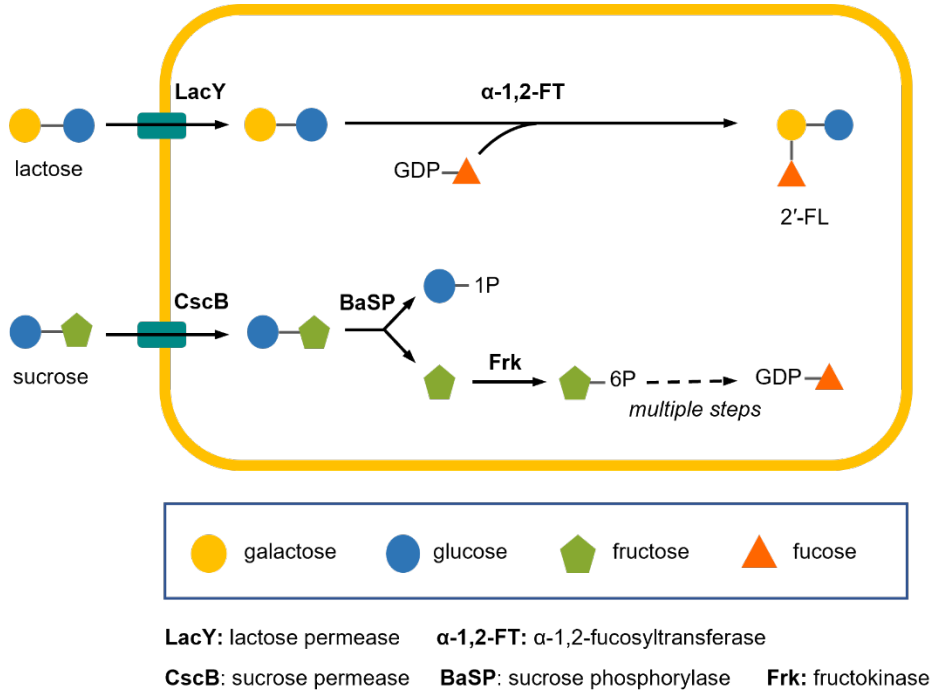


**Figure 2** *The role of the five introduced genes in directing the biosynthesis of 2′-FL in the production strain. LacY and CscB allow entry of sucrose and lactose into the cell; BaSP and Frk facilitate the conversion of sucrose into GDP-fucose; and α-1,2-FT catalyses the conversion of lactose and GDP-fucose into 2′-FL.*

The genome- and plasmid-inserted genes were all chemically synthesised and codon-optimised as required. This ensured that no genetic material, other than the desired genes, could be introduced into the production strain.

In addition to the introduced genes, a total of sixteen genes were fully or partially removed from the genome using standard molecular biology techniques. These deletions were made to increase the efficiency of 2′-FL production by:

- preventing the breakdown of lactose;
- preventing the formation of undesired by-products; and
- increasing the amount of GDP-fucose in the cells.

***Characterisation of introduced DNA***

PCR, Sanger sequencing, and whole genome sequencing (WGS) were used to verify the presence of the inserted DNA in the production strain. The sequencing data confirmed the production strain contained the intended genomic insertions as well as the expression plasmid. No antibiotic resistance markers are present in the final production strain.

***Genetic stability and inheritance of the introduced DNA***

Data was provided showing the production of 2′-FL after multiple fermentation runs and from growth studies performed in minimal media. These data showed that production of 2′-FL by the production strain was stable over a period of 6 days, confirming the stability and inheritance of the inserted DNA over this period.

### 3.1.3 Conclusion

*E. coli* K-12 has a long history of use for the production of recombinant proteins and other products and poses no risks to humans. No safety concerns arising from the gene donors were identified.

Characterisation of the GM production strain confirmed that all introduced genes were both genetically stable and functional.

On the basis of the data provided, no potential safety concerns were identified in the assessment of the 2′-FL production strain INB-2FL_03.

## 3.2 Toxicology assessment

### 3.2.1 Previous FSANZ safety assessments of 2′-FL

A range of toxicity and clinical studies on 2′-FL have previously been reviewed by FSANZ as part of applications A1265, A1251, A1233, A1190, and A1155.

In summary, these assessments found 2′-FL to be structurally and chemically identical to the form present naturally in human milk. As such, no differences in pharmacokinetics between the naturally occurring and manufactured form of 2′-FL is expected. Data indicate that intestinal absorption is limited, and a significant proportion of 2′-FL reaches the large intestine where it is fermented by the microbiota or excreted unchanged in the faeces. Toxicity studies indicated that 2′-FL is not mutagenic or genotoxic *in vitro* and does not produce adverse effects in oral studies using neonatal animals. In human clinical studies, consumption of infant formula containing 2′-FL was safe and well tolerated (FSANZ 2019b, FSANZ 2020, FSANZ 2021, FSANZ 2022a, FSANZ 2022b, FSANZ 2023).

### 3.2.2 Newly available data

The applicant submitted a number of new toxicity and clinical studies; however some of these were excluded from this assessment for the following reasons:

- study already evaluated in previous FSANZ safety assessments; and/or
- study population not relevant, and/or
- study experimental infant formula contained other substances in addition to 2′-FL, compared to the control formula, and as such the safety outcomes related to the experimental formula could not be attributed directly to 2′-FL.

For the present application, the following studies were evaluated:

- Two studies in neonatal rats and piglets, and
- Two human clinical studies of tolerance and gut microbiome.

No additional relevant toxicity or human clinical studies were identified in a search of the published literature.

#### 3.2.2.1 Toxicological studies with commercially available 2′-FL

***Short-term toxicity studies conducted with 2'-FL***

*Effect of supplementation with select human milk oligosaccharides on artificially reared newborn rats (Wang et al. 2022). Regulatory status: non-GLP; Canadian Council on Animal Care guidelines. Ethical approval: University of Calgary Animal Care Committee.*

Two commercially available human milk oligosaccharides (2′-FL and 3′-SL) were fed to rats, using an artificial rearing system, from postnatal days 4 to 21. Basal rat milk substitute served as the control diet. Fifty four male Sprague Dawley rats at 4 days of age were randomly assigned to one of four rat milk substitute groups: control, 2′-FL (1.2 g/L), 3′-SL (1.2 g/L), or 2′-FL (0.6 g/L) +3′-SL (0.6 g/L) (*n*=13-14 per group). Groups received allocated test item for 18 days; test items were prepared with the basal milk substitute.

Rat milk substitutes were prepared every three days. Rats were cannulated through the lining of the cheek and the milk substitutes were delivered with the same flow and speed, adjusted based on age and average body weight (intakes of 2′-FL and 3′-SL were not calculated).

Pups were weighed twice daily, bowel movements and food intake behaviours were monitored daily, through the 18 day artificial rearing. Three faecal samples for each pup were collected by homogenising the faeces collected on days 5-7, 12-14, and 19-21, respectively. At 18 days of age pups underwent an oral glucose tolerance test and at study termination, fat mass and lean mass of pups were measured, along with the Lee obesity index. At necropsy, blood, intestinal tissues and caecal digesta samples were collected, and the brain, liver and caecum weighed. Total bacterial DNA was isolated from the faecal samples and purified DNA and faecal microbiota were quantified.

A total of 39 out of 54 pups survived until weaning which did not differ according to treatment; and there was no effect of either human milk oligosaccharide on mortality. There were no significant differences in daily body weight gain, Lee index, blood glucose concentrations, body composition (fat mass and lean mass), organ weights, and composition of faecal microbiota, between treatment and control groups. The authors concluded that there were no differences in growth performance, body composition, or organ weights in pups fed 2′-FL and/or 3′-SL.

*Evaluation of 2'-FL and Bifidobacterium longum subspecies infantis on growth, organ weights, and intestinal development of piglets (Daniels et al. 2022). Regulatory status: non-GLP; non-guideline. Ethical approval: University of Illinois Institutional Animal Care and Use Committee*

Milk replacer diets supplemented with 2′-FL and/or *Bifidobacterium longum* subspecies *infantis* Bi-26 (Bi-26) were fed to piglets using a neonatal piglet rearing system, from postnatal day 2 until postnatal day 34 or 35. Sixty-three male, naturally farrowed, piglets were randomly assigned to a 2 x 2 factorial design to receive milk replacer (control) or milk replacer with 1 g/L 2′-FL *ad libitum* (intakes of 2′-FL were not calculated). The 2′-FL supplied was Care4U 2′-fucosyllactose. Piglets within each group were then further randomised to receive either no probiotic (glycerol stock) or probiotic (Bi-26), once daily, orally (via syringe during postnatal days 2-12 and via milk bowl during postnatal days 13-33/34) (resulting in four treatment groups).

All piglets were monitored continuously for animal health and well-being, using in-cage cameras. Once daily, individual piglets were weighed and food intake assessed. Twice daily, individual piglets were assessed for health checks (faecal consistency, body condition, visual inspection of feeding system, lethargy, evidence of vomiting). Ten piglets were removed from

the study due to a failure to thrive, unrelated to treatments.

At study termination (postnatal day 34 or 35), piglets were sacrificed and the brain and liver were weighed, the intestines were cut into segments (duodenum, jejunum, ileum, ascending colon, and rectum) for histomorphological analyses. Mucosal scrapings were taken from the jejunum and ileum for measurement of disaccharidase activity. Luminal contents were assessed for dry matter, DNA extraction and abundance of Bi-26.

Formula intake was well tolerated and did not differ between treatments. Total body weight gain showed a trend towards greater gain ($p$ = 0.075) in the control group versus the other groups. There were no differences in the brain, liver or intestinal weights between all treatment groups. Intestinal morphology did not differ between treatment groups, although ileal crypt depth was increased ($p$ = 0.040) in the 2′-FL group and decreased ($p$ = 0.001) in the Bi-26 group. There were no differences in lactase activity between all treatment groups; however, sucrase activity was increased ($p$ = 0.01) in the ileum in the 2′-FL groups. Luminal contents were similar between all treatment groups with respect to dry matter and quantification of Bi-26. The authors concluded that supplementation with 2′-FL and/or Bi-26 was well tolerated in piglets, with no adverse effects observed in the intestine, or on intestinal, liver or brain growth.

#### 3.2.2.2 Human studies with 2′-FL

For the present application, two new clinical studies were evaluated. The methodology used in these studies is detailed in Attachment 1. Safety outcomes are reviewed below.

*Effects of addition of 2-FL to infant formula on growth and specific pathways of utilisation by Bifidobacterium in healthy term infants (Wallingford et al. 2022)*

Healthy term infants ≤28 days of age were recruited and randomised to a control group or test group. A group of exclusively fed breastfed infants of the same age was also enrolled as a reference group. The test formula was identical to the control formula except for the addition of 2′-FL at 1 g/L. Infant formula was given from enrolment for 16 weeks. The two formulas (control and test) and the human milk (breastfed) were fed to infants *ad libitum*, as their sole source of nutrition.

There were no significant differences in the occurrence of adverse events in the test group compared to the control group; however there was a slightly greater incidence of gastrointestinal disorders in the two formula groups compared to the reference breastfed group. The predominant adverse events in the three dietary groups were gastrointestinal disorders (22.6%), infections and infestations (19.9%), and skin and subcutaneous tissue disorders (13.1%). The authors concluded that the addition of 2′-FL to infant formula had no effect on the incidence of adverse events in infants.

*Safety and efficacy of a probiotic-containing infant formula supplemented with 2'-FL: a double-blind randomised controlled trial (Alliet et al. 2022)*

Healthy term infants ≤ 14 days of age were recruited and randomised to a control group or test group. A group of exclusively fed breastfed infants of the same age was also enrolled as a reference group. The test formula was identical to the control formula except for the addition of 2′-FL at 1 g/L. Infant formula was given from enrolment until 6 months of age. The two formulas (control and test) and the human milk (breastfed) were fed to infants *ad libitum*, as their sole source of nutrition, up to at least 4 months of age.

Stool frequency and consistency were comparable between test and control groups. There were no differences between the three groups for difficulty in passing stools. Gastrointestinal

symptoms/behaviours were comparable between all groups. Reported adverse events were similar between all groups, however only four (2.8%) adverse events (no detail provided) were physician-confirmed to be study-product related, with these all occurring in the test group. The authors concluded that the 2′-FL-supplemented formula was well tolerated and safe.

### 3.2.3 Safety assessments by other agencies

As noted in previous assessments, the European Food Safety Authority (EFSA) has assessed 2′-FL as a novel food ingredient and issued scientific opinions on the safety of a mixture of 2′-FL/DFL. Uses evaluated included addition to infant formula, follow-on formula, and in food supplements for infants (EFSA 2015, EFSA 2019; EFSA 2022). EFSA concluded that 2′-FL is safe for use alone or in combination with DFL, under the proposed conditions of use.

The US Food and Drug Administration (FDA) has responded that it has 'no questions' to self-assessments that 2′-FL and a 2′-FL/DFL mixture are Generally Recognized as Safe (GRAS) (FDA 2018a, FDA, 2020).

### 3.2.4 Summary of the toxicology assessment

Based on previous FSANZ applications of 2′-FL and the toxicological assessment in the present application, it was concluded that there are no public health and safety concerns associated with 2′-FL produced from the new genetically modified source organism that is the subject of this application.

## 3.3 Microbiology assessment

FSANZ has undertaken microbiological risk and health benefit assessment on a number of previous applications in regards to production and addition of 2′-FL to infant formula products: A1155, A1190, A1233, A1251 and A1265 (FSANZ 2019b; FSANZ 2021; FSANZ 2022a; FSANZ 2022b; FSANZ 2023).

Based on these previous microbiological assessments, given the identical chemical structure and that the applicant has not requested any change in the maximum permitted level of 2′-FL added to infant formula products, FSANZ has concluded that there are no public health and safety concerns. The associated health benefits from the addition of 2′-FL to infant formula products for infants remain the same: (1) an anti-pathogenic effect; (2) immunomodulation and (3) development of the gut microbiome through supporting growth of *Bifidobacteria* spp.

## 3.4 Nutrition assessment

### 3.4.1 Approach for the nutrition assessment

The objective of the nutrition assessment is to determine the effect (if any) of the addition of 2′-FL to infant formula products on infant growth. Schedule 26 of the Code permits 2′-FL produced by several source organisms (as described in Section 1 above) for addition to infant formula products at a maximum permitted level of 96 mg/100 kJ, equivalent to 2.4 g/L. The applicant has requested the use of a genetically modified (GM) strain of *E. coli* K-12 for the production of 2′-FL but has not requested a change to the maximum permitted level.

The effect of addition of 2′-FL to infant formula products on growth has been assessed in applications A1155, A1190, A1233, A1251 and A1265 (FSANZ 2019b; FSANZ 2020; FSANZ 2021; FSANZ 2022a; FSANZ 2022b; FSANZ 2023). The studies assessed for these

applications used several infant growth endpoints including mean length, weight and head circumference, body weight, mean weight gain per day, fat mass index, weight velocity, and height and weight z-scores. The present nutrition assessment evaluated the effect of 2′-FL in infant formula products on infant growth, and considered the effect on weight gain (g/day) which has an associated clinically relevant threshold for determining effects on infant growth. A difference of more than 3 g/day in weight gain over a three to four month period, between birth and four months of age is considered clinically relevant (American Academy of Pediatrics 1988). The nutrition assessment also considered weight-for-age z-scores across the intervention period, where this data was available. The World Health Organization (WHO) Child Growth Standards outline z-scores from birth to five years of age for both sexes, against which weight-for-age data can be evaluated (WHO 2008).

The applicant provided ten human clinical studies, two of which were relevant to the nutrition assessment (Alliet et al. 2022; Wallingford et al. 2022). FSANZ also undertook a literature search in Pubmed on 2 June 2023 to identify any additional relevant studies using the search terms “2′FL or 2′-FL or 2′-fucosyllactose or 2′fucosyllactose” and “milk or breast or formula” and “anthropometric or weight or growth or development” and “child or infant for baby or maternal”. No additional relevant studies were identified.

Information regarding the selection of included studies is provided in Section 2.2.2. A summary of study parameters for the included studies is in Table A-1.

### 3.4.2 Previous FSANZ assessments of 2′-FL

FSANZ has previously assessed the effect of the addition of 2′-FL to infant formula products in five applications: A1155: *2′-FL and LNnT in infant formula and other products*; A1190: *2′-FL in infant formula and other products*; A1233: *2′-FL from new GM source for infant formula*; A1251: *2′-FL combined with galacto-oligosaccharides and/or inulin-type fructans in infant formula products* and A1265: *2′-FL/DFL, LNT, 6′-SL sodium salt and 3′-SL sodium salt for use as nutritive substances in infant formula products* (FSANZ 2019b; FSANZ 2020; FSANZ 2021; FSANZ 2022a; FSANZ 2022b, FSANZ 2023).

The assessment and review of A1155 FSANZ considered three infant cohort studies (Sprenger et al. 2017; Larsson et al. 2019; Lagström et al. 2020) and five clinical trials in infants (Marriage et al. 2015; Kajzer et al. 2016; Puccio et al. 2017; Storm et al. 2019; Román et al. 2020). These studies measured several infant growth endpoints, including mean length, weight and head circumference, mean weight gain per day, fat mass index, weight velocity, and height and weight z-scores. Based on the available evidence it was concluded that the addition of 2′-FL and lacto-N-neotetraose (LNnT) to infant formula products at levels normally found in human milk should not affect growth.

In A1190, FSANZ assessed four publications that studied the effect of infant formula products containing 2′-FL on infant growth (Reverri et al. 2018; Berger et al. 2020; Leung et al. 2020; Ramirez-Farias et al. 2021). These studies used several infant growth endpoints, including body weight and weight-for-age z-scores. Following consideration of the additional evidence, FSANZ maintained the conclusion that, compared to control formula, no difference in growth was observed in infants fed formula with added 2′-FL. In A1233, FSANZ noted no new information was available that indicated a need to change the conclusions of A1155 and A1190.

In A1251, FSANZ considered one new clinical study in infants (Vandenplas et al. 2020) which investigated equivalence in mean weight gain per day in formula containing 2′-FL, short-chain galacto-oligosaccharides (scGOS) and long-chain fructo-oligosaccharides (lcFOS) compared to control formula. The assessment concluded that, based on available evidence, no difference in growth is likely to occur in infants fed infant formula products that

contain 2′-FL, scGOS and/or lcFOS at the permitted levels.

In A1265, FSANZ considered three clinical studies in infants (Parschet et al. 2021; Cohen 2022; Lasekan et al. 2022) that studied difference in mean weight gain per day as the primary outcome for experimental formulas that contained 2′-FL and other HiMOs including a mixture of 2′-FL and difucosyllactose (DFL), LNnT, 6′-sialyllactose (6′-SL) sodium salt and 3′-sialyllactose (3′-SL) sodium salt. The assessment concluded that infants achieve normal growth when fed infant formula products containing HiMOs at levels that are normally present in human milk.

### 3.4.3 New studies on the effect of 2′-FL on infant growth

Alliet et al. (2022) assessed the growth, gastrointestinal tolerance, and gut microbiome outcomes of infants fed an experimental formula containing 2′-FL in a double-blinded, randomised controlled study of 289 infants, in five study centres in Belgium and two study centres in Italy. Healthy infants (37-42 weeks gestation, birth weight 2500-4500 g) aged up to 14 days old were randomly assigned to experimental or control groups, with stratification by study centre, sex, and mode of delivery. Of 144 infants in the experimental group, 47.9% were male, 97.9% were Caucasian (remaining 2.1% not specified), and the average age at enrolment was 6.8 days. Of 145 infants in the control group, 46.9% were male, 95.2% were Caucasian (remaining 4.8% not specified), and the average age at enrolment was 6.7 days. A non-randomised breastfed reference group was also included, although study authors noted this was to provide a comparison for the study's investigation of gut microbiome and intestinal maturation outcomes.

The duration of the intervention was approximately 180 days, and both infant formula groups were required to exclusively consume study formulas until four months of age. The experimental group received a standard bovine milk based formula supplemented with 1.0 g/L 2′-FL and containing *L. reuteri,* a common probiotic used in infant formula. The control group received the standard bovine milk based formula containing *L. reuteri* but not supplemented with 2′-FL. The study pre-specified a non-inferiority margin of - 3 g/day for comparing mean weight gain per day between the experimental, control and breastfed reference groups.

Mean daily body weight gain (g/day) between recruitment and four months of age was the primary endpoint. The differences in weight gain was analysed using analysis of covariance, adjusted for baseline weight, sex, mode of delivery (vaginal, Caesarean), and study centre. The least square mean weight gain per day in the intention-to-treat populations for the experimental and control groups was 29.15 (standard deviation (SD): 7.8) g/day and 28.89 (SD: 8.5) g/day respectively. The per-protocol analysis reported least squares mean weight gain per day for the experimental and control groups was 29.13 (SD: 7.0) g/day and 28.81 (SD: 7.3) g/day, respectively.

The difference in mean weight gain per day between the experimental and control groups for intention-to-treat population was 0.26 g/day (95% CI: [-1.26, 1.79], p= 0.736), and for the per-protocol analysis was 0.32 g/day (95% CI: [-1.33,1.96], p= 0.704). The difference in mean weight gain per day between the experimental and control groups, as well as the upper and lower bounds of the 95% confidence intervals for those means, in both the intention-to-treat population and the per-protocol analysis, was smaller than the non-inferiority margin of - 3 g/day. The mean weight gain per day for the breastfed reference group was not reported.

Weight-for-age z-scores for all groups were calculated at baseline, 1, 2, 3, 4 and 6 months of age, and were within one standard deviation of the mean between groups. The normal range is defined as up to two standard deviations below the median and up to one standard

deviation above the median (WHO 2008). Study authors stated that no statistical differences between experimental, control and breastfed reference group z-scores were observed, however data was not provided. Data for weight-for-age z-scores were extracted using WebPlotDigitizer Version 4.6 (Rohatgi 2022; Table 2).

**Table 2.** *Z-scores for weight-for-age at baseline (≤14 days), 1, 2, 3, 4 and 6 months of age in intention-to-treat experimental and control populations, and breastfed reference groups (Alliet et al. 2022)*

| Age | Experimental<br>Z-score (mean (SD)) | Control<br>Z-score (mean (SD)) | Breastfed<br>Z-score (mean (SD)) |
|---|---|---|---|
| **Baseline (≤14 days)** | -0.29 (0.89) | -0.19 (0.82) | -0.09 (0.84) |
| **1 month** | -0.20 (0.75) | -0.10 (0.78) | -0.09 (0.90) |
| **2 months** | -0.10 (0.81) | -0.10 (0.75) | -0.29 (1.03) |
| **3 months** | -0.10 (0.88) | -0.10 (0.80) | -0.30 (1.01) |
| **4 months** | 0.00 (0.99) | 0.00 (0.83) | -0.18 (1.18) |
| **6 months** | 0.11 (0.94) | 0.11 (0.77) | -0.10 (1.23) |

A limitation of the study was that it was powered to detect a non-inferiority margin of up to - 3 g/day in mean daily weight gain, whereas the clinically relevant threshold is a difference of ± 3 g/day (American Academy of Pediatrics 1988).

Wallingford et al. (2022) investigated the effect of 2′-FL on infant growth and microbiota outcomes, comparing an experimental group fed a commercial infant formula supplemented with 1.0g/L 2′-FL with a control group fed the commercial formula without 2′-FL, in eight study centres in the United States (US) and one study centre in Honduras. A breastfed reference group was also included. Healthy infants (37-42 weeks gestation, with Appearance, Pulse, Grimace, Activity and Respiration (APGAR) scores of 7 or greater at birth) were randomly assigned to experimental and control groups. Of 66 infants in the experimental group, 48% were male, the average age at enrolment was 14.1 days, 80% of infants were white and 11% were Black or African American. Of the 66 infants in the control group, 42% were male, 74% were white and 14% were Black or African American and the average age at enrolment was 14.3 days.

The duration of the intervention was approximately 16 weeks, and the experimental and control formulas were required to be the sole source of nutrition from enrolment for the duration of the study. The study pre-specified a non-inferiority margin of - 3 g/day for comparing mean weight gain per day between the experimental, control and breastfed reference groups.

The primary growth endpoint was weight gain per day from baseline to the conclusion of the study. The study reported least squares mean weight gain in the per-protocol population of 30.6 g/day (standard error (SE): 1.0 g/day; 95% CI: [28.6, 32.6]) for the experimental group, 30.3 g/day (SE: 1.2 g/day; 95% CI: [27.9, 32.7]) for the control group, and 28.6 g/day (SE: 0.9 g/day; 95% CI: [27.0, 30.2]) for the breastfed reference group. The least square mean difference in weight gain for the per-protocol analysis was 0.3 g/day (95% CI: [-2.8, 3.4]) between the experimental and control groups, and 2.0 g/day (95% CI: [-0.6, 4.6]) between

the experimental and breastfed reference groups. For the intention-to-treat population, authors noted that the lower bounds of the 95% confidence intervals for the least squares mean difference in weight gain between experimental and control group was -2.3 g/day, and between experimental and breastfed group was - 1.4 g/day. The lower bounds of all 95% confidence intervals for difference in least squares mean weight gain per day in both the intention-to-treat and the per-protocol analysis, between the experimental and control groups and between experimental and breastfed reference groups, were smaller than the non-inferiority margin of - 3 g/day.

A limitation of the study was that it was powered to detect a non-inferiority margin of - 3 g/day, and not the clinically relevant difference ± 3 g/day (American Academy of Pediatrics 1988). The upper bounds of the 95% confidence intervals for the intention-to-treat population were also not reported and therefore no conclusions could be drawn on whether these were within the clinically relevant threshold of no more than 3 g/day difference. Another limitation was that randomisation to experimental and control formula groups was not stratified by demographics such as sex, ethnicity, study centre or mode of delivery. Authors reported that mean weight gain per day in the formula-fed groups at the Honduras study centre was slightly more than those for US-based study centres. While specific data for this difference was not presented, authors reported the difference to be smaller than the non-inferiority margin of - 3 g/day, and smaller than the difference between the breastfed reference groups in Honduras compared to the US.

### 3.4.4 Key findings of the nutrition assessment

FSANZ has previously assessed the effect of the addition of 2′-FL to infant formula products in five applications: A1155, A1190, A1233, A1251, and A1265. These applications collectively assessed sixteen studies which investigated infant growth using various endpoints including body weight, mean weight gain per day, anthropometric z-scores, fat mass index, and weight velocity. These assessments concluded that the addition of 2′-FL to infant formula products does not pose a risk to normal growth of infants at levels typically found in human milk.

Schedule 26 of the Code currently permits the addition of 2′-FL produced by different source organisms to infant formula products at a maximum permitted level of 96 mg/100 kJ, equivalent to 2.4 g/L. The applicant did not request changes to the maximum permitted level.

The present nutrition assessment considered two infant clinical trials that were published since the previous FSANZ assessments (Alliet et al. 2022; Wallingford et al. 2022). Both studies investigated the effects of infant formula products supplemented with 1.0 g/L 2′-FL on infant mean weight gain per day, and reported the difference in mean weight gain per day between the experimental and control groups was less than the clinically relevant threshold of ± 3 g/day. However some limitations were noted in the design of the studies.

Based on the available evidence, no difference in growth is likely to occur in infants fed formula containing 2′-FL at currently permitted levels, compared with infants fed formula that does not contain 2′-FL. Considering the newly available evidence for this assessment in addition to the body of evidence considered in previous FSANZ assessments of 2′-FL (FSANZ 2019b; FSANZ 2020; FSANZ 2021, FSANZ 2022a; FSANZ 2022b; FSANZ 2023), the nutrition assessment concludes that the addition of 2′-FL to infant formula products is unlikely to pose a risk to the normal growth of infants.

# 4 Conclusions

Schedule 26 of the Code currently permits the addition of 2′-FL from different source

organisms to infant formula products. The maximum permitted level is 96 mg/100 kJ, equivalent to 2.4 g/L. The purpose of the present assessment is therefore to assess the safety of 2′-FL produced by the new production strain. The applicant's 2′-FL is chemically and structurally identical to the naturally occurring substance in human milk.

The *E. coli* K-12 host organism has a long history of use for the production of recombinant proteins and poses no risks to humans. Characterisation of the GM production strain confirmed that all introduced genes were genetically stable and functional.

FSANZ has previously determined that there are no safety concerns associated with the addition of 2′-FL to infant formula products at concentrations up to 2.4 g/L. Newly available information did not indicate a reason to change this conclusion. No microbiological safety concerns were identified.

Intestinal absorption of human milk oligosaccharides (HMOs) is limited and a significant proportion reach the large intestine where they are fermented by the microbiota or excreted unchanged in the faeces. As the applicant's 2′-FL is identical to naturally occurring HMOs it is not anticipated that there will be any significant differences in pharmacokinetics between naturally occurring and manufactured forms of these substances. The nutrition assessment concluded that the addition of 2′-FL to infant formula products is unlikely to pose a risk to the normal growth of infants.

Based on the available toxicological and nutritional data, there are no public health and safety concerns associated with the addition of 2′-FL (from the new source organism) to infant formula products at the proposed use levels.

# 5 References


Alliet P, Vandenplas Y, Roggero P, Jespers SNJ, Peeters S, Stalens JP, Kortman GAM, Amico M, Berger B, Sprenger N, Cercamondi CI, Corsello G (2022) Safety and efficacy of a probiotic-containing infant formula supplemented with 2′-fucosyllactose: a double-blind randomized controlled trial. Nutr J 21: 11 https://doi.10.1186/s12937-022-00764-2.

American Academy of Pediatrics (1988) Clinical testing of infant formulas with respect to nutritional suitability for term infants: Report to the FDA. Elk Grove Village, IL: AAP: Committee on Nutrition. https://wayback.archive-it.org/7993/20170722090324/https://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/InfantFormula/ucm170649.htm.

Bachmann BJ (1996) Derivations and genotypes of some mutant derivatives of *Escherichia coli* K-12. In: Neidhardt FC (ed) *Escherichia coli* and *Salmonella*: Cellular and Molecular Biology, 2nd. ASM Press, Washington DC, pp 2460–2488.

Berger PK, Plows JF, Jones RB, Alderete TL, Yonemitsu C, Poulsen M, Ryoo JH, Peterson BS, Bode L, Goran MI (2020) Human milk oligosaccharide 2′-fucosyllactose links feedings at 1 month to cognitive development at 24 months in infants of normal and overweight mothers. PLoS One 15(2): e0228323.

Blattner FR, Plunkett G 3rd, Bloch CA, Perna NT, Burland V, Riley M, Collado-Vides J, Glasner JD, Rode CK, Mayhew GF, Gregor J, Davis NW, Kirkpatrick HA, Goeden MA, Rose DJ, Mau B, Shao Y (1997) The complete genome sequence of *Escherichia coli* K-12. Science 277(5331): 1453-62. doi: 10.1126/science.277.5331.1453.

Cohen SS (2022) Growth, safety and efficacy of a starter infant formula, follow-up formula, and growing-up milk supplemented with a blend of five human milk oligosaccharides: a double-blind, randomized, controlled trial. Second generation HMOs blend study: 12 month report. . EpidStrategies, a division of ToxStrategies, Inc. epub.(Study No: 16.24.INF; NPDI Code No: DNUT-109692).

Daniels VC, Monaco MH, Wang M, Hirvonen J, Jensen HM, Ouwehand AC, Mukherjea R, Dilger RN, Donovan SM (2022) Evaluation of 2′-Fucosyllactose and *Bifidobacterium longum* subspecies *infantis* on growth, organ weights, and intestinal development of piglets. Nutrients 14: 199-2011.

EFSA (2015) Safety of 2′-O-fucosyllactose as a novel food ingredient pursuant to Regulation (EC) No 258/97. EFSA Journal 13: 4184 https://doi.org/10.2903/j.efsa.2015.4184.

EFSA (2019) Scientific opinion on the safety of 2′-fucosyllactose/difucosyllactose mixture as a novel food pursuant to Regulation (EU) 2015/2283. EFSA Journal 17(6): e05717 https://doi.org/10.2903/j.efsa.2019.5717.

EFSA (2022) Scientific opinion on the safety of the extension of use of 2′-fucosyllactose/difucosyllactose (2′-FL/DFL) mixture and lacto-N-tetraose (LNT) as novel foods in food supplements for infants pursuant to Regulation (EU) 2015/2283. EFSA Journal 20(3): e07140

https://doi.org/10.2903/j.efsa.2022.7140.

EPA (1997) Final risk assessment of *Escherichia coli* K-12 derivatives. Environmental Protection Agency, Washington DC, USA.

FDA (2014) Agency response letter to GRAS Notice No. GRN 546: 2′-O-fucosyllactose. US Food and Drug Administration, Center for Food Safety and Applied Nutrition, Office of Food Additive Safety, Maryland, USA https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=546&sort=GRN_No&order=DESC&startrow=1&type=basic&search=546.

FDA (2015) Agency response letter to GRAS Notice No. GRN 571: 2′-Fucosyllactose. US Food and Drug Administration, Center for Food Safety and Applied Nutrition, Office of Food Additive Safety, Maryland, USA https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=571&sort=GRN_No&order=DESC&startrow=1&type=basic&search=571.

FDA (2018a) Agency response letter to GRAS Notice No. GRN 735: 2′-Fucosyllactose. US Food and Drug Administration, Center for Food Safety and Applied Nutrition, Office of Food Additive Safety, Maryland, USA https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=735.

FDA (2018b) Agency response letter to GRAS Notice No. GRN 749: 2′-O-fucosyllactose. US Food and Drug Administration, Center for Food Safety and Applied Nutrition, Office of Food Additive Safety, Maryland, USA https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=749&sort=GRN_No&order=DESC&startrow=1&type=basic&search=749.

FDA (2020) Agency response letter to GRAS Notice No. GRN 815: 2′-fucosyllactose and difucosyllactose. US Food and Drug Administration, Center for Food Safety and Applied Nutrition, Office of Food Additive Safety, Maryland, USA https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=815.

FSANZ (2019a) Application A1155 - 2′-FL and LNnT in infant formula and other products. Supporting Document 1 at Second Call for Submissions. Report prepared by Food Standards Australia New Zealand, Canberra. www.foodstandards.gov.au/code/applications/Documents/A1155_SD1_Risk%20assessment%20-%202nd%20CFS.pdf

FSANZ (2019b) Application A1155 - 2′-FL and LNnT in infant formula and other products. Supporting Document 1 at Approval. Report prepared by Food Standards Australia New Zealand, Canberra. www.foodstandards.gov.au/code/applications/Documents/A1155%20SD1%20at%20approval.pdf.

FSANZ (2020) Review of Application A1155 – 2′-FL and LNnT in infant formula and other products. Supporting Document 1. Review of new toxicology, clinical safety, growth and development studies. Report prepared by Food Standards Australia New Zealand, Canberra. https://www.foodstandards.gov.au/code/applications/Documents/A1155%20Review%20SD1%20Safety%20and%20growth%20.pdf.

FSANZ (2021) Application A1190 - 2′-FL and LNnT in infant formula and other products. Supporting Document 1 at First Call for Submissions. Risk and safety assessment. Report prepared by Food Standards Australia New Zealand, Canberra. https://www.foodstandards.gov.au/code/applications/Documents/A1190_SD1.pdf.

FSANZ (2022a) Application A1233 - 2′-FL from new GM source for infant formula. Supporting Document 1 – Food technology and safety assessment at approval. Report prepared by Food Standards Australia New Zealand, Canberra. https://www.foodstandards.gov.au/code/applications/Documents/A1233%20SD1%20at%20approval.pdf.

FSANZ (2022b) Application A1251 - 2′-FL combined with galacto-oligosaccharides and/or insulin-type fructans in infant formula products. Supporting Document 1 – Food technology and safety assessment at approval. Report prepared by Food Standards Australia New Zealand, Canberra. https://www.foodstandards.gov.au/code/applications/Documents/A1251%20SD1.pdf.

FSANZ (2023) Application A1265 - 2′-FL/DFL, LNT, 6′-SL sodium salt and 3′-SL sodium salt for use as nutritive substances in infant formula products. Supporting Document 1 at First Call for Submissions. Risk assessment. Report prepared by Food Standards Australia New Zealand, Canberra. https://www.foodstandards.gov.au/code/applications/Documents/A1265%20SD1%20(1).pdf.

Huang C-J, Lin H, Yang X (2012) Industrial production of recombinant therapeutics in *Escherichia coli* and its recent advancements. J Ind Microbiol Biotechnol 39:383–399 https://pubmed.ncbi.nlm.nih.gov/22252444/.

JECFA (1991) Evaluation of certain food additives and contaminants. Thirty-seventh report of the Joint FAO/WHO Expert Committee on Food Additives. Technical Report No. 806. World Health Organization, Geneva, Switzerland.

Jozala AF, Geraldes DC, Tundisi LL, Feitosa VdA, Breyer CA, Cardoso SL, Mazzola PG, Oliveira-Nascimento Ld, Rangel-Yagui CdO, Magalhães PdO, Oliveira MAd, Pessoa A (2016) Biopharmaceuticals from microorganisms: From production to purification. Braz J Microbiol 47 Suppl 1: 51–63 https://pubmed.ncbi.nlm.nih.gov/27838289/.

Kajzer J, Oliver JS, Marriage BJ (2016) Gastrointestinal tolerance of formula supplemented with oligosaccharides. The FASEB Journal 30: Abstract 671.4.

Lagström, H, Rautava S, Ollila H, Kaljonen A, Turta O, Mäkelä J, Bode L (2020) Associations between human milk oligosaccharides and growth in infancy and early childhood. The American Journal of Clinical Nutrition 111(4): 769-778.

Larsson MW, Lind MV, Laursen RP, Yonemitsu C, Larnkjær A, Mølgaard C, Michaelsen KF, Bode L (2019) Human milk oligosaccharide composition is associated with excessive weight gain during exclusive breastfeeding - an explorative study. Front Pediatr 7:297.

Lasekan J, Choe Y, Dvoretskiy S, Devitt A, Zhang S, Mackey A, Wulf K, Buck R, Steele C, johnson M, Baggs G (2022) Growth and gastrointestinal tolerance in healthy term infants fed milk-based infant formula supplemented with five human milk oligosaccharides (HMOs): a randomized multicenter trial. Nutrients. 14(13):265 https://pubmed.ncbi.nlm.nih.gov/35807803/.

Leung TF, Ulfman LH, Chong MKC, Hon KL, Khouw IMSL, Chan PKS, Delsing DJ, Kortman GAM, Bovee-Oudenhoven IMJ (2020) A randomized controlled trial of different young child formulas on upper respiratory and gastrointestinal tract infections in Chinese toddlers. Pediatr Allergy Immunol 31:745-754.

Marriage BJ, Buck RH, Goehring KC, Oliver JS, Williams JA (2015) Infants fed a lower calorie formula with 2′-FL show growth and 2′-FL uptake like breast-fed infants. J Pediatr Gastroenterol Nutr 61:649–658.

NIH (2019) NIH Guidelines for Research involving recombinant or synthetic nucleic acid molecules. Department of Health and Human Services, National Institutes of Health, US. https://osp.od.nih.gov/wp-content/uploads/NIH_Guidelines.pdf

Parschat K, Melsaether C, Japelt KR, Jennewein S (2021) Clinical evaluation of 16-Week supplementation with 5HMO-mix in healthy-term human infants to determine tolerability, safety, and effect on growth. Nutrients. 13(8): 2871 https://pubmed.ncbi.nlm.nih.gov/34445031/.

Puccio G, Alliet P, Cajozzo C, Janssens E, Corsello G, Sprenger N, Wernimont S, Egli D, Gosoniu L, Steenhout P (2017) Effects of infant formula with human milk oligosaccharides on growth and morbidity: A Randomized Multicenter Trial. J Pediatr Gastroenterol Nutr 64:624–631.

Ramirez-Farias C, Baggs GE, Marriage BJ (2021) Growth, tolerance, and compliance of infants fed an extensively hydrolyzed infant formula with added 2′-FL fucosyllactose (2′-FL) human milk oligosaccharide. Nutrients 13:186.

Reverri EJ, Devitt AA, Kajzer JA, Baggs GE, Borschel MW (2018) Review of the clinical experiences of feeding infants formula containing the human milk oligosaccharide 2′-fucosyllactose. Nutrients 10(10): 1346 https://pubmed.ncbi.nlm.nih.gov/30241407/.

Rohatgi A (2022) WebPlotDigitizer. http://automeris.io/WebPlotDigitizer. Accessed 31 July 2023.

Román E, Moreno Villares JM, Domínguez Ortega F, Carmona Martínez A, Picó Sirvent L, Santana Sandoval L, Casas Rivero J, Alshweki A, Cercamondi C, Dahbane S, Vidal Guevara ML (2020) Real-world study in infants fed an infant formula with two human milk oligosaccharides. Nutr Hosp 37(4): 698-706.

Sahl JW, Morris CR, Rasko DA (2013) Comparative genomics of pathogenic *Escherichia coli*. In: Donnenberg MS (ed) *Escherichia coli*: Pathotypes and principles of pathogenesis, 2nd edition. Academic Press, Amsterdam, pp. 21–43

Sprenger N, Le Lee Y, Castro CA de, Steenhout P, Thakkar SK (2017) Longitudinal change of selected human milk oligosaccharides and association to infants' growth, an observatory, single center, longitudinal cohort study. PLoS One 12(2):e0171814 https://pubmed.ncbi.nlm.nih.gov/28182762/.

Storm HM, Shepard J, Czerkies LM, Kineman B, Cohen SS, Reichert H, Carvalho R (2019) 2′-fucosyllactose is well tolerated in a 100% whey, partially hydrolyzed infant formula with Bifidobacterium lactis: a randomized controlled trial. Glob Pediatr Health https://doi.org/10.1177/2333794X19833995.

Vandenplas Y, de Halleuz V, Arciszewska M, Lach P, Pokhylko V, Klymenko V, Schoen S, Abrahamse-Berkeveld M, Mulder KA, Rubio RP (2020) A partly fermented infant formula with postbiotics including 3′-GL, specific oligosaccharides, 2′-FL, and milk fat supports adequate growth, is safe and well-tolerated in healthy term infants: a double-blind, randomised, controlled, multi-country trial, Nutrients 12: 3560.

Wallingford JC, Neve-Myers P and Barber CM (2022) Effects of addition of 2-fucosyllactose to infant formula on growth and specific pathways of utilisation by Bifidobacterium in healthy term infants. Frontiers in Nutrition 9: 961526.

Wang W, Mu C, Cho NA et al. (2022) Effect of supplementation with select human milk oligosaccharides on artificially reared newborn rats. British Journal of Nutrition 128(10): 1906-1906.

WHO (2008) Child Growth Standards Training Course on Child Growth Assessment. World Health Organization, Geneva, Switzerland https://www.who.int/tools/child-growth-standards Accessed 31 July 2023.

# Attachment 1

**Table A-1 Summary of human clinical trials**

| Study design | Infant formula composition | Study population and allocation | Outcomes investigated | Other |
|---|---|---|---|---|
| ***Alliet et al. (2022)*** | | | | |
| Double-blind randomised controlled trial.<br><br>Breastfed reference group was not randomised.<br><br>Computer generated randomisation, stratified by centre, sex, and mode of delivery.<br><br>Conducted across 7 study sites; 5 in Belgium, 2 in Italy. | Experimental group: control formula supplemented with 1g/L 2′-FL.<br><br>Control group: standard cow-milk based whey-dominant formula, containing *L. reuteri*. DSM 17938 at 1 x $10^7$ CFU/g.<br><br>Source of 2′-FL: not provided. | Healthy term infants (37-42 weeks of gestation) ≤ 14 days of age, birth weight 2,500-4,500g.<br><br>Fed control or experimental formulas from enrolment for approximately 180 days; sole source of nutrition until at least 4 months of age. Progressive introduction of complementary foods or liquids permitted after 4 months of age.<br><br><u>Enrolled: 349</u><br>Control: 145<br>Experimental: 144<br>Breastfed reference group: 60<br><br><u>Completed: 224</u><br>Control: 89<br>Experimental: 100<br>Breastfed reference group: 35 | Growth:<br>Primary outcome: weight gain per day for formula-fed infants, between baseline/commencement of feeding, and four months of age.<br>Secondary outcomes: length, head circumference, z-scores for weight, head circumference and length,.<br><br>Safety/tolerance:<br>Records of adverse events collected by parents and/or confirmed by physicians. For example, spitting-up/vomiting, flatulence, crying, fussiness, sleep duration.<br><br>Other:<br>Stool samples collected at baseline, and 1, 2, and 3 months, for fecal microbiota, metabolism and DNA analysis. | The study was registered at clinicaltrials.gov (# NCT03090360).<br><br>Primary analysis: analysis of covariance. Analysis of intention-to-treat and per-protocol population groups conducted.<br><br>Approximately 36% of enrolled infants dropped out of the study: 30% from experimental group, 39% from control group, and 41% from breastfed reference group.<br><br>Of total drop-outs, about half (18% of the enrolled infants) were due to parents withdrawing consent without explanation.<br><br>Conflict of interest: study was funded by Nestle Nutrition, Societe des Produits Nestle S.A., and four authors are current employees of the study sponsor. One other author is employed by NIZO Food Research BV. |

| Study design | Infant formula composition | Study population and allocation | Outcomes investigated | Other |
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| ***Wallingford et al. (2022)*** | | | | |
| Double-blind randomised controlled trial (test and control formula groups were randomised and reference group was non-randomised).<br><br>Computer generated randomisation.<br><br>Slight excess of female infants in each group.<br><br>Conducted over 9 study sites; 8 in United States, 1 in Honduras. | Experimental group: control formula supplemented with 1g/L 2′-FL.<br><br>Control group:<br>A commercial whey-dominant cow's milk-based infant formula.<br><br>Source of 2′-FL: Jennewein (now owned by CHR Hansen). | Healthy term infants (37-42 weeks of gestation; birth weight between 5$^{th}$ and 95$^{th}$ percentiles and APGAR scores of ≥7); ≤ 28 days of age.<br><br>Fed experimental or control formulas from enrolment for 16 weeks as a sole source of nutrition.<br><br>Enrolled: 221<br>Control: 66 (F:M ratio: 38:28)<br>Experimental: 66 (F:M ratio: 34:32)<br>Breastfed reference group: 89 (F:M ratio: 47:42)<br><br>Completed: 176<br>Control: 41<br>Experimental: 56<br>Breastfed reference group: 79 | Growth:<br>Primary outcome: weight gain, measured as total grams gained over the 16 week feeding period.<br>Secondary outcomes: length gain, head circumference.<br><br>Safety/tolerance:<br>Occurrence of adverse events (defined as any medical event whether or not it was considered product related), feeding compliance determined from caregiver diaries and scheduled in-person/phone visits.<br><br>Other:<br>Stool samples collected by caregivers for faecal microbiome analysis, including expression of microbial genes that metabolise 2′-FL. | The study was performed in compliance with applicable US FDA regulations, the Declaration of Helsinki, ICH Good Clinical Practice Guidelines.<br><br>Primary analysis: analysis of covariance. Analysis of intention-to-treat and per-protocol population groups conducted.<br><br>The primary reasons for dropping out of the study were:<br>Request from caregiver: experimental (44%); control (60%)<br>Subjects lost to follow up experimental (20%); control (28%)<br>Adverse events (experimental (20%); control (16%)) (vomiting, abdominal pain, gastroesophageal reflux, constipation, spitting up).<br><br>2′-FL level in experimental formula was based on a mean value for human milk (authors acknowledge that the level might have been too low to generate any differences).<br><br>Reported adverse events were not correlated to 2′-FL.<br><br>Conflict of interest: study was funded by Perrigo Nutritionals and one author is an employee of Perrigo Nutritionals. In addition, another author is an employee of Nutrispectives LLC. |