APPLICATION TO AMEND THE *AUSTRALIA AND NEW ZEALAND FOOD STANDARDS CODE* TO ALLOW FOR THE USE OF 2'-FUCOSYLLACTOSE PRODUCED USING GENE TECHNOLOGY FOR USE AS A NUTRITIVE SUBSTANCE IN INFANT FORMULA PRODUCTS

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Application to Amend the *Australia and New Zealand Food Standards Code* to Allow for the Use of 2'-Fucosyllactose Produced Using Gene Technology for Use as a Nutritive Substance in Infant Formula Products

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GENERAL REQUIREMENTS

In accordance with Section 3.1.1 – General Requirements of the *Food Standards Australia New Zealand Application Handbook* (FSANZ, 2019a), the following general information must be provided:

1. Format of the application
2. Applicant details
3. Purpose of the application
4. Justification for the application
5. Information to support the application.
6. Assessment procedure
7. Confidential commercial information
8. Other confidential information
9. Exclusive capturable commercial benefit
10. International and other national standards
11. Statutory declaration
12. Checklist

Each point is addressed in the following subsections.

1. Format of the Application

1.1 Information Related to Changes to Standard 2.9.1 – Infant Formula Products

This application for an amendment to Standard 2.9.1 and related Schedules (*i.e.,* Schedules 3, 26, and 29) is prepared pursuant to Section 3.3.3 (Substances Used for a Nutritive Purpose) of the *Food Standards Australia New Zealand Application Handbook* (FSANZ, 2019a). In addition, based on recommendations from Food Standards Australia New Zealand (FSANZ), relevant sections of Section 3.3.2 (Processing Aids) and 3.6.2 (Special Purpose Food – Infant Formula Products) have been included herein.

At the beginning of each section of this application, the information that must be addressed therein is specified in detail. Additionally, an executive summary for the application has been provided as a separate electronic document with this application. The application has been prepared in English and submitted electronically, as required within the *Food Standards Australia New Zealand Application Handbook* (FSANZ, 2019a).

2. Applicant Details

Kyowa Hakko Bio Co., Ltd. (Kyowa) is a manufacturer of biotechnology and fermentation products, established in 2008 with the goal of bringing to market an array of useful chemical substances produced *via* fermentation technologies. The contact details for the person responsible for this application are listed below:

3. Purpose of the Application

This application is being submitted to FSANZ. The applicant, Kyowa Hakko Bio Co., Ltd. (Kyowa), is seeking to amend Schedules 26 ("Foods Produced using Gene Technology") and 29 ("Special Purpose Foods – Infant Formula Products – substances permitted for use as nutritive substances") of the *Food Standards Code* (the Code) to permit an alternative genetically modified source organism (*i.e.,* a modified strain of *Escherichia coli* W) for the production by fermentation of 2'-fucosyllactose (2'-FL) for use as a nutritive substance in infant formula products. Kyowa is also requesting an amendment to Schedule 3 of the Code to reference or include a specification for this source of 2'-FL.

Kyowa's 2'-FL is manufactured by fermentation using a genetically modified strain of *E. coli* W. The 2'-FL ingredient is secreted into the media, isolated, and purified. The final 2'-FL ingredient contains ≥82% 2'-FL and minor levels of other carbohydrates, such as D-lactose, L-fucose, fucosylgalactose, difucosyllactose, and D-glucose and D-galactose. Kyowa's 2'-FL is structurally and chemically identical to 2'-FL ingredients that are currently permitted in the Code, included in Regulation (EU) 2017/2470 establishing the Union list of novel foods.[1] (hereafter referred to as the EU Union List), as well as to 2'-FL that is naturally present in human breast milk.

[1] Commission Implementing Regulation (EU) 2017/2470 of 20 December 2017 establishing the Union list of novel foods in accordance with Regulation (EU) 2015/2283 of the European Parliament and of the Council on novel foods. OJ L 351, 30.12.2017, p. 72–201. Available online: http://data.europa.eu/eli/reg_impl/2017/2470/2024-01-10 (current consolidated version: 10/01/2024).

Kyowa intends to include 2’-FL as an ingredient in infant formula products. The safety and suitability of 2'-FL as an ingredient in infant formula products has been assessed previously by FSANZ (Applications A1155, A1190, A1233, A1251, A1265, and A1277), with no evidence of safety or nutritional concerns identified. 2'-FL is included in Schedule 29 of the Code, Section S29-5 (“Infant Formula Products – substances permitted for use as nutritive substances”), with permitted source organisms including modified strains of *E. coli* K-12 and *E. coli* BL21 and is included in Schedule 26 of the Code under the category, “Food produced using gene technology of microbial origin.” The use of 2'-FL in infant formula products is specified in Standard 2.9.1 of the Code (“Infant Formula Products”).

4. Justification for the Application

The use of 2’-FL in Australia and New Zealand in infant formula products as a nutritive substance is permitted under Schedule 29-5; this permission was initially included as a result of Application A1155. Kyowa produces 2'-FL by microbial fermentation using a non-toxigenic and non-pathogenic strain of *E. coli* W. As this source organism is not listed in Schedule 26 of the Code as a permitted source organism for 2'-FL, Kyowa is seeking to amend Schedules 3, 26, and 29 of the Code to include their modified strain of *E. coli* W as an alternative genetically modified source organism for 2'-FL.

4.1 Regulatory Impact Information (Costs and Benefits of Application)

4.1.1 Impact on the Consumer

Consumers are expected to benefit from increased availability of infant formula products containing 2'-FL produced using an alternative fermentation source. The proposed amendment to the Code is not expected to impose any additional economic costs on consumers.

4.1.2 Impact on the Industry

Food product manufacturers are expected to benefit from increased availability of 2'-FL produced using an alternative fermentation source, ultimately, enabling market competition and benefitting Australia/New Zealand consumers. The proposed amendment to the Code is not expected to impose any additional economic costs on industry.

4.1.3 Impact on the Government

Government agencies are expected to benefit from increased availability of 2'-FL produced using an alternative fermentation source. Approval of the proposed amendment to the Code, to permit the use of Kyowa’s 2'-FL produced using an alternative fermentation source organism in Australia/New Zealand, is not expected to have any negative impact on government agencies.

4.1.4 Impact on International Trade

Kyowa’s 2'-FL produced using a modified strain of *E. coli* W has received a letter of “no questions” from the United States (U.S.) Food and Drug Administration (FDA) regarding the Generally Recognized as Safe (GRAS) status of the ingredient for use in a variety of food and beverage products, including infant formula. A favourable European Food Safety Authority (EFSA) scientific opinion regarding the use of Kyowa’s 2’-FL as a novel food ingredient has been published, and the evaluation of Kyowa’s 2’-FL as a novel food ingredient in the United Kingdom (UK) is currently underway. Approval of Kyowa’s 2'-FL produced using a modified strain of *E. coli* W for use in infant formula products in Australia and New Zealand will promote international trade and reduce technical barriers to trade while continuing to protect public health and safety.

5. Information to Support the Application

Detailed technical information related to the safety of an Enzyme Processing Aid is provided in Section A of this application in accordance with Section 3.3.2 of the FSANZ Application Handbook (FSANZ, 2019a). Information related to Substances Used for a Nutritive Purpose is provided in Section B in accordance with Section 3.3.3 of the FSANZ Application Handbook. Section C of this application addressed Section 3.6.2 of the FSANZ Application Handbook, Special Purpose Food – Infant Formula Products.

Information is provided in this application to enable the objectives specified in Section 18 of the *Food Standards Australia New Zealand Act*, which includes:

- The protection of public health and safety;
- The provision of adequate information relating to food to enable consumers to make informed choices; and
- The prevention of misleading or deceptive conduct.

Literature Search Strategy

To identify publications relevant to the safety of 2'-FL published since the most recent approvals by FSANZ for 2'-FL produced by fermentation using a genetically modified production organism (Applications A1233 and A1265; covering the period from 2017 to 2022), a comprehensive search of the published scientific literature was conducted in October 2023 according to the literature search strategy described below. As detailed in Section B, no newly published studies were identified that indicated the potential for allergic, toxic, or adverse health effects related to consumption of 2'-FL.

6. Assessment Procedure

The applicant believes the most appropriate assessment procedure for the application herein relates to Standard 2.9.1 – Infant Formula Products of the Code; specifically, amending Schedule 26-3 to add their modified strain of *E. coli* W as a production organism. Considering that Kyowa's 2'-FL produced using a genetically modified strain of *E. coli* W is identical to 2'-FL naturally present in human milk, and that the safety of 2'-FL has been assessed previously by FSANZ, this application is expected to fall under the General Procedure (Subdivision D of the *Food Standards Australia New Zealand Act*), Cost Category Level 2.

7. Confidential Commercial Information (CCI)

The applicant requests that the specific information related to the construction of the production organism and the final 2'-FL ingredient be considered confidential commercial information (CCI) and informs FSANZ in writing in Table A.7-1.

Table A.7-1 Information Requested to be Considered as Confidential

Section(s)	Description	Justification
Contact Details	Contact details for applicant and person responsible for the dossier.	The contact details for the applicant and person responsible for the dossier are sensitive and should be treated as confidential. Disclosure of this information is not required for the safety assessment of 2'-FL.
Appendices C, E, and I	Raw materials and processing aids used in the production process. Detailed description of the production process. Detailed description of the production microorganism, including the name of the production strain.	The documents included in these Appendices contain data considered confidential and proprietary (*i.e.*, detailed description of the production process, detailed HACCP plan, detailed description of the genetic modification process) as these have been developed by the applicant. The raw materials and processing aids are a crucial part of the confidential and proprietary manufacturing process. The production microorganism is a key component of the manufacturing process, and the name of the production strain is requested to be kept confidential. The applicant has invested considerable financial investment in the development of the production strain. The data in these Appendices are of significant commercial value to the applicant. The publication of these data would provide a competitive advantage to other manufacturers.
Appendix D – Identification Reports	Analytical reports for the identification of 2'-FL.	This Appendix contains unpublished data from analytical studies on 2'-FL. It is therefore of significant commercial value to the applicant and should remain confidential and proprietary.
Appendix J – Analytical Methods	Details pertaining to proprietary analytical methods.	This Appendix includes full details of the unpublished analytical methods developed by the applicant. Analytical method development was conducted with considerable financial investment by the applicant. It is therefore of significant commercial value to the applicant and should remain confidential and proprietary.
Appendix K – Laboratory Accreditation Certificates	Laboratory Accreditation Certificates and system suitability method description.	The laboratory accreditation certificates should remain confidential to the applicant. Its confidentiality has no bearing on the safety assessment of 2'-FL. The system suitability method has been developed by Kyowa and is of considerable commercial value.
Appendix F – Analytical Data	Certificates of Analysis and Compositional Analytical Reports.	The Certificates of Analysis and the compositional analytical reports contain unpublished analytical data and sensitive information. They are considered proprietary and should therefore remain confidential. The applicant requests that the lot numbers of the samples described in the Certificates of Analysis and Study Reports are kept confidential as disclosure of lot numbers and knowledge of lot numbering rules could permit competitors to estimate production capacity. The data and information within this Appendix are of significant commercial value to the applicant.
Appendix G – Solubility Test Report	Full description of the solubility study conducted with 2'-FL.	This report contains unpublished data from analytical studies on 2'-FL. It is therefore of significant commercial

Table A.7-1 Information Requested to be Considered as Confidential

Section(s)	Description	Justification
		value to the applicant and should remain confidential and proprietary.
Appendix H – Stability Data	Stability studies of 2'-FL.	The stability studies report contains unpublished data regarding 2'-FL and other sensitive information. It is therefore of significant commercial value to the applicant should remain confidential.
Appendix M – Toxicology Study Reports	Details pertaining to study methodology and results.	This Appendix contains unpublished data regarding 2'-FL and other sensitive information. The entire study reports are, therefore, of significant commercial value to the applicant and should remain confidential.

2'-FL = 2'-fucosyllactose; HACCP = Hazard Analysis and Critical Control Points.

The applicant requests that the above data and information be considered CCI by FSANZ due to its proprietary nature that is of significant commercial value to the applicant. Non-confidential descriptions of the CCI are provided in the respective sections of this application.

8. Other Confidential Information

The applicant notes that the Applicant/Manufacturer and Contact Person details are sensitive and not required for the evaluation of the safety of the ingredient, and requests that these be treated as confidential.

9. Exclusive Capturable Commercial Benefit (ECCB)

Due to the nature of Kyowa's modified *E. coli* W production strain technology, it is assumed that only the applicant will be able to commercially benefit from the production of 2'-FL using this specific strain in Australia and New Zealand upon approval of this application. Therefore, the application would confer exclusive capturable commercial benefit (ECCB) in accordance with Section 8 of the *Food Standards Australia New Zealand Act*.

Exclusive permission is sought for the use of Kyowa's modified strain of *E. coli* W to produce 2'-FL by fermentation.

10. International and Other National Standards

Kyowa intends to market 2'-FL, produced by fermentation using a modified strain of *E. coli* W, as an ingredient in infant formula products in accordance with use levels previously evaluated and approved by FSANZ (*i.e.,* as detailed in Schedule 29-5). Kyowa has received a letter of "no questions" from the U.S. FDA regarding the GRAS status of their 2'-FL produced by fermentation using a modified strain of *E. coli* W (GRN 1051 – U.S. FDA, 2023a).

2'-FL produced by chemical synthesis or microbial fermentation has been authorized for use in several food applications under Regulation (EU) 2017/2470 establishing the Union list of novel foods.[2] In general, 2'-FL is permitted for use in infant formula and follow-on formula (maximum level of 1.2 g/L), baby foods and beverages for young children (levels ranging between 1.2 and 12 g/kg or L), conventional foods (levels ranging between 1.2 and 400 g/kg or L), and foods for special medical purposes (according to particular nutritional requirements). 2'-FL is also permitted in food supplements as defined in Directive 2002/46/EC,[3] excluding food supplements for infants (1.2 g/day for young children and 3.0 g/day for the general population).

In the U.S., several GRAS notifications for 2'-FL were identified upon searching the U.S. FDA's inventory of GRAS Notices (GRNs), 13 of which were filed with no objections (GRNs 546, 571, 650, 735, 749, 852, 897, 929, 932, 1014, 1034, 1051, 1060, and 1091 – U.S. FDA, 2015a,b, 2016, 2018a,b, 2019a, 2020a, 2021a,b, 2022a,b, 2023a,b,c), 1 of which is listed as pending (GRN 1157– U.S. FDA, 2024), and 4 of which were withdrawn (GRNs 859, 924, 987, 1040 – U.S. FDA, 2019b, 2020b, 2022,c,d). Notifications filed with no objections are for synthetic 2'-FL (GRN 546 – U.S. FDA, 2015a), and for 2'-FL produced by fermentation from genetically modified strains of *E. coli* BL21, (GRN 571, 929, 1014– U.S. FDA, 2015b, 2020b, 2022a), *E. coli* K-12 (GRNs 650, 735, 749, 852, 897, 1034, and 1060 – U.S. FDA, 2016, 2018a,b, 2019a, 2020a, 2022b, 2023b), or *Corynebacterium glutamicum* (GRN 932 – U.S. FDA, 2021). It should be noted that GRAS notifications for 2'-FL and difucosyllactose (2'-FL/DFL), and for 2'-FL and lacto-N-fucopentaose, have also been filed with no objections (GRN 815, 1035 – U.S. FDA, 2019c, 2023d). Kyowa has received a letter of "no questions" from the U.S. FDA (GRN 1051). 2'-FL is generally intended for use as an ingredient in term infant formula and toddler formula at a level of up to 2.4 g/L, in baby foods and beverages for young children at levels ranging between 1.2 and 57 g/kg or L, and in conventional foods at levels ranging between 1.2 and 600 g/kg or g/L.

Six applications for 2'-FL (alone or with other ingredients) have been evaluated and approved by FSANZ, as shown in Table 10-1, below. The nature of these applications is summarized in Table 10-1. Although the applicants requested varying levels of 2'-FL in infant formula products (ranging from 1.2 to 2 g/L), FSANZ authorized a common level of 2.4 g/L, which is consistent with international authorizations and would provide greater compatibility with a greater range of overseas food standards.

Table 10-1 Food Standards Australia New Zealand Approvals for 2'-FL

Application Number	Applicant	Ingredient	Source	Purity	Authorized Food Uses and Use Levels (g/kg or g/L)[a]
A1155	Glycom A/S	2'-FL (alone or with LNnT)	Fermentation *Escherichia coli* (strains SCR6 and MP572)	≥94% 2'-FL and ≥92.0% LNnT (dry matter basis)	Permitted in infant formula products[b] at a maximum level of 96 mg/100 kJ (2.4 g/L) 2'-FL alone, or at a combined maximum level of 96 mg/100 kJ (2.4 g/L) which contains not more than 24 mg/100 kJ LNnT (0.6 g LNnT/L).
A1190	Jennewein Biotechnologie, GmbH	2'-FL	Fermentation [*E. coli* BL21 (DE3) #1540]	≥90%	Permitted in infant formula products[b] at a maximum level of 96 mg 2'-FL/100 kJ (2.4 g/L).
A1233	Friesland Campina	2'-FL	Fermentation (*E. coli* K-12 E997)	≥90%	Permitted in infant formula products[b] at a maximum level of 96 mg 2'-FL/100 kJ (2.4 g/L).
A1251	Nutricia Australia Pty	2'-FL (with FOS and/or ITF	Fermentation [*E. coli* BL21 (DE3) #1540]	≥90%	Permitted in infant formula products[b] at maximum levels of up to 2.4 g 2'-FL/L and 8 g scGOS and lcFOS (9:1 ratio).

[2] Commission Implementing Regulation (EU) 2017/2470 of 20 December 2017 establishing the Union list of novel foods in accordance with Regulation (EU) 2015/2283 of the European Parliament and of the Council on novel foods. OJ L 351, 30.12.2017, p. 72–201. Available online: http://data.europa.eu/eli/reg_impl/2017/2470/2024-01-10 (current consolidated version: 10/01/2024).

[3] Directive 2002/46/EC of the European Parliament and of the Council of 10 June 2002 on the approximation of the laws of the Member States relating to food supplements. OJ L 183, 12.7.2002, p. 51–57. Available online: http://data.europa.eu/eli/dir/2002/46/2024-02-06 (current consolidated version: 06/02/2024)..

Table 10-1 Food Standards Australia New Zealand Approvals for 2'-FL

Application Number	Applicant	Ingredient	Source	Purity	Authorized Food Uses and Use Levels (g/kg or g/L)[a]
	Ltd and Chr. Hansen A/S				
A1265	Glycom A/S	2'-FL (with DFL, LNnt, LNT, 6'-SL, and 3'-SL)	Fermentation (modified *E. coli* K-12)	≥92.0% (sum of HMOs) ≥85.0% (2'-FL and DFL)	Permitted in infant formula products[b] at a maximum level of 0.15 g/100 kJ (combined maximum for all HMOs). May be used with GOS and/or ITF.
A1277	Inbiose N.V.	2'-FL	Fermentation (modified *E. coli* K-12)	≥94%	Permitted in infant formula products[b] at a maximum level of 96 mg 2'-FL/100 kJ (2.4 g/L).

2'-FL = 2'-fucosyllactose; 3'-SL = 3'-sialyllactose; 6'-SL = 6'- sialyllactose; DFL = difucosyllactose; FOS = fructooligosaccharides; GOS = galactooligosaccharides; HMO = human milk oligosaccharides; ITF = inulin-type fructans; lcFOS = long-chain fructooligosaccharides; LNnT = lacto-*N*-tetraose; LNT = lacto-*N*-tetraose; scGOS = short-chain galactooligosaccharides.
[a] Authorized under Standard 1.5.2, Schedule 26 (https://www.legislation.gov.au/Details/F2022C00560) and Standard 2.9.1, Schedule 29 (https://www.legislation.gov.au/F2015L00463/latest/text).
[b] Infant formula product means a product based on milk or other edible food constituents of animal or plant origin which is nutritionally adequate to serve by itself either as the sole or principal liquid source of nourishment for infants, depending on the age of the infant (https://www.legislation.gov.au/F2015L00409/latest/text) and includes term, pre-term, and follow-on formulas.

11. Statutory Declaration

A signed Statutory Declaration for Australia is provided as Appendix A.

12. Checklist

A completed checklist relating to the information required for submission with this application is provided in Appendix B.

A. INFORMATION RELATED TO THE SAFETY OF AN ENZYME PROCESSING AID

In accordance with Section 3.3.2 – Processing Aids of the *Food Standards Australia New Zealand Application Handbook* (FSANZ, 2019a), the following technical information is provided:

1. Information related to the safety of an enzyme processing aid – Information on the potential toxicity of the enzyme processing aid (Section 3.3.2, C.2 of the Handbook);

2. Information related to the safety of an enzyme processing aid – Information on the potential allergenicity of the enzyme processing aid (Section 3.3.2, C.3 of the Handbook);

3. Additional information related to the safety of an enzyme processing aid derived from a microorganism – Information on the source microorganism (Section 3.3.2, D.1 of the Handbook);

4. Additional information related to the safety of an enzyme processing aid derived from a microorganism – Information on the pathogenicity and toxicity of the source microorganism (Section 3.3.2, D.2 of the Handbook);

5. Additional information related to the safety of an enzyme processing aid derived from a microorganism – Information on the genetic stability of the source organism (Section 3.3.2, D.3 of the Handbook); and

6. Additional information related to the safety of an enzyme processing aid derived from a genetically-modified microorganism – Information on the methods used in the genetic modification of the source organism (Section 3.3.2, E.1 of the Handbook).

Each point is addressed in the following subsections.

A.1 Information on the Potential Toxicity of the Enzyme Processing Aid

A.1.1 Information on the Enzyme's Prior History of Human Consumption and its Similarity to Proteins with a History of Safe Human Consumption

The U.S. FDA concluded that there are no questions regarding the GRAS status of Kyowa's 2'-FL, produced using the same production organism as described herein, for use in various foods, beverages, infant formula, and enteral tube feeding formulas (GRN 1051). EFSA also has concluded that Kyowa's 2'-FL, produced using the same production organism as described herein, is safe under the proposed conditions of use in a variety of foods including infant formula (EFSA, 2023). However, consumer exposure to Kyowa's microbial processing aid used in the manufacture of 2'-FL is not expected, as the organism remains intact during the fermentation process and is inactivated and removed during sterilization and purification steps. Furthermore, the results of analytical testing (summarized in Section B.2.3.1) demonstrate that the manufacturing organism and DNA derived therefrom are not present in the final 2'-FL ingredient.

Details pertaining to the donor organisms used in the modification of *E. coli* W to produce Kyowa's production strain are considered to be CCI and are detailed in Appendix C **[CONFIDENTIAL AND PROPRIETARY]**.

A.1.2 Information on Any Significant Similarity Between the Amino Acid Sequence of the Enzyme and that of Known Protein Toxins

Details on the molecular characterization of the genetic modification in the new organism are provided in Appendix C **[CONFIDENTIAL AND PROPRIETARY]**. The toxicity of the newly predicted open reading frame (ORF) products was searched using blastp2.9.0 with an E-value cut-off of 0.05 and the BLOSUM62 algorithm. A BLAST search was conducted on all non-redundant GenBank CDS (translations+PDB+SwissProt+PIR+PRF) excluding environmental samples from WGS projects (Molecule Type:Protein Update date:2021/08/02 Number of sequences:420374447). No similarities were observed between the newly predicted ORF products (proteins 1 to 5) and existing proteins in the database, except for 1 hypothetical protein from *Citrobacter* for protein 4. From these results, it was concluded that if the newly predicted ORF products were translated to proteins, the proteins would not be related to toxins. The full results of BLAST search are presented in Appendix C **[CONFIDENTIAL AND PROPRIETARY]**.

In summary, all the amino acid sequences and predicted protein products were found to be non-toxic by BLAST search. As there is no significant similarity between the amino acid sequence of the enzyme processing aid and any known toxin proteins, and the genetically modified production strain remains intact throughout the fermentation process and is not present in Kyowa's final 2'-FL product (as summarized in Section B.2.3.1), information on the stability of the enzyme processing aid to degradation in the gastrointestinal tract and acute or short-term oral studies in rodents have not been conducted and are not presented herein.

A.2 Information on the Potential Allergenicity of the Enzyme Processing Aid

A.2.1 The Source of the Enzyme Processing Aid

The production strain contains 1 heterologous gene sequence (encoding α-1,2 fucosyltransferase) originating from *Helicobacter mustelae* (ATCC 43772 – ATCC, 2021a), which is a gastric pathogen of ferrets (Fox *et al.,* 1990). The α-1,2 fucosyltransferase gene has been cloned from chromosomal DNA and mutated using site-directed mutagenesis according to Kamada and Koizumi (2007), producing a protein that has α-1,2 fucosyltransferase activity but whose amino acid sequence has at least 1 amino acid that has been deleted, substituted, or added. Although the gene encoding α-1,2 fucosyltransferase is cloned from chromosomal DNA from a pathogenic strain, no unspecified DNA is expected to be associated with the transfer of the gene encoding α-1,2 fucosyltransferase, as the DNA insert is well-characterized and is confirmed to consist of the desired sequence only. Furthermore, the expression product, α-1,2 fucosyltransferase, has a well-defined function in the biosynthesis of 2'-FL and is not associated with any potential toxicity or pathogenic traits of the donor organism. Details on the molecular characterization of the genetic modification in the new organism, as well as details of the regulatory sequences used to express the α-1,2-fucosyltransferase gene, are provided in Appendix C **[CONFIDENTIAL AND PROPRIETARY]**.

A.2.2 An Analysis of Similarity Between the Amino Acid Sequence of the Enzyme and that of Known Allergens

The allergenicity of the newly predicted ORF protein products was also analyzed using AllergenOnline (Goodman *et al.,* 2016). AllergenOnline Version 16 includes 1,956 sequences from 778 taxonomic-protein groups that are accepted with evidence of allergic serum IgE-binding and/or biological activity. The E-value cut-off was set to 0.01 according to the China National Standard. No allergenic epitopes or sequences were identified. The full results of the AllergenOnline analysis are presented in Appendix C **[CONFIDENTIAL AND PROPRIETARY]**.

In summary, several unintended ORFs in the heterologous inserted genomic loci were identified. However, all the amino acid sequences and predicted protein products were found to be non-allergenic by AllergenOnline searches.

As the enzyme processing aid is not derived from an allergenic source, there is no significant similarity between the amino acid sequence of the enzyme and that of a known allergen, and the genetically modified production strain remains intact throughout the fermentation process and is not present in Kyowa's final 2'-FL product, information on the stability of the enzyme processing aid to degradation in the gastrointestinal tract and specific serum screening are not considered relevant and are not presented herein.

A.3 Additional Information Related to the Safety of an Enzyme Processing Aid Derived from a Microorganism

A.3.1 Information on the Source Microorganism

Kyowa's 2'-FL ingredient is manufactured by fermentation using a genetically modified strain of *E. coli* W. The *E. coli* W strain has a history of use in the production of food ingredients, as summarized in Section A.3.2. The current taxonomic classification of the host strain, *E. coli* W, is summarized in Table A.3.1-1.

Table A.3.1-1 Characteristics of the Host Organism *Escherichia coli* W

Family	Enterobacteriaceae
Genus	*Escherichia*
Species	*Escherichia coli*
Subspecies	Not applicable
Strain	*E. coli* strain W
Culture Collection	American Type Culture Collection
Deposition Number[a]	ATCC 9637

ATCC = American Type Culture Collection.
[a] https://www.atcc.org/products/all/9637.aspx.

A.3.2 Information on the Pathogenicity and Toxicity of the Source Microorganism

Kyowa's 2'-FL ingredient is produced by fermentation using a genetically engineered strain of *E. coli* W. *E. coli* W is 1 of 4 strains designated safe for laboratory use (K-12, B, C, and W). These 4 strains and their derivatives are designated as Risk Group 1 or Biosafety Level 1 organisms in biological safety guidelines (Archer *et al.,* 2011; ATCC, 2021b,c) as they are well-characterized and do not cause disease in healthy adult humans (NIH, 2019), and do not colonise the human gut (Bauer *et al.,* 2008). *E. coli* W was first isolated from the soil of a cemetery near Rutgers University around 1943 by Selman A. Waksman (Archer *et al.,* 2011). Early reported uses of *E. coli* W are related to the strain's susceptibility to streptomycin and other antibiotics; later uses include a wide variety of industrial and research applications (Archer *et al.,* 2011).

The *E. coli* W strain is a Gram-negative, rod-shaped, facultative anaerobe that has been used in the industrial production of amino acids for foods, feeds, medicines, and various other applications for nearly 80 years (Archer *et al.,* 2011; UniProt, 2021). Compared to other Risk Group 1 *E. coli* strains (K-12, B, and C), *E. coli* W has a larger genome (the chromosome is 4,900,968 base pairs and encodes 4,764 ORFs), belongs to phylogroup B1 rather than A (both of which are classified as non-pathogenic commensal strains), grows faster, and utilizes a wider range of carbon sources including—unlike the other 3 Risk Group 1 strains—sucrose (Archer *et al.,* 2011; UniProt, 2021). The *E. coli* W strain has been deposited in the American Type Culture Collection (ATCC) (ATCC 9637 – ATCC, 2021b), and its genome has been sequenced, annotated, and compared to other safe *E. coli* strains and group B1 commensal/pathogenic *E. coli* strains (Archer *et al.,* 2011). Although *E. coli* W has genes that encode pathogenicity determinants, these have been mutationally inactivated or are missing key components required for pathogenicity, similar to other safe strains (Archer *et al.,* 2011). Genomic analyses also confirmed the lack of genes encoding toxins that can be secreted. As such, *E. coli* W is non-pathogenic and non-toxigenic.

Further details on the host strain (*E. coli* W), the modifications made to it to produce the manufacturing organism, and confirmation of the identity of the production strain are provided in Appendix C **[CONFIDENTIAL AND PROPRIETARY]**.

A.3.3 Information on the Genetic Stability of the Source Organism

The genes introduced into the production strain are required for the manufacture of 2'-FL. The modifications to the host strain, *E. coli* W, were introduced by homologous recombination, which contributes to the stability of the modifications. The manufacturing process for 2'-FL begins with the use of a frozen cell culture, further contributing to the maintenance of genetic stability. Furthermore, the fermentation process is controlled using various parameters (*i.e.*, pH, temperature) as indicators, which also contribute to the genetic stability of the production organism. As noted in Section B.2.4.2.2 the genetic stability from a minimum of 3 cell passages from the master and working cell bank is verified based on 2'-FL production, cell growth, oxygen consumption, and other functional parameters indicating a change in cell culture behaviour.

Kyowa assessed the stability of the heterologous gene introduced into the *E. coli* W production organism. The production organism was passaged for 5 generations, with the structure of the genomic DNA analyzed using PCR for each passage generation. The results, shown in Appendix C **[CONFIDENTIAL AND PROPRIETARY]**, indicate no changes in the genomic structure and demonstrate that the production strain is genetically stable.

A.4 Additional Information Related to the Safety of an Enzyme Processing Aid Derived from a Genetically Modified Microorganism

A.4.1 Information on the Methods Used in the Genetic Modification of the Source Microorganism

The production microorganism is a genetically engineered strain of *E. coli* W. The production strain was optimised to produce 2'-FL from glucose and lactose *via* a series of enzymatic reactions which are carried out during a fermentation process. Target genes are cloned by PCR from chromosomal DNA of defined donor organisms and fused to a constitutive promoter originating from *E. coli* W and expressed at the insertion loci. Desired host modifications are introduced to the *E. coli* W strain in a stepwise manner for the construction of the production strain.

In all instances, genetic modifications were achieved using a modified lambda Red recombination system (Datsenko and Wanner, 2000), a common technique used to make targeted genetic modifications in *E. coli* at loci specified by flanking homology regions including insertions, deletions, and point mutations (Murphy, 1998; Yu *et al.,* 2000; Sharan *et al.,* 2009). Lambda Red recombination genes are expressed from the Red recombinase pKD46 plasmid under the inducible arabinose promoter (P_{araB}) containing a temperature-sensitive replicon (Datsenko and Wanner, 2000; GenBank Accession No. AY048746 – NCBI, 2021). Following expression of the recombinase enzymes, linear DNA substrates are introduced by electroporation, and recombination is catalyzed by the Lambda-derived proteins (Sharan *et al.,* 2009).

Further details on the modification and characterization of the production strain are provided in Appendix C **[CONFIDENTIAL AND PROPRIETARY]**, which was prepared in accordance with EFSA's *Guidance on the risk assessment of genetically modified microorganisms and their products intended for food and feed use* (EFSA, 2011).

B. INFORMATION RELATED TO SUBSTANCES USED FOR A NUTRITIVE PURPOSE

In accordance with Section 3.3.3 – Substances used for a nutritive purpose of the *Food Standards Australia New Zealand Application Handbook* (FSANZ, 2019a), the following information is provided:

1. Information on the use of the nutritive substance (Section 3.3.3, Part A of the Handbook)
2. Technical information on the use of the nutritive substance (Section 3.3.3, Part B of the Handbook)
3. Information related to the safety of the nutritive substance (Section 3.3.3, Part C of the Handbook)
4. Information on dietary intake of the nutritive substance (Section 3.3.3, Part D of the Handbook)
5. Information related to the nutritional impact of a nutritive substance other than vitamins and minerals (Section 3.3.3, Part F of the Handbook)
6. Information related to potential impact on consumer understanding and behaviour (Section 3.3.3, Part G of the Handbook)

Each point is addressed in the following subsections.

B.1 Information on the Purpose of the Use of the Nutritive Substance

Schedule 26 of the Code permits the addition of 2'-FL produced by several genetically modified organisms to infant formula products at levels up to 96 mg/100 kJ (2.4 g/L). The nutritive purpose of 2'-FL as a component of infant formula products has been assessed in applications A1155, A1190, A1233, A1251, A1265, and A1277 (FSANZ, 2019b, 2021, 2022a,b, 2023, 2024).

Kyowa's intended nutritive purpose for the addition of 2'-FL to infant formula products is identical to the nutritive purpose of the 2'-FL ingredients previously assessed by FSANZ in Applications A1155, A1190, A1233, A1251, A1265, and A1277. The addition of 2'-FL to infant formula products allows these products to resemble the composition of human milk more closely. In previous evaluations, FSANZ has concluded that the beneficial physiological effects of 2'-FL as a component of infant formula products include contributing to a healthy intestinal microbiota (*i.e.,* promoting a bifidogenic effect), limiting binding of pathogenic strains of *Campylobacter jejuni* to intestinal epithelial cells, and having a beneficial role in infant growth and development.

In the current application, Kyowa is requesting permission for the use of a new genetically modified source organism for 2'-FL, but is not requesting changes to the currently permitted uses or use levels of 2'-FL.

B.2 Technical Information on the Use of the Nutritive Substance

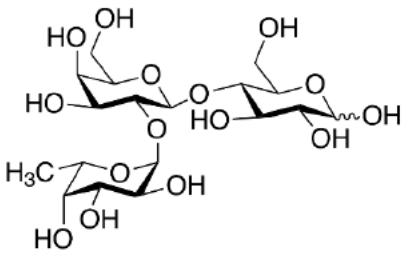
Kyowa intends to market 2'-FL for use as a nutritive substance in infant formula products. No changes to the currently permitted uses and use levels for 2'-FL in the Code are sought, other than the inclusion of a new genetically modified source organism.

B.2.1 Information to Enable Identification of the Nutritive Substance

2'-FL is a fucosylated oligosaccharide composed of 3 monosaccharides, namely L-fucose, D-galactose, and D-glucose. As D-lactose is composed of D-galactose and D-glucose, 2'-FL can also be described as composed of the disaccharide D-lactose and the monosaccharide L-fucose, linked by an *alpha* (1→2) bond. The trisaccharide was first isolated and identified from human breast milk in the 1950s using chromatographic techniques (Polonowski and Montreuil, 1954; Kuhn *et al.,* 1955; Montreuil, 1956). 2'-FL is the second most abundant free soluble glycan in human milk, following lactose (Castanys-Muñoz *et al.,* 2013). 2'-FL has since been detected in the milk of various mammalian species, although it is most abundant in human milk, and either absent or present at low levels in bovine milk and milk from other domestic commercial animals (summarized in Castanys-Muñoz *et al.* [2013]).

Kyowa's 2'-FL manufactured by microbial fermentation using a genetically modified strain of *E. coli* W contains by specification ≥82% 2'-FL, with lesser amounts of D-lactose (≤5%); fucosylgalactose and difucosyllactose (each ≤3%); and L-fucose, and D-glucose and D-galactose (≤1%). The identity information about the 2'-FL ingredient is provided in Table B.2.1-1, below.

Table B.2.1-1 Identity of 2'-Fucosyllactose Produced by Microbial Fermentation of a Genetically Modified Strain of *Escherichia coli* W

Common Name	2'-Fucosyllactose; 2'-*O*-fucosyllactose
Source	*E. coli* W expressing α-1,2-fucosyltransferase from *H. mustelae*
Marketing Name	2'-Fucosyllactose
International Union of Pure and Applied Chemistry (IUPAC) Name	(2*S*,3*S*,4*R*,5*S*,6*S*)-2-[(2*S*,3*R*,4*S*,5*R*,6*R*)-4,5-dihydroxy-6-(hydroxymethyl)-2-[(2*R*,3*S*,4*R*,5*R*)-4,5,6-trihydroxy-2-(hydroxymethyl)oxan-3-yl]oxyoxan-3-yl]oxy-6-methyloxane-3,4,5-triol
Chemical Abstracts Service (CAS) Number	41263-94-9
Synonyms and Abbreviations	α-L-Fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-D-glucopyranose; 6-deoxy-*beta*-L-galacto-hexopyranosyl-(1→2)-*beta*-D-galacto-hexopyranosyl-(1→4)-D-gluco-hexopyranose; 2'-FL; 2-FL; 2FL
Trade Name	2'-Fucosyllactose; 2'-*O*-fucosyllactose
Molecular and Structural Formulae; Stereochemistry	$C_{18}H_{32}O_{15}$
Molecular Mass	488.44 g/mol
Chemical Structure	

2'-FL = 2'-fucosyllactose.

B.2.2 Information on the Chemical and Physical Properties of the Nutritive Substance

B.2.2.1 Information Confirming the Chemical and Structural Identity of the Nutritive Substance

The chemical structure of 2'-FL has been structurally characterized, along with other human milk oligosaccharides (HMOs), by coupling chromatographic separation techniques with more sensitive analytical techniques, such as nuclear magnetic resonance (NMR) spectroscopy (Jenkins *et al.,* 1984; Ishizuka *et al.,* 1999; Rundlöf and Widmalm, 2001; Urashima *et al.,* 2002, 2004, 2005; Almond *et al.,* 2004; Wada *et al.,* 2008) and mass spectrometry (Fura and Leary, 1993; Asres and Perreault, 1996; Perreault and Costello, 1999). The structure of 2'-FL has also been elucidated using X-ray crystallography (Kuhn *et al.,* 1956; Svensson *et al.,* 2002).

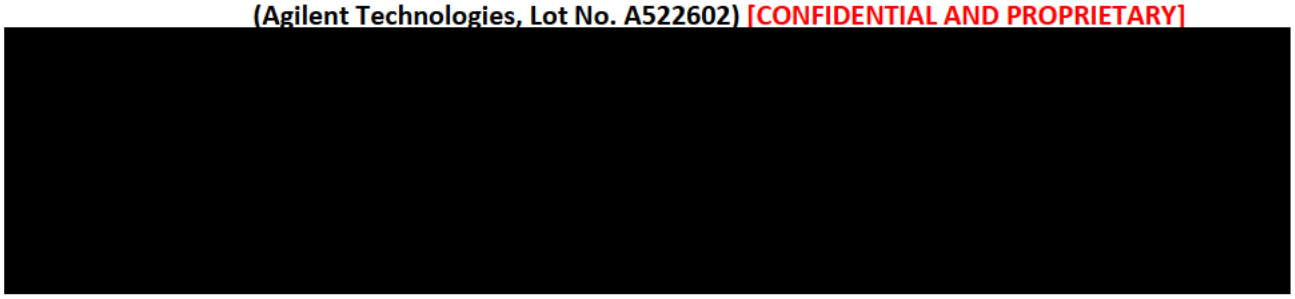
The chemical and structural identity of Kyowa's 2'-FL (produced according to the same manufacturing method and using the same production organism but using an early-development preliminary fermentation media; Lot A) was confirmed against 2'-FL isolated from human milk (Agilent technologies, Lot No. A522602) by liquid chromatography–mass spectrometry (LC-MS), proton nuclear magnetic resonance spectroscopy (^{1}H NMR), and carbon-13 nuclear magnetic resonance spectroscopy (^{13}C NMR). The representative LC-MS chromatogram and mass spectrum of Kyowa's 2'-FL ingredient demonstrating its purity and identity against 2'-FL obtained from human milk are presented in Figures B.2.2.1-1 and B.2.2.1-2, below. A summary of the mass spectroscopy (MS) peak assignments and accurate mass (m/z) of the fragments is provided in Table B.2.2.1-1, below. The full analytical report and the Certificates of Analysis for the standards are provided in Appendix D **[CONFIDENTIAL AND PROPRIETARY]**, while a comparison of the early-development preliminary and current fermentation media is presented in Appendix E **[CONFIDENTIAL AND PROPRIETARY]**. A comparison of the compositional analytical data for 6 lots of Kyowa's 2'-FL produced with early-development preliminary fermentation media and 3 lots of Kyowa's 2'-FL produced with the current fermentation media (Lots H, I, and J) is provided in Appendix F **[CONFIDENTIAL AND PROPRIETARY]** to demonstrate the compositional equivalence of the final product.

Batch analyses of 3 lots of Kyowa's 2'-FL produced by fermentation using the current fermentation media, with a genetically modified strain of *E. coli* W, demonstrate that it is a high-purity product (~91 to 94% 2'-FL) with low levels of other structurally related saccharides (see Appendix F **[CONFIDENTIAL AND PROPRIETARY]**).

Figure B.2.2.1-1 Chromatogram of 2'-Fucosyllactose Produced with a Genetically Modified Strain of *Escherichia coli* W (Lot A) Compared to Standard Isolated from Human Milk (Agilent Technologies, Lot No. A522602) [CONFIDENTIAL AND PROPRIETARY]

Figure B.2.2.1-2 Mass Spectrum and Estimated Compositional Formula of 2'-Fucosyllactose (Lot A) Produced with a Genetically Modified Strain of *Escherichia coli* W Compared to Standard Isolated from Human Milk (Agilent Technologies, Lot No. A522602) [CONFIDENTIAL AND PROPRIETARY]

Table B.2.2.1-1 Summary of Information from Mass Spectroscopy Spectra of Kyowa's 2'-Fucosyllactose (Lot A) and Analytical Standard of 2'-Fucosyllactose (Agilent Technologies, Lot No. A522602) [CONFIDENTIAL AND PROPRIETARY]

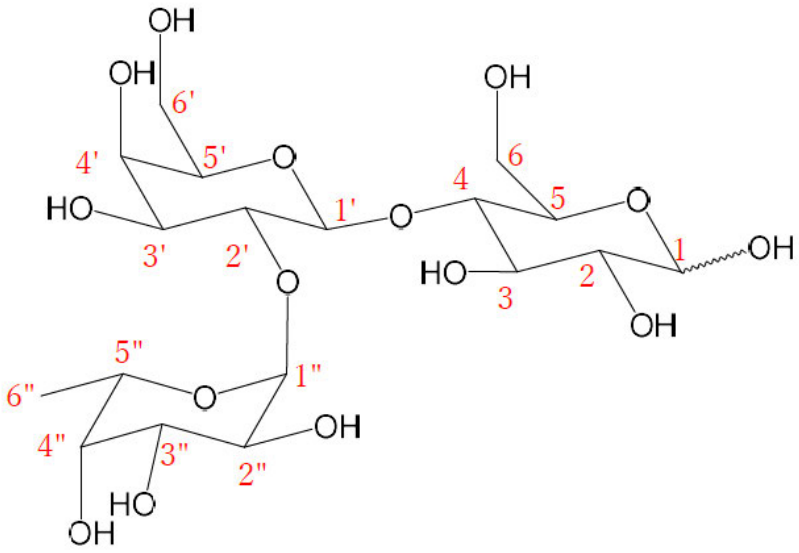


Kyowa conducted 2-dimensional NMR on 2'-FL (produced according to the same manufacturing method and using the same production organism but using an early-development preliminary fermentation media; Lot A) to confirm that the L-fucosyl moiety is linked to D-galactose through an α-(1"-2') bond and to confirm that the bond between D-galactose and D-glucose is β-(1'-4). The structure of 2'-FL, along with carbon assignments, is provided in Figure B.2.2.1-3 below. The bond between the L-fucose and D-galactose occurs between the 1" carbon of fucose and the 2' carbon of galactose. This bond is confirmed to be in the *alpha* configuration, as the J value of the 1" position hydrogen at multiplicity d was 2.4 Hz. The bond is also confirmed to be bound in the *alpha* configuration at (1"-2') by the correlation between ^{1}H at the 1" position and ^{13}C at the 2' position in the heteronuclear multiple bond correlation (HMBC) spectrum (see Figure B.2.2.1-4, below).

The bond between D-galactose and D-glucose occurs between the 1' carbon of galactose and the 4 carbon of glucose (see Figure B.2.2.1-3, below). This bond is confirmed to be in the *beta* configuration, as the J value of the 1' hydrogen at multiplicity d is 7.6 Hz. The bond is also confirmed to be bound in the *beta* configuration at (1'-4) by the correlation between ^{1}H at the 1" position and ^{13}C at the 4' position in the HMBC spectrum (see Figure B.2.2.1-4, below).

The full analytical report is provided in Appendix D **[CONFIDENTIAL AND PROPRIETARY]**.

Figure B.2.2.1-3 Structure of 2'-Fucosyllactose



Kyowa also conducted HMBC on a 3'-FL standard obtained from Carbosynth Ltd, UK (Batch OF056731501) in order to differentiate 2'-FL from its constitutional isomer 3-fucosyllactose (3-FL), in which the L-fucosyl moiety is linked through an α-(1"-3') bond to D-glucose. As demonstrated in Figure B.2.2.1-4, in 2'-FL, the HMBC spectra of ^{1}H at the 1" position and ^{1}H at the 1' position correlate with ^{13}C at the 2' position. In contrast, as demonstrated in Figure B.2.2.1-5, in 3-FL, the HMBC spectra correlate the ^{1}H at the 1" position and the ^{1}H at the 1' position with the ^{13}C at the 3' position. These differences in HMBC spectra permit the differentiation between 2'-FL and 3-FL.

The full analytical report and the Certificates of Analysis for the standard are presented in Appendix D **[CONFIDENTIAL AND PROPRIETARY]**.

Figure B.2.2.1-4 Heteronuclear Multiple Bond Correlation (HMBC) Spectra of 2'-Fucosyllactose (Lot A) [CONFIDENTIAL AND PROPRIETARY]

Figure C.2.2.1-5 Heteronuclear Multiple Bond Correlation (HMBC) Spectra of 3-Fucosyllactose (Carbosynth Ltd, UK) [CONFIDENTIAL AND PROPRIETARY]

Kyowa conducted LC-MS and liquid chromatography with tandem mass spectrometry (LC-MS/MS) on 3 lots of the final 2'-FL ingredient that is the subject of this application (Lots H, I, and J) and compared the results to those from 1 lot of 2'-FL produced according to the same manufacturing method and using the same production organism but using an early-development preliminary fermentation media (Lot A) to demonstrate that the products are the same. The LC-MS spectra demonstrating the fragmentation pattern and the relative peak assignments for 2'-FL Lot A (produced with early-development preliminary fermentation media) and 2'-FL Lot H (produced with current fermentation media) are provided in Figures B.2.2.1-6 and B.2.2.1-7, respectively, below. The LC-MS/MS spectra demonstrating the fragmentation pattern and the relative peak assignments for 2'-FL Lot A and 2'-FL Lot H are provided in Figures B.2.2.1-8 and B.2.2.1-9, respectively, below. In Figure B.2.2.1-10, the LC-MS/MS spectra from Lot A and Lots H, I, and J are overlaid. The results confirm that Kyowa's 2'-FL produced using the early-development preliminary and current fermentation media are the same.

The full analytical report and Certificates of Analysis are presented in Appendix D **[CONFIDENTIAL AND PROPRIETARY].**

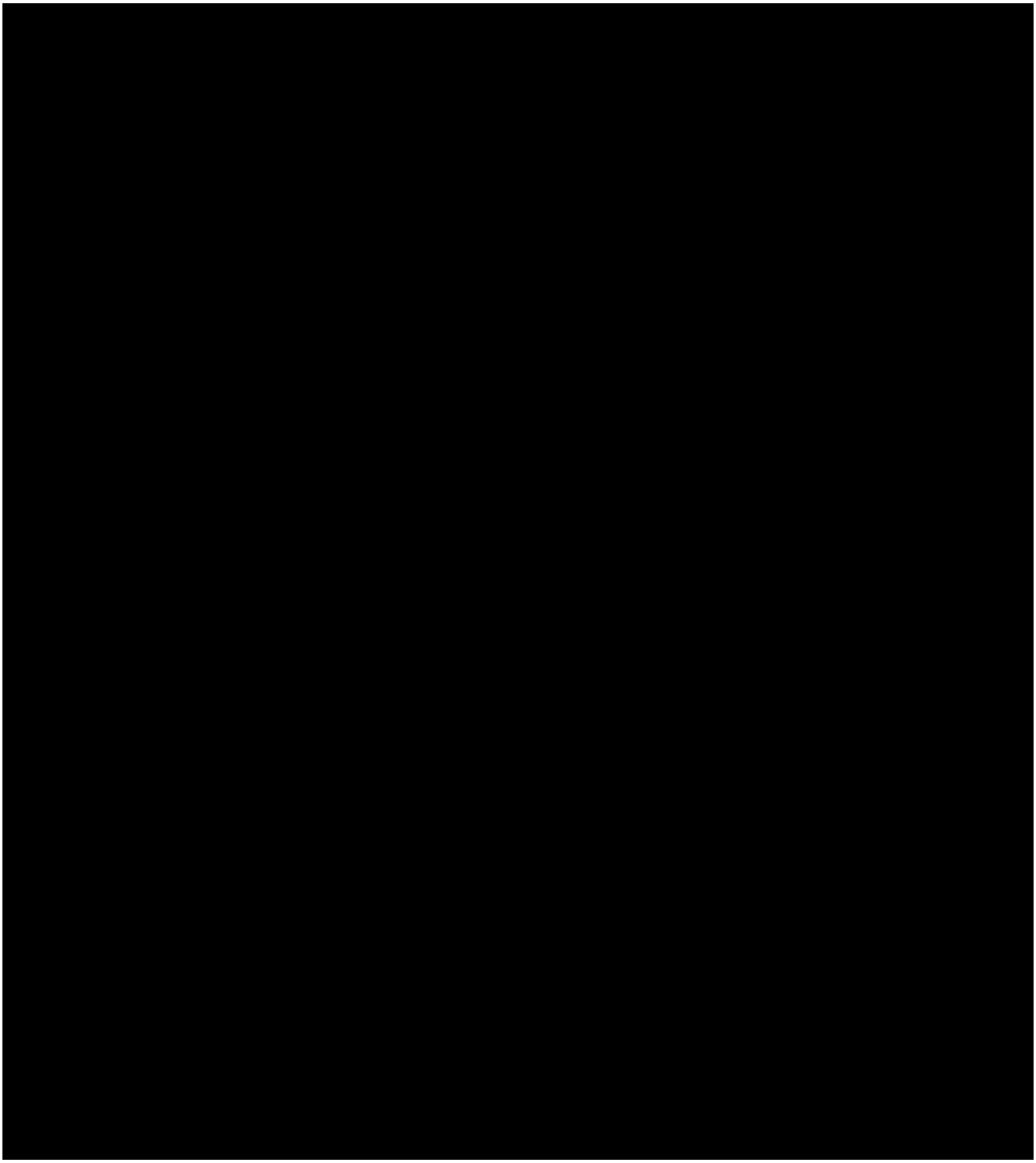
Figure B.2.2.1-6 Liquid Chromatography–Mass Spectrometry (LC-MS) Spectra for 2'-FL (Lot A)
[CONFIDENTIAL AND PROPRIETARY]

Figure B.2.2.1-7 Liquid Chromatography–Mass Spectrometry (LC-MS) Spectra for 2'-FL (Lot H)
[CONFIDENTIAL AND PROPRIETARY]

Figure B.2.2.1-8 Liquid Chromatography with Tandem Mass Spectrometry (LC-MS/MS) Spectra for 2'-Fucosyllactose (Lot A) [CONFIDENTIAL AND PROPRIETARY]

Figure B.2.2.1-9 Liquid Chromatography with Tandem Mass Spectrometry (LC-MS/MS) Spectra for 2'-Fucosyllactose (Lot TK H) [CONFIDENTIAL AND PROPRIETARY]

Figure B.2.2.1-10 Comparison of Liquid Chromatography with Tandem Mass Spectrometry (LC-MS/MS) Spectra from 2'-Fucosyllactose (2'-FL) Lot A and 2'-FL Lots H, I, and J [CONFIDENTIAL AND PROPRIETARY]



Kyowa also conducted ^{1}H NMR and ^{13}C NMR on 3 lots of 2'-FL that is the subject of the current application (Lots H, I, and J) and compared the results to those from 1 lot of 2'-FL produced according to the same manufacturing method and using the same production organism but using an early-development preliminary fermentation media (Lot A) to demonstrate that the products are the same.

In Figure B.2.2.1-11, below, the ^{1}H NMR spectra from Lot A and Lots H, I, and J are overlaid. In Figure B.2.2.1-12, the ^{13}C NMR spectra from Lot A and Lots H, I, and J are overlaid. The results confirm that Kyowa's 2'-FL produced using the early-development preliminary and current fermentation media are the same. The full analytical report and Certificates of Analysis are presented in Appendix D **[CONFIDENTIAL AND PROPRIETARY]**.

Figure B.2.2.1-11 Comparison of Proton Nuclear Magnetic Resonance Spectroscopy (^{1}H NMR) Spectra from 2'-Fucosyllactose (2'-FL) Lot A and 2'-FL Lots H, I, and J [CONFIDENTIAL AND PROPRIETARY]

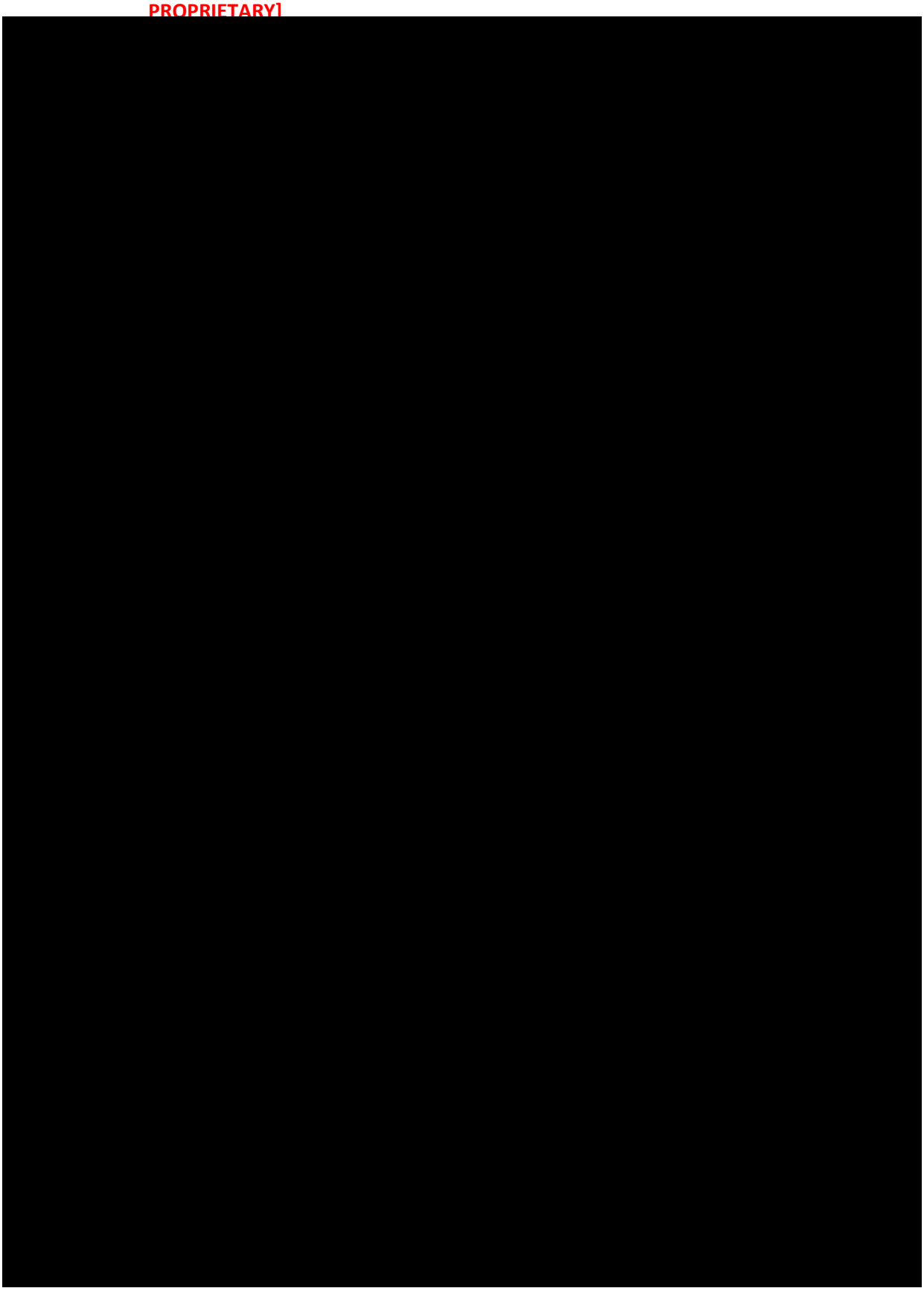
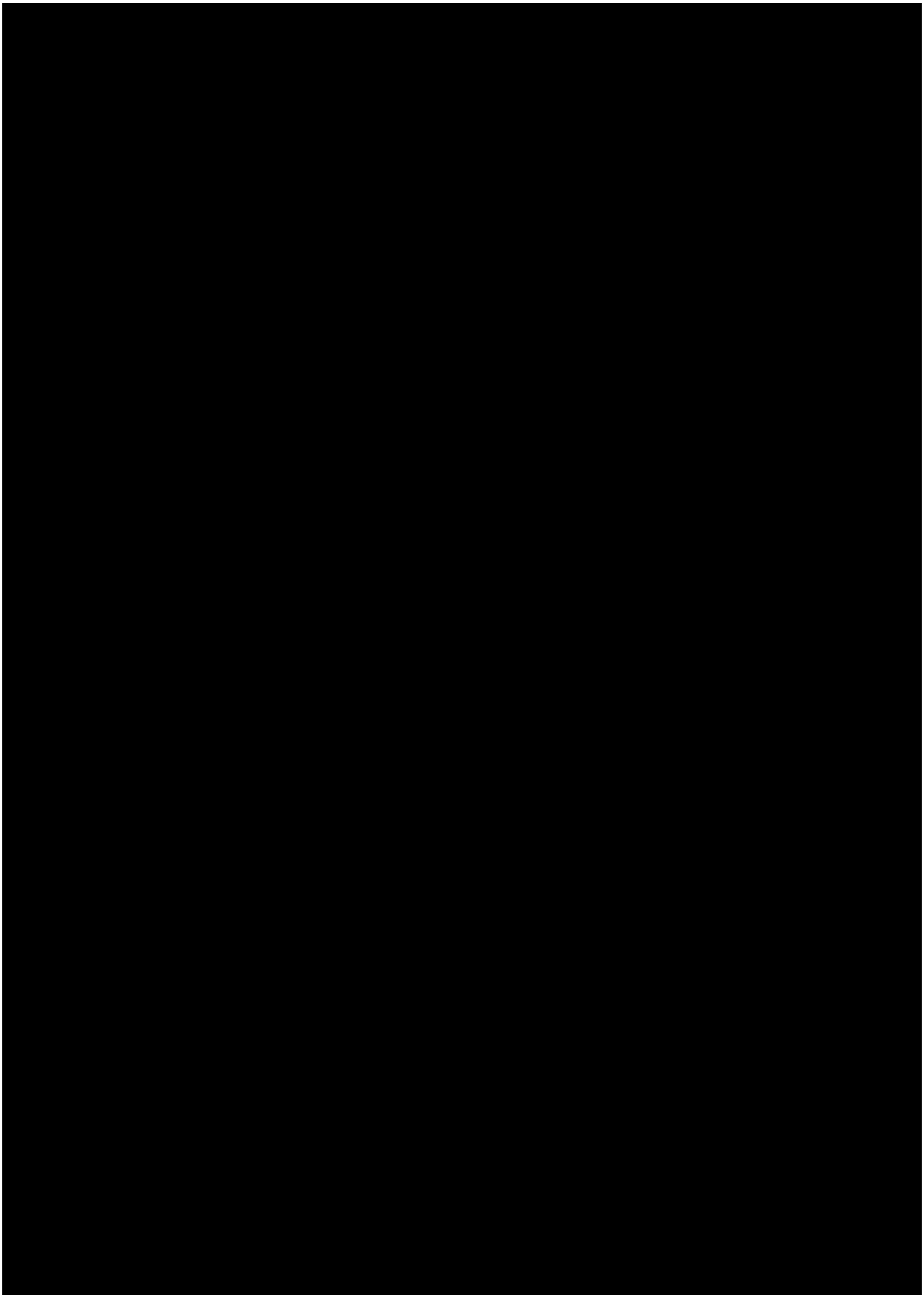


Figure B.2.2.1-12 Comparison of Carbon-13 Nuclear Magnetic Resonance Spectroscopy (^{13}C NMR) Spectra from 2'-Fucosyllactose (2'-FL) Lot A and 2'-FL Lots H, I, and J [CONFIDENTIAL AND PROPRIETARY]



B.2.2.2 Solubility

Kyowa has conducted a solubility test without ultrafiltration on the final 2'-FL powdered product (Lot D) in accordance with Organisation for Economic Co-operation and Development (OECD) Test Guideline (TG) 105 (*Flask method*; OECD, 1995). Study methodology and results are provided in Appendix G **[CONFIDENTIAL AND PROPRIETARY]** and demonstrate that Kyowa's final 2'-FL ingredient has a water solubility of 739 g/L.

Kyowa also has conducted a solubility test with ultrafiltration on 5 representative batches of the final 2'-FL powdered product (Lots A, D, H, I, and J) in accordance with OECD TG 105 (OECD, 1995). Study methodology and results are provided in Appendix G **[CONFIDENTIAL AND PROPRIETARY]**. The results are presented in Table B.2.2.2-1, below and demonstrate that Kyowa's final 2'-FL ingredient has a water solubility range of approximately 440 to 453 g/L.

Table B.2.2.2-1 Solubility Analysis of 5 Representative Batches of 2'-Fucosyllactose

Parameter	Manufacturing Lot No.				
	A	D	H	I	J
Solubility (g/L)[a]	446	445	453	440	443

[a] Average of measurements taken at 24, 48, and 72 hours.

B.2.2.3 Particle Size

Appraisal routes to confirm whether or not a material contains a fraction of small particles are provided in EFSA's *Guidance on technical requirements for regulated food and feed product applications to establish the presence of small particles including nanoparticles* (EFSA, 2021). Solubility in water assessed using OECD Test Guideline 105 (OECD, 1995) or equivalent is one appraisal route, and it is stated in the EFSA Scientific Committee (EFSA, 2021) guidance, "*If the solubility of the substance in water is equal to or higher than 33.3 g/L, no additional assessment for the fraction of small particles is needed*."

Kyowa has assessed the solubility of 2'-FL using the flask method test of OECD Test Guideline 105 (see Table B.2.2.2-1, above) and concluded the water solubility of 2'-FL to be approximately 440 to 453 g/L which is significantly greater than 33.3 g/L (OECD, 1995). Therefore, the criterion for solubility has been met and no additional assessment for the fraction of small particles within 2'-FL is needed to confirm particle size.

B.2.2.4 Stability

Kyowa's proposed addition to the Code of a new microbial source of production for 2'-FL has no effect on the chemical or structural identity of the 2'-FL in the final ingredient. Furthermore, the contents of additional carbohydrates in Kyowa's 2'-FL produced using a genetically modified strain of *E. coli* W are comparable to those of the other 2'-FL ingredients currently permitted under Schedule 29-5 of the Code.

Kyowa has investigated the stability of the 2'-FL ingredient under accelerated storage conditions (see Section B.2.2.4.1, below) and normal storage conditions (see Section B.2.2.4.2) to assess the physicochemical and biochemical stability of the ingredient and to investigate the potential degradation products. Microbiological stability of the 2'-FL final ingredient has been addressed through the investigation of water activity and analyses after long-term storage for 30 months and storage under accelerated conditions for 6 months (see Section C.2.2.4.3, below). Stability in final food matrices has been assessed using publicly available information on other 2'-FL preparations (see Section C.2.2.4.4).

B.2.2.4.1 Accelerated Storage Conditions

Although the change in the production organism is not expected to affect the stability of the final product, Kyowa has conducted a study to assess the physicochemical and biochemical bulk stability of 5 lots of 2'-FL produced according to the same manufacturing method and using the same production organism but using an early-development preliminary fermentation medium, under accelerated conditions (temperature of 40 ± 2°C; 75 ± 5% relative humidity) over a 6-month period. 2'-FL was stored in polyethylene bags within an aluminium foil bag, which are similar packaging materials to those intended for storage and distribution of the commercial product. The results are shown in Table B.2.2.4.1-1, below. 2'-FL was stable throughout the 6-month storage period and remained within specification limits with no significant change in physicochemical parameters (appearance, colour, pH, water activity) or biochemical parameters (purity, carbohydrate profile, and water content). Details pertaining to methodology and results are provided in Appendix H [CONFIDENTIAL AND PROPRIETARY].

Table B.2.2.4.1-1 Summary of Accelerated Stability Testing (40 ± 2°C; 75 ± 5% Relative Humidity) for 2'-Fucosyllactose Produced with a Genetically Modified Strain of *Escherichia coli* W

Parameter	Specification	Storage Time (months)			
		0	2	4	6
Lot B					
Appearance	Powder	Complies	Complies	Complies	Complies
Colour	White to off-white	Complies	Complies	Complies	Complies
Purity (dwb%)	≥82	92	91	91	91
Water (w/w%)	≤9.0	3.9	3.5	3.5	3.5
Water activity (Aw)	NA	0.25	-	-	0.18
pH (25°C; 5% solution)	4.0 to 9.0	6.4	6.5	7.0	6.5
D-Lactose (w/w%)[a]	≤5	2.7	2.7	2.7	2.7
L-Fucose (w/w%)[a]	≤1	0.1	0.1	0.1	0.1
D-Glucose and D-Galactose (w/w%)[a]	≤1	0.1	0.1	0.1	0.1
Fucosyl-Galactose (w/w%)[a]	≤3	0.5	0.5	0.5	0.5
Difucosyllactose (w/w%)[a]	≤3	1.4	1.4	1.4	1.5
Lot C					
Appearance	Powder	Complies	Complies	Complies	Complies
Colour	White to off-white	Complies	Complies	Complies	Complies
Purity (dwb%)	≥82	92	93	93	93
Water (w/w%)	≤9.0	3.9	3.6	3.6	3.6
Water activity (Aw)	NA	0.25	-	-	0.22
pH (25°C; 5% solution)	4.0 to 9.0	6.2	6.5	6.7	6.6
D-Lactose (w/w%)[a]	≤5	2.3	2.3	2.3	2.4
L-Fucose (w/w%)[a]	≤1	0.1	0.1	0.1	0.1
D-Glucose and D-Galactose (w/w%)[a]	≤1	0.1	0.1	0.1	0.1
Fucosyl-Galactose (w/w%)[a]	≤3	0.4	0.4	0.4	0.4
Difucosyllactose (w/w%)[a]	≤3	0.9	0.9	0.9	0.9
Lot D					
Appearance	Powder	Complies	Complies	Complies	Complies

Table B.2.2.4.1-1 Summary of Accelerated Stability Testing (40 ± 2°C; 75 ± 5% Relative Humidity) for 2'-Fucosyllactose Produced with a Genetically Modified Strain of *Escherichia coli* W

Parameter	Specification	Storage Time (months)			
		0	2	4	6
Colour	White to off-white	Complies	Complies	Complies	Complies
Purity (dwb%)	≥82	91	95	95	93
Water (w/w%)	≤9.0	2.7	2.8	2.9	2.9
Water activity (Aw)	NA	0.14	-	-	<0.11 (LOD)
pH (25°C; 5% solution)	4.0 to 9.0	5.7	6.0	6.1	6.2
D-Lactose (w/w%)[a]	≤5	2.4	2.4	2.3	2.4
L-Fucose (w/w%)[a]	≤1	0.1	0.1	0.2	0.2
D-Glucose and D-Galactose (w/w%)[a]	≤1	0.2	0.2	0.2	0.2
Fucosyl-Galactose (w/w%)[a]	≤3	0.9	0.9	0.9	1.0
Difucosyllactose (w/w%)[a]	≤3	1.0	1.0	1.0	1.1
Lot E					
Appearance	Powder	Complies	Complies	Complies	Complies
Colour	White to off-white	Complies	Complies	Complies	Complies
Purity (dwb%)	≥82	96	96	95	95
Water (w/w%)	≤9.0	2.8	2.8	3.2	2.9
Water activity (Aw)	NA	0.14	-	-	<0.11 (LOD)
pH (25°C; 5% solution)	4.0 to 9.0	6.1	6.2	5.8	6.2
D-Lactose (w/w%)[a]	≤5	2.1	2.2	2.1	2.1
L-Fucose (w/w%)[a]	≤1	0.1	0.1	0.1	0.1
D-Glucose and D-Galactose (w/w%)[a]	≤1	≤0.05	≤0.05	≤0.05	≤0.05
Fucosyl-Galactose (w/w%)[a]	≤3	0.1	0.2	0.2	0.2
Difucosyllactose (w/w%)[a]	≤3	1.1	1.1	1.1	1.1
Lot F					
Appearance	Powder	Complies	Complies	Complies	Complies
Colour	White to off-white	Complies	Complies	Complies	Complies
Purity (dwb%)	≥82	94	95	94	94
Water (w/w%)	≤9.0	2.3	2.4	2.6	2.5
Water activity (Aw)	NA	0.12	-	-	<0.11 (LOD)
pH (25°C; 5% solution)	4.0 to 9.0	6.2	6.0	6.2	6.1
D-Lactose (w/w%)[a]	≤5	2.3	2.3	2.3	2.3
L-Fucose (w/w%)[a]	≤1	0.1	0.1	0.2	0.2
D-Glucose and D-Galactose (w/w%)[a]	≤1	0.1	0.1	0.1	0.1
Fucosyl-Galactose (w/w%)[a]	≤3	0.8	0.8	0.8	0.9
Difucosyllactose (w/w%)[a]	≤3	1.0	1.0	1.0	1.1

- = batches not analyzed or planned to be analyzed at this timepoint; dwb = dry weight basis; LOD = limit of detection; NA = not applicable.

[a] Limit of quantification = 0.05 % (w/w).

B.2.2.4.2 Normal Storage Conditions

The recommended storage conditions for 2'-FL are at room temperature. A study was conducted by Kyowa to assess the physicochemical and biochemical stability of 1 lot of 2'-FL produced using a genetically modified strain of *E. coli* W (Lot A; produced according to the same manufacturing method and using the same production organism but using an early-development preliminary fermentation medium) under standard room-temperature conditions (25 ± 2°C; 60 ± 5% relative humidity). 2'-FL was stored in polyethylene bags within an aluminium foil bag, which are similar packaging materials used for storage and distribution of the commercial product. The duration of the study was 36 months (*i.e.*, the proposed shelf life of 2'-FL), with analyses conducted at 0, 2, 4, 6, 9, 12, 18, 24, 30, and 36 months.

The results shown in Table B.2.2.4.2-1, below, demonstrate that 2'-FL was stable throughout the 36-month storage period and remained within specification limits, with no significant change in physicochemical (appearance, colour, pH, water activity) or biochemical (purity, carbohydrate profile, and water content) parameters. Although increases in pH can be seen, neutral sugars such as 2'-FL do not have buffering capacity, and thus, fluctuations in pH are likely to occur. Nonetheless, Kyowa's 2'-FL remained within specification limits for pH through 36 months of storage. The results of this study support a 36-month shelf life, which is the proposed shelf life for Kyowa's 2'-FL ingredient. See Appendix H **[CONFIDENTIAL AND PROPRIETARY]** for details pertaining to the study plan, methodology, and results.

Table B.2.2.4.2-1 Summary of Stability Testing of 1 Lot of 2'-Fucosyllactose (Lot A) produced with a Genetically Modified Strain of *Escherichia coli* W Under Standard Conditions (25 ± 2°C; 60 ± 5% Relative Humidity)

Parameter	Specification	Storage Time (months)									
		0	2	4	6	9	12	18	24	30	36
Appearance	Powder	Complies	Complies	Complies	Complies	Complies	Complies	Complies	Complies	Complies	Complies
Colour	White to off-white	Complies	Complies	Complies	Complies	Complies	Complies	Complies	Complies	Complies	Complies
Purity (dwb%)	≥82	92	92	92	91	96	94	93	94	92	97
Water (w/w%)	≤9.0	5.0	3.9	3.8	3.7	3.7	3.5	4.2	3.8	3.9	3.9
Water activity (Aw)	NA	0.22	-	-	0.26	-	0.19	-	0.23	-	-
pH (25°C; 5% solution)	4.0 to 9.0	6.3	6.6	7.8	7.8	8.2	7.8	7.9	6.8	8.1	7.6
D-Lactose (w/w%)[a]	≤5	3.1	2.6	2.8	2.8	3.0	2.7	3.0	3.0	3.1	2.9
L-Fucose (w/w%)[a]	≤1	≤0.05	≤0.05	≤0.05	≤0.05	≤0.05	≤0.05	0.06	0.06	0.06	0.06
D-Glucose and D-Galactose (w/w%)[a]	≤1	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Fucosyl-Galactose (w/w%)[a]	≤3	0.8	0.7	0.7	0.7	0.8	0.7	0.8	0.8	0.8	0.8
Difucosyllactose (w/w%)[a]	≤3	0.5	0.4	0.5	0.5	0.5	0.5	0.5	0.5	0.6	0.5

"-" = not measured; dwb = dry weight basis; NA = not applicable.

[a] Limit of quantification = 0.05 % (w/w).

B.2.2.4.3 Microbiological Stability

In an EFSA BIOHAZ Panel Opinion on factors that affect microbial survival and growth in composite products and foods in general, EFSA noted that foods with measured water activity of <0.88 generally "*do not permit growth of or toxin formation by food-borne pathogenic bacteria*" (EFSA, 2012). Kyowa therefore measured the water activity of 2'-FL after 0 and 6 months of storage under accelerated conditions (40°C; 75% relative humidity), and after 0, 6, 12, and 24 months of storage under standard conditions (25°C; 60% relative humidity). As shown in Tables B.2.2.4.1-1 and C.2.2.4.2-1, above, the water activity of 2'-FL was considerably lower than 0.88 at all timepoints of evaluation and conditions of storage, with values not exceeding 0.26. The low water content of the analyzed batches of 2'-FL indicate also that the storage packaging prevents water absorption by the 2'-FL ingredient. Based on the low water content and water activity values, microbial growth or toxin formation in Kyowa's 2'-FL ingredient is unlikely. See Appendix H **[CONFIDENTIAL AND PROPRIETARY]** for details pertaining to the study plan, methodology, and results to date.

To confirm the microbiological stability of Kyowa's 2'-FL, microbiological analysis was conducted on Lot A following storage for 30 months in the real-time stability study described above in Section B.2.2.4.2 and on Lots B and C following storage for 6 months as in the accelerated stability study described above in Section B.2.2.4.1. As shown in Table B.2.2.4.3-1, below, there were no microbiological contaminants detected and the results remained within specification limits following 30 months of storage under normal conditions and 6 months under accelerated conditions, demonstrating the microbiological stability of Kyowa's 2'-FL. See Appendix H **[CONFIDENTIAL AND PROPRIETARY]** for the Certificates of Analysis.

Table B.2.2.4.3-1 Microbiological Stability Following Storage Under Standard Conditions (25 ± 2°C; 60 ± 5% Relative Humidity) for 30 Months and Accelerated Conditions (40 ± 2°C; 75 ± 5% Relative Humidity) for 6 Months

Storage Conditions		Standard Conditions (25 ± 2°C; 60 ± 5% Relative Humidity) for 30 Months	Accelerated (40 ± 2°C; 75 ± 5% Relative Humidity) for 6 Months	
Parameter	**Specification**	**Lot A**	**Lot B**	**Lot C**
Aerobic plate count	≤1,000 CFU/g	< 10 CFU/g	< 10 CFU/g	< 10 CFU/g
Yeasts and moulds	≤100 CFU/g	< 100 CFU/g	< 100 CFU/g	< 100 CFU/g
Salmonella	Negative in 100 g	NT	Negative	Negative
Enterobacteriaceae	Negative in 10 g	NT	Negative	Negative
Cronobacter spp. (Enterobacter sakazakii)	Negative in 100 g	NT	Negative	Negative
Listeria monocytogenes	Negative in 25 g	NT	Negative	Negative
Bacillus cereus (CFU/g)	≤50	NT	<10 CFU/g	<10 CFU/g

CFU = colony-forming units; NT = not tested.

B.2.2.4.4 Stability in Intended Food Uses

As discussed above, Kyowa's proposed amendment to the microbial source of production for 2'-FL has no effect on the chemical or structural identity of the 2'-FL in the final ingredient. Furthermore, the contents of additional carbohydrates in Kyowa's 2'-FL produced using a genetically modified strain of *E. coli* W are comparable to those of the other 2'-FL ingredients included in the EU Union List and in the Code (synthetic or microbial source). Therefore, no changes are expected with respect to the stability profile of Kyowa's 2'-FL ingredient compared to the ingredients currently included when incorporated into food matrices.

The stability of 2'-FL in matrices representative of the authorized uses in Schedule 29-5 has previously been assessed by the EFSA (2015) and FSANZ as part of applications for chemically identical 2'-FL produced synthetically or by fermentation using other microbial sources. Specifically, no significant losses of 2'-FL were observed when 2'-FL was added to infant formula (stored for 3 years at temperatures of 4, 20, 30, and 37°C), yogurt (stored for 21 days at 4°C), citrus juice (stored for 28 days at 4°C), or ready-to-drink flavoured milk (stored for 14 days [pasteurized] or 28 days [ultra-high temperature–treated] at 4°C). The EFSA (2015) concluded that the "*data provide sufficient information with respect to the stability of the NFI* [novel food ingredient]."

On the basis that Kyowa's 2'-FL is structurally and chemically identical to other authorized sources of 2'-FL in the EU Union List and in the Code, and that the additional carbohydrate profile is similar, the results of the stability studies conducted with other 2'-FL ingredients can be considered representative of the stability of Kyowa's 2'-FL ingredient. Therefore, stability data under the intended conditions of use conducted with Kyowa's 2'-FL ingredient are not considered necessary due to the existence of previously evaluated stability data demonstrating the stability of 2'-FL under the intended conditions of use (which are the same uses as those intended for Kyowa's 2'-FL ingredient).

B.2.3 Information on the Impurity Profile

Kyowa's 2'-FL is a white to off-white powder with a purity of at least 82% 2'-FL. Specified limits for water content and ash in Kyowa's 2'-FL are ≤9.0 w/w% and ≤0.5 w/w%, respectively. Kyowa has established limits for potential impurities of the production process, including D-lactose (≤5%), L-fucose (≤1%), difucosyllactose (≤3%), fucosylgalactose (≤3%), and D-glucose and D-galactose (≤1% combined). These specifications are equal to or lower than the specification limits for the same compounds in other 2'-FL ingredients included in the EU Union List and in Schedule 3 of the Code.

Kyowa's specified limits for lead, arsenic, cadmium, and mercury of ≤0.1 mg/kg (individually), and iron of ≤10 mg/kg, also are equal to or less than limits for heavy metals in other 2'-FL ingredients currently included in Schedule 3 of the Code, and are within the limits for heavy metals listed in Section S3-4 of Schedule 3 of the Code.

Kyowa's specifications for residual proteins (≤100 mg/kg) and residual endotoxins (≤10 endotoxin units [E.U.]/mg) also are equal to or less than specifications for these parameters for other 2'-FL ingredients included in the EU Union List and in Schedule 3 of the Code.

Further information on the specifications for identity and purity of Kyowa's 2'-FL, and analytical data supporting consistent adherence to these specifications, are provided in Section B.2.4.

B.2.3.1 Absence of Production Organism and DNA

Kyowa's final 2'-FL ingredient was assessed for residual viable cells of the production organism using a culture method conducted in accordance with EFSA's *Guidance on the characterization of microorganisms used as feed additives or as production organisms* (EFSA, 2018). Briefly, 10 g samples of 3 lots of 2'-FL produced according to the same manufacturing method and using the same production organism but using an early-development preliminary fermentation medium (Lots D, E, and F) were inoculated (500 µL/plate, 20 plates per sample) and cultured in triplicate in violet red bile dextrose (VRBD) agar medium at 37°C for 3 days. The production organism cultured in LB medium at 30°C for 16 hours was diluted, suspended in sterile water, inoculated to VRBD agar (500 µL/plate), and incubated at 37°C for 3 days and overnight was used as a positive control. The results of this test (detailed in Appendix F **[CONFIDENTIAL AND PROPRIETARY]**) demonstrate that the production strain was detected in the positive control and the number of detected cells was greater than 25, which meets the requirements in EFSA's Guidance. In the tests on the 2'-FL samples, viable cells from the production strain were not detected (colony count of 0 in all samples), which confirms the absence of viable cells from the production strain in the final product. The full report is presented in Appendix F **[CONFIDENTIAL AND PROPRIETARY]**.

To confirm the absence of residual production organism–derived DNA in the final product, Kyowa conducted a quantitative PCR analysis using 3 lots of 2'-FL produced according to the same manufacturing method and using the same production organism but using an early-development preliminary fermentation medium (assayed in triplicate). The analysis was conducted in accordance with EFSA's *Guidance on the characterization of microorganisms used as feed additives or as production organisms* (EFSA, 2018). The quantitative PCR assay was conducted using primers specific to the production organism, with DNA extracted from the production organism used as a positive control. The results of this test (detailed in Appendix F **[CONFIDENTIAL AND PROPRIETARY]**) demonstrate that there is no detectable residual DNA (detection limit of 5 µg/kg, or 5 ppb) in the final 2'-FL ingredient.

As indicated in Section B.2.4, below, the production organism is removed during the purification processes of the manufacturing process by a combination of microfiltration, filtration through cationic and anionic exchange resins, and ultra-filtration (molecular weight cut-off of 6,000 Da). The absence of the production organism in the final 2'-FL ingredient is further demonstrated by the lack of detection of Enterobacteriaceae (the family to which *E. coli* W belongs) in microbiological batch analyses according to internationally recognized methods International Organization for Standardization (ISO) 21528-1:2017) (see Table B.2.5.2.1-1).

B.2.4 Manufacturing Process

B.2.4.1 Non-Confidential Description of the Production Process

Kyowa's 2'-FL produced by a genetically modified strain of *E. coli* W is manufactured using food-grade raw materials and processing aids in accordance with a detailed Hazard Analysis and Critical Control Points (HACCP) plan using a 2-step process that includes fermentation and purification steps. In the first step, the production microorganism is cultured in chemically defined nutrient media under sterile conditions in tightly controlled conditions. In the main fermentation process, the production microorganism uses glucose and lactose to synthesize 2'-FL, which is excreted into the media. In the second step, 2'-FL is isolated and purified from the fermentation medium using a series of filtration and cationic and anionic exchange steps, followed by concentration and spray-drying to obtain the final 2'-FL product. A detailed description of the manufacturing process is provided in Appendix E **[CONFIDENTIAL AND PROPRIETARY]**.

B.2.4.2 Manufacturing Process

B.2.4.2.1 Raw Materials and Processing Aids

The raw materials and processing aids used in the production of 2'-FL, and specifications for the filters used, are provided in Appendix E **[CONFIDENTIAL AND PROPRIETARY]**. All raw materials and processing aids used in the production of 2'-FL are food-grade quality or of a higher standard. Glucose and lactose are the main carbon sources added to the fermentation medium during the fermentation process.

B.2.4.2.2 Production Method

The manufacture of 2'-FL by fermentation with a genetically modified strain of *E. coli* W involves 2 main steps: fermentation and purification. The manufacturing process is controlled by a HACCP plan which is described in detail in Appendix I **[CONFIDENTIAL AND PROPRIETARY]**. Each of the 2 steps is briefly described below, along with a schematic overview of the fermentation and purification processes (see Figure B.2.4.2.2-1).

Fermentation Process

The fermentation process to produce 2'-FL is conducted using chemically defined nutrient media under sterile conditions. A master frozen cell bank is prepared for the modified *E. coli* W production strain. Cells from the master cell bank are inoculated to produce the working frozen cell bank. Cells from the working cell bank are then inoculated to produce the flask seed culture. The genetic stability from a minimum of 3 cell passages from the master and working cell bank is verified based on 2'-FL production, cell growth, oxygen consumption, and other functional parameters indicating a change in cell culture behaviour.

The cells from the working cell bank are inoculated to flasks and cultured. Subsequently, the cell cultures are transferred to the factory seed medium; the process conditions of this fermentation step are tightly controlled. The seed culture step is complete when a specific optical density is reached.

In the main fermentation, the medium is first inoculated with the production strain factory seed culture and fermented in the presence of glucose. Following the depletion of glucose in the culture medium, lactose and glucose are fed to the culture medium. The main fermentation is maintained at a constant temperature until the completion of feeding. During the feeding step, the production strain takes up the lactose and glucose for the synthesis of 2'-FL, which is excreted into the media. As with the initial fermentations, the process conditions of the main fermentation are tightly controlled. The production of 2'-FL is stopped *via* heat treatment (sterilization), after which the broth is cooled and acidified.

Purification Process

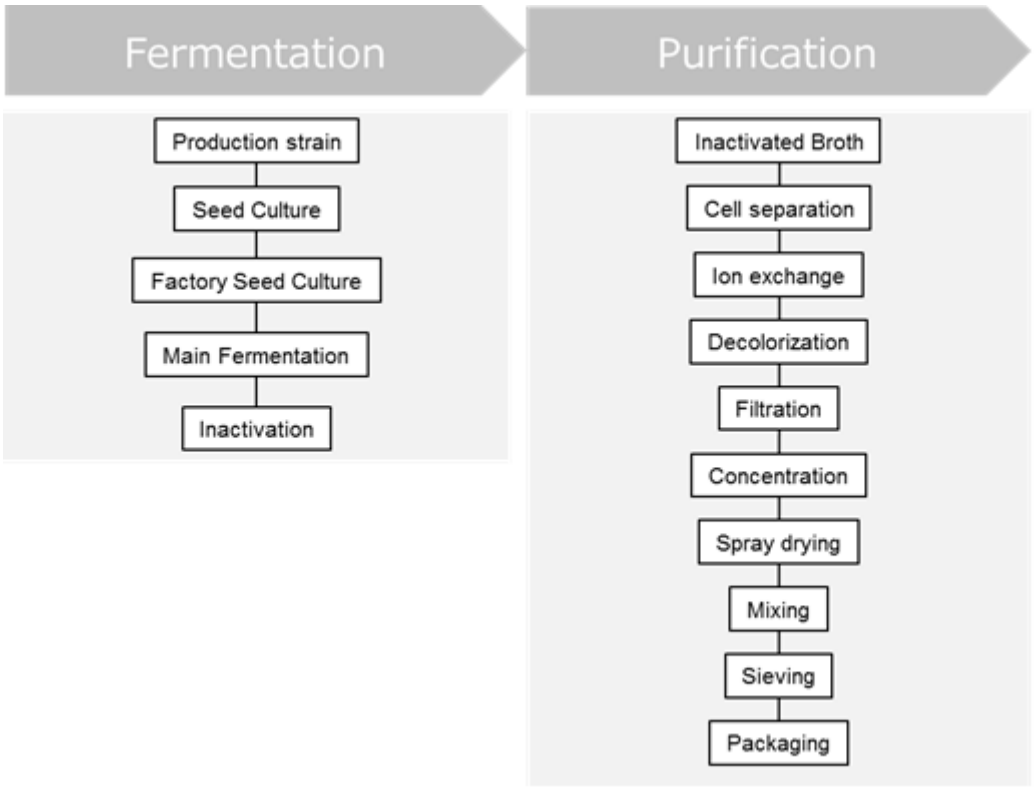
The intact production organism cells are removed *via* microfiltration. The obtained solution is then passed through a series of cationic resin and anionic resin ion exchangers to remove cations, anions, minerals, and organic impurities. The pH of the effluent is adjusted, and the solution is concentrated. The concentrated solution is decolourised with activated carbon, and then filtered in a series of filtration steps using a microfiltration membrane and an ultra-filtration membrane to remove potential endotoxins, as well as any residual protein, organic impurities, and production organism not removed by the cationic/anionic exchange resins. The obtained solution is then further concentrated, spray-dried, mixed using an air blender, and then passed through a sieve to remove foreign materials to obtain the final 2'-FL product as outlined in Figure B.2.4.2.2-1.

Storage and Packaging

The final 2'-FL ingredient is packaged directly in a clean room using aluminium bags with low-density polyethylene (LDPE)/linear low-density polyethylene (LLDPE) that are highly shielding against moisture. The packaged 2'-FL product is stored at 25°C and 60% RH or lower.

Following packaging, a sample bag is taken and analyzed for all specification parameters to confirm that the final 2'-FL product meets the required specifications. Only lots that meet all specification parameters are released for shipment and sale.

Figure B.2.4.2.2-1 Schematic Overview of the Fermentation and Purification Processes for 2'-Fucosyllactose Produced Using a Genetically Modified Strain of *Escherichia coli* W



B.2.4.2.3 Quality Control

The manufacturing process for 2'-FL produced by microbial fermentation of a genetically modified strain of *E. coli* W is controlled by a HACCP plan which is described in detail in Appendix I **[CONFIDENTIAL AND PROPRIETARY].** A HACCP plan for the liquid glucose starting material, and a decision tree to identify operational prerequisite programs (OPRPs) and critical control points (CCPs), are also provided in Appendix I **[CONFIDENTIAL AND PROPRIETARY]**. The manufacturing facility has been certified to comply with the requirements of Food Safety System Certification 22000 (FSSC 22000). See Appendix I **[CONFIDENTIAL AND PROPRIETARY]** for the FSSC 22000 certificate.

The production process includes 2 CCPs, consisting of monitoring of the membrane filtration to ensure no bacterial contamination and X-ray inspection to ensure the absence of foreign materials in the final 2'-FL ingredient. The product specifications for the reverse osmosis (RO) system and the ultrafiltration membrane used in the water purification are provided in Appendix E **[CONFIDENTIAL AND PROPRIETARY]**.

B.2.5 Specification for Identity and Purity

B.2.5.1 Specifications

Kyowa's proposed qualitative and quantitative specifications for identity and purity of 2'-FL produced with a genetically modified strain of *E. coli* W are presented in Table B.2.5.1-1, below, and compared to the current specifications included in the EU Union List of novel foods and in Schedule 3 of the Code for 2'-FL ingredients from other sources. Parameters were selected based on the analytical compositional data provided in Section B.2.5.2 and to ensure control of levels of potential contaminants, including residual proteins, residual endotoxins, heavy metals, and microbiological parameters.

Kyowa's final product is a white to off-white powder with a purity of at least 82% 2'-FL as determined by an in-house method confirmed to be suitable (high-performance liquid chromatography with pulsed amperometric detection [HPLC-PAD]).[4] The proposed purity specification for Kyowa's 2'-FL produced using a genetically modified strain of *E. coli* W is similar to the purity of 2'-FL included in the EU Union List produced using a genetically modified strain of *E. coli* K-12 (*i.e.*, ≥82% *versus* ≥83%); lower than the purity specifications for 2'-FL ingredients produced using a genetically modified strain of *E. coli* K-12 included in Schedule 3 of the Code; and lower than 2'-FL produced using modified *E. coli* BL21 or from chemical synthesis included in the EU Union List (*i.e.*, ≥90 to ≥95% purity).

Kyowa also has established limits for potential impurities of the production process, including D-lactose (≤5%), L-fucose (≤1%), difucosyllactose (≤3%), fucosylgalactose (≤3%), and D-glucose and D-galactose (≤1% combined) (all determined by HPLC-PAD). These specifications are equal to or lower than the specification limits for the same compounds in other 2'-FL ingredients (synthetic and microbial source) included in the EU Union List and in Schedule 3 of the Code (see Table B.2.5.1-1, below). Lactose, fucose, and difucosyllactose are naturally occurring components of human milk: fucosylgalactose is a naturally occurring breakdown product of fucosylated oligosaccharides; galactose is a naturally occurring breakdown product of lactose; and glucose is a naturally occurring breakdown product of lactose, a common dietary component, and serves as a starting material for the biosynthesis of 2'-FL. The exposures to these carbohydrates from the intended uses of Kyowa's 2'-FL ingredient (*i.e.,* those currently permitted under Schedule 26-3 of the Code) are expected to be insignificant compared to background exposures and are not expected to pose safety concerns. In addition, residual proteins are specified to be ≤100 mg/kg (determined by the Bradford assay) in Kyowa's final 2'-FL ingredient; this specification is equal to specifications for residual proteins for other 2'-FL ingredients (synthetic and microbial source) included in the EU Union List and in Schedule 3 of the Code. Kyowa's specification for residual endotoxins (≤10 E.U./mg; determined with Section 4.01, kinetic-turbidimetric method, of the *Japanese Pharmacopoeia*, 17th Edition) is identical to the specification for this parameter for synthetic and *E. coli* K-12–derived 2'-FL as included in the EU Union List, and to *E. coli* K-12– and *E. coli* BL21–derived 2'-FL in Schedule 3 of the Code.

[4] Details of Kyowa's in-house HPLC-PAD method are provided in Appendix K **[CONFIDENTIAL AND PROPRIETARY]**.

The specified limit for water content in Kyowa's 2'-FL ingredient is ≤9.0 w/w% as determined by Karl-Fischer titration (as specified in Section 2.48 of the *Japanese Pharmacopoeia*, 17th Edition). The ash component of the final ingredient is specified to be ≤0.5 w/w%. The final ingredient is specified to have a pH between 4.0 and 9.0 when analyzed in 5% solution at 25°C. The specification limits for lead, arsenic, cadmium, and mercury of ≤0.1 mg/kg (individually), and iron of ≤10mg/kg, in the final ingredient are equal to or less than limits for heavy metals in other 2'-FL ingredients currently included in Schedule 3 of the Code and are within the limits for heavy metals listed in Section S3-4 of Schedule 3 of the Code. Methods of analysis were obtained from the *United States Pharmacopeia* or the *Japanese Pharmacopeia* or were developed and concluded to be suitable by Kyowa. Internal methods, including identification and quantification of 2'-FL, are provided in Appendix J **[CONFIDENTIAL AND PROPRIETARY]**. Documentation of the suitability of the HPLC methods is provided in Appendix J **[CONFIDENTIAL AND PROPRIETARY]**.

Table B.2.5.1-1 Proposed Specifications for Kyowa's 2'-Fucosyllactose (2'-FL) Produced with a Genetically Modified Strain of *Escherichia coli* W Compared to Current Specifications for 2'-FL in the EU Union List and in Schedule 3 of the Food Standards Australia New Zealand *Food Standards Code*

Parameter	Kyowa's Proposed Specifications for 2'-FL	Method	Existing Specifications for 2'-FL in the EU Union List		Existing Specifications in Schedule 3 of the FSANZ *Food Standards Code*		
			Synthetic	Genetically Modified Strain of *E. coli* K-12	S3-47 - Genetically Modified Strain of *E. coli* K-12	S3-40 – 2'-FL Sourced from *E. coli* K-12	S3-45 – 2'-FL sourced from *E. coli* BL21
Definition							
Chemical name	α-L-Fucopyranosyl-(1→2)-β-d-galactopyranosyl-(1→4)-d-glucopyranose	-	α-L-Fucopyranosyl-(1→2)-β-d-galactopyranosyl-(1→4)-d-glucopyranose	α-L-Fucopyranosyl-(1→2)-β-d-galactopyranosyl-(1→4)-d-glucopyranose	α-L-Fucopyranosyl-(1→2)-β-d-galactopyranosyl-(1→4)-d-glucopyranose	α-L-fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-D-glucopyranose	α-L-fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-D-glucopyranose
Chemical formula	$C_{18}H_{32}O_{15}$	-	$C_{18}H_{32}O_{15}$	$C_{18}H_{32}O_{15}$	$C_{18}H_{32}O_{15}$	$C_{18}H_{32}O_{15}$	$C_{18}H_{32}O_{15}$
CAS No.	41263-94-9	-	41263-94-9	41263-94-9	41263-94-9	41263-94-9	41263-94-9
Molecular weight	488.44 g/mol	-	488.44 g/mol	488.44 g/mol	488.44 g/mol	-	-
Source	Genetically modified strain of *E. coli* W		Synthetic	Genetically modified strain of *E. coli* K-12	Genetically modified strain of *E. coli* BL21	*E. coli* K12	*E. coli* BL21
Description	2'-FL is a white to off-white powder that is produced by a microbial process.	-	2'-FL is a white to off-white powder that is produced by a chemical synthesis process.	2'-FL is a white to off-white crystalline powder that is produced by a microbial process.	2'-FL is a white to off-white powder and the liquid concentrate (45% ± 5% w/v) aqueous solution is a colourless to slight yellow clear aqueous solution. 2'-FL is produced by a microbiological process.	-	-

Table B.2.5.1-1 Proposed Specifications for Kyowa's 2'-Fucosyllactose (2'-FL) Produced with a Genetically Modified Strain of *Escherichia coli* W Compared to Current Specifications for 2'-FL in the EU Union List and in Schedule 3 of the Food Standards Australia New Zealand *Food Standards Code*

Parameter	Kyowa's Proposed Specifications for 2'-FL	Method	Existing Specifications for 2'-FL in the EU Union List		Existing Specifications in Schedule 3 of the FSANZ *Food Standards Code*		
			Synthetic	Genetically Modified Strain of *E. coli* K-12	S3-47 - Genetically Modified Strain of *E. coli* K-12	S3-40 – 2'-FL Sourced from *E. coli* K-12	S3-45 – 2'-FL sourced from *E. coli* BL21
Appearance	Powder	Visual observation	-	-	-	White to off-white powder or agglomerates	A white to ivory powder, or a colourless to slightly yellow liquid
Colour	White to off-white	General Notice, JP[a]	-	-	-	-	-
Purity							
Purity (2'-FL)	≥82 dwb%	HPLC-PAD (internal method)	≥95%	≥83%	≥90%	≥94.0%	≥90.0%
Water	≤9.0 w/w%	JP 2.48[a]	≤9.0%	≤9.0%	≤9.0% (powder)	≤5.0%	≤9.0% (for powder; NA for liquid product)
Solids	-	-	-	-	-	-	45% ± 5% (w/v; dry matter in water; NA for powder product)
Ash	≤0.5 w/w%	JP 2.44[a]	≤0.2%	≤2.0%	≤0.5%	≤1.5%	≤0.5%
Residual protein	≤100 mg/kg (0.01%)	Bradford assay	≤0.01%	≤0.01%	≤0.01%	≤0.01%	≤0.01%
pH (25˚C; 5% solution)	4.0 to 9.0	JP 2.54[a]	3.2 to 7.0	3.0 to 7.5	-	3.2 to 5.0	-
Acetic acid	-	-	≤0.3%	≤1.0%	-	≤1.0%	-
Residual solvents	-	-	≤50.0 mg/kg singly; ≤200 mg/kg in combination[b]	-	-	-	-

Table B.2.5.1-1 Proposed Specifications for Kyowa's 2'-Fucosyllactose (2'-FL) Produced with a Genetically Modified Strain of *Escherichia coli* W Compared to Current Specifications for 2'-FL in the EU Union List and in Schedule 3 of the Food Standards Australia New Zealand *Food Standards Code*

Parameter	Kyowa's Proposed Specifications for 2'-FL	Method	Existing Specifications for 2'-FL in the EU Union List		Existing Specifications in Schedule 3 of the FSANZ *Food Standards Code*		
			Synthetic	Genetically Modified Strain of *E. coli* K-12	S3-47 - Genetically Modified Strain of *E. coli* K-12	S3-40 – 2'-FL Sourced from *E. coli* K-12	S3-45 – 2'-FL sourced from *E. coli* BL21
Other Carbohydrates							
D-lactose	≤5 dwb%	HPLC-PAD (internal method)	≤1.0%	≤10.0%	≤5.0%	≤3.0%	≤5.0%
L-fucose	≤1 dwb%	HPLC-PAD (internal method)	≤1.0%	≤2.0%	≤3.0%	≤1.0%	≤3.0%
3-Fucosyllactose	-	-	-	-	≤5.0%	-	≤5.0%
Fucosylgalactose	≤3 dwb%	-	-	-	≤3.0%	-	≤5.0%
Difucosyl-d-lactose isomers	-	-	≤1.0%	-	-	-	-
Difucosyllactose (difucosyl-d-lactose)	≤3 dwb%	HPLC-PAD (internal method)	-	≤5.0%	≤5.0%	≤1.0%	-
D-glucose and D-galactose	≤1 dwb%	HPLC-PAD (internal method)	-	-	≤3.0% (individually)	-	≤3.0% (individually)
2'-Fucosyl-d-lactulose	-	-	≤0.6%	≤1.5%	-	≤1.0%	-
Sum of saccharides (2′-Fucosyllactose, D-Lactose, L-Fucose, Difucosyl-D-lactose, 2′-Fucosyl-D-lactulose)	-	-	-	≥90%		≥96.0%	-
Heavy Metals							
Arsenic	≤0.1 mg/kg	USP 233[c]	-	-	≤0.2 mg/kg	-	≤0.2 mg/kg
Cadmium	≤0.1 mg/kg	USP 233[c]	-	-	≤0.1 mg/kg	-	≤0.1 mg/kg
Lead	≤0.1 mg/kg	USP 233[c]	-	-	≤0.02 mg/kg	≤0.1 mg/kg	≤0.02 mg/kg
Mercury	≤0.1 mg/kg	USP 233[c]	-	-	≤0.5 mg/kg	-	≤0.5 mg/kg
Palladium	-	-	≤0.1 mg/kg	-	-	-	-
Nickel	-	-	≤3.0 mg/kg	-	-	-	-

Table B.2.5.1-1 Proposed Specifications for Kyowa's 2'-Fucosyllactose (2'-FL) Produced with a Genetically Modified Strain of *Escherichia coli* W Compared to Current Specifications for 2'-FL in the EU Union List and in Schedule 3 of the Food Standards Australia New Zealand *Food Standards Code*

Parameter	Kyowa's Proposed Specifications for 2'-FL	Method	Existing Specifications for 2'-FL in the EU Union List				Existing Specifications in Schedule 3 of the FSANZ *Food Standards Code*		
			Synthetic		Genetically Modified Strain of *E. coli* K-12		S3-47 - Genetically Modified Strain of *E. coli* K-12	S3-40 – 2'-FL Sourced from *E. coli* K-12	S3-45 – 2'-FL sourced from *E. coli* BL21
Iron	≤10 mg/kg	AOAC (2019) 999.10 and 2011.14	-		-		-	-	-
Microbial Parameters									
Aerobic plate count	≤1,000 CFU/g	ISO 4833-1:2013	≤500 CFU/g (mesophilic)		≤3,000 CFU/g (mesophilic)		-	-	-
Total plate count	-	-	-		-		≤10,000 CFU/g (powder); ≤5,000 CFU/g (liquid)	≤500 CFU/g	≤10,000 CFU/g (powder) or ≤5,000 CFU/g (liquid)
Mould	≤100 CFU/g (as yeasts and moulds)	ISO 21527-2:2008	≤10 CFU/g	≤100 CFU/g	CFU/g: ≤100 (powder); ≤50 (liquid)	≤10 CFU/g	≤100 CFU/g (powder) or 50 CFU/g (liquid)		
Yeasts				≤100 CFU/g		≤10 CFU/g			
Salmonella	Negative in 100 g	ISO 6579-1:2017	-		-		Negative/100 g (powder); negative/200 mL (liquid)	Absent in 25 g	Absent in 100 g (powder) or 200 mL (liquid)
Enterobacteriaceae	Negative in 10 g	ISO 21528-1:2017	-		-		Absent in 11 g (includes coliforms)	Absent in 10 g	Absent in 11 g (powder) or 22 mL (liquid) (includes coliforms)
Cronobacter spp. *(Enterobacter sakazakii)*	Negative in 100 g	ISO 22964:2017	-		-		Negative/100 g (powder); Negative/200 mL (liquid)	Absent in 10 g	Absent in 100 g (powder) or 200 mL (liquid)

Table B.2.5.1-1 Proposed Specifications for Kyowa's 2'-Fucosyllactose (2'-FL) Produced with a Genetically Modified Strain of *Escherichia coli* W Compared to Current Specifications for 2'-FL in the EU Union List and in Schedule 3 of the Food Standards Australia New Zealand *Food Standards Code*

Parameter	Kyowa's Proposed Specifications for 2'-FL	Method	Existing Specifications for 2'-FL in the EU Union List		Existing Specifications in Schedule 3 of the FSANZ *Food Standards Code*		
			Synthetic	Genetically Modified Strain of *E. coli* K-12	S3-47 - Genetically Modified Strain of *E. coli* K-12	S3-40 – 2'-FL Sourced from *E. coli* K-12	S3-45 – 2'-FL sourced from *E. coli* BL21
Listeria monocytogenes	Negative in 25 g	ISO 11290-1:2017	-	-	-	Absent in 25 g	-
Bacillus cereus	≤50 CFU/g	ISO 7932:2004	-	-	-	≤50 CFU/g	-
Residual endotoxins	≤10 E.U./mg	JP 4.01 (kinetic-turbidimetric method)[a]	≤10 E.U./mg	≤10 E.U./mg	≤100 E.U./g (powder); ≤100 E.U./mL (liquid)	≤10 E.U./mg	≤10 E.U./mg
GMO detection	-	-	-	-	-	-	Not detected

- = not specified; 2'-FL = 2'-fucosyllactose; CAS = Chemical Abstracts Service; CFU = colony-forming units; dwb = dry weight basis; E.U. = endotoxin units; EU = European Union; FSANZ = Food Standards Australia New Zealand; GMO = genetically modified organism; HPLC-PAD = high-performance liquid chromatography with pulsed amperometric detection; ISO = International Organization for Standardization; JP = *Japanese Pharmacopoeia*; NA = not applicable; USP = *United States Pharmacopoeia*.

[a] Method is consistent with the compendial method specified in 17th edition of the *Japanese Pharmacopeia* (2016).

[b] Methanol, 2-propanol, methyl acetate, and acetone.

[c] Method is consistent with the compendial method specified in the *United States Pharmacopeia* 35th revision (2011).

B.2.5.2 Analytical Data

Kyowa's 2'-FL produced with a genetically modified strain of *E. coli* W is a purified carbohydrate ingredient consisting primarily of 2'-FL, with lesser amounts of D-lactose, fucosylgalactose, difucosyllactose, L-fucose, and D-glucose and D-galactose. Quantitative compositional data on 2'-FL are provided below, as well as data on chemical and microbiological contaminants.

Data are presented for 3 batches manufactured by fermentation using a genetically modified strain of *E. coli* W. The results are presented and discussed in Section B.2.5.2.1, and Certificates of Analysis are provided in Appendix F **[CONFIDENTIAL AND PROPRIETARY]**. Methods of analysis were obtained from the *United States Pharmacopeia* or the *Japanese Pharmacopeia* or were developed internally by Kyowa and confirmed to be suitable, as no standardised methods were available. Full descriptions of internal methods, including references and limits of detection or limits of quantification, are provided in Appendix J **[CONFIDENTIAL AND PROPRIETARY]**. Analyses were conducted at Kyowa's Technical Research Laboratories or Japan Food Research Laboratories (JFRL). Accreditation documents for JFRL are provided in Appendix K **[CONFIDENTIAL AND PROPRIETARY]**.

B.2.5.2.1 Batch Analyses

Analytical results for 3 batches of Kyowa's 2'-FL ingredient manufactured by fermentation using a genetically modified strain of *E. coli* W (Lots H, I, and J) are presented in Table B.2.5.2.1-1 (Certificates of Analysis and HPLC-PAD chromatograms are provided in Appendix F **[CONFIDENTIAL AND PROPRIETARY]**). The lack of detection of heavy metals and microbiological contamination confirms that the purification processes in place are effective in removing these contaminants. A comparison of the compositional analytical data for 6 lots of Kyowa's 2'-FL produced with early-development preliminary fermentation media and 3 lots of Kyowa's 2'-FL produced with the current fermentation media (Lots H, I, J) is provided in Appendix F **[CONFIDENTIAL AND PROPRIETARY]** to demonstrate the compositional equivalence of the final product.

Methods of analysis were obtained from U.S. or *Japanese Pharmacopeia* or were developed internally by Kyowa and confirmed to be suitable, as no standardised methods were available. Internal methods, including identification and quantification of 2'-FL, are provided in Appendix J **[CONFIDENTIAL AND PROPRIETARY]**. Confirmation of the identity of Kyowa's 2'-FL produced by microbial fermentation, and demonstration of chemical equivalence to 2'-FL isolated from human milk, are presented in Section B.2.2 and Appendix D **[CONFIDENTIAL AND PROPRIETARY]**.

Table B.2.5.2.1-1 Summary of Batch Analyses for the Final 2'-Fucosyllactose Powdered Ingredient Produced with a Genetically Modified Strain of *Escherichia coli* W

Specification Parameter	Specification	Methods of Analysis	Manufacturing Lot		
			H	I	J
Properties					
Appearance	Powder	Visual observation	Complies	Complies	Complies
Colour	White to off-white	Visual observation	Complies	Complies	Complies
pH (25˚C; 5% solution)	4.0 to 9.0	JP 2.54[a]	6.1	6.0	6.2
Purity					
Identification	RT of standard ± 3%	HPLC-PAD (internal method)	NR	NR	NR
Purity (dwb%)	≥82	HPLC-PAD (internal method)	94	93	91
Water (w/w%)	≤9.0	JP 2.48[a]	4.7	5.3	4.5
Ash (w/w%)	≤0.5	JP 2.44[a]	0.1	0.2	0.1
Residual protein (mg/kg)	≤100	Bradford	≤100[b]	≤100[b]	≤100[b]
Other Carbohydrates					
D-lactose (dwb%)	≤5	HPLC-PAD (internal method)[c]	1.1	1.1	1.1
L-fucose (dwb%)	≤1	HPLC-PAD (internal method)[c]	≤0.05	≤0.05	≤0.05
D-glucose and D-galactose (dwb%)	≤1	HPLC-PAD (internal method)[c]	0.3	0.3	0.3
Fucosylgalactose (dwb%)	≤3	HPLC-PAD (internal method)[c]	1.0	1.0	1.0
Difucosyllactose (dwb%)	≤3	HPLC-PAD (internal method)[c]	0.4	0.4	0.4
Heavy Metals					
Arsenic (mg/kg)	≤0.1	Based on AOAC (2019) 999.10 and 2011.14[d]	<0.01	<0.01	<0.01
Cadmium (mg/kg)	≤0.1	Based on AOAC (2019) 999.10 and 2011.14[e]	<0.01	<0.01	<0.01
Lead (mg/kg)	≤0.1	Based on AOAC (2019) 999.10 and 2011.14[f]	<0.02	<0.02	<0.02
Mercury (mg/kg)	≤0.1	Based on US EPA, February 2007, Method 7473[g]	<0.004	<0.004	<0.004
Iron (mg/kg)	≤10	Based on AOAC (2019) 999.10 and 2011.14[h]	1	<0.1	<0.70

Table B.2.5.2.1-1 Summary of Batch Analyses for the Final 2'-Fucosyllactose Powdered Ingredient Produced with a Genetically Modified Strain of *Escherichia coli* W

Specification Parameter	Specification	Methods of Analysis	Manufacturing Lot		
			H	I	J
Microbial Parameters					
Aerobic plate count (CFU/g)	≤1,000	ISO 4833-1:2013[i]	<10	<10	<10
Yeasts and moulds (CFU/g)	≤100	ISO 21527-2:2008[j]	<10	<10	<10
Salmonella	Negative in 100 g	ISO 6579-1:2017[k]	Negative	Negative	Negative
Enterobacteriaceae	Negative in 10 g	ISO 21528-1:2017[l]	Negative	Negative	Negative
Cronobacter spp. *(Enterobacter sakazakii)*	Negative in 100 g	ISO 22964:2017[m]	Negative	Negative	Negative
Listeria monocytogenes	Negative in 25 g	ISO 11290-1:2017[m]	Negative	Negative	Negative
Bacillus cereus (CFU/g)	≤50	ISO 7932:2004[n]	<10	<10	<10
Residual endotoxins (E.U./mg)	≤10	JP 4.01 (kinetic-turbidimetric method)[o]	<0.0001563	<0.0001563	<0.0001563

2'-FL = 2'-fucosyllactose; AOAC = Association of Official Analytical Chemists; CFU = colony-forming units; dwb = dry weight basis; E.U. = endotoxin units; HPLC-PAD = high-performance liquid chromatography with pulsed amperometric detection; ISO = International Organization for Standardization; JP = *Japanese Pharmacopeia*; LOD = limit of detection; LOQ = limit of quantification; NR = not reported; ppm = part per million; RT = retention time; U.S. EPA = United States Environmental Protection Agency.

[a] Method is consistent with the compendial method specified in 17th edition of the *Japanese Pharmacopeia* (2016).

[b] Evaluated using a limit test at 100 ppm.

[c] LOQ = 0.05 % (w/w).

[d] Method based on AOAC (2019) 999.10 and 2011.14. LOD = 0.01 mg/kg; LOQ = 0.03 mg/kg.

[e] Method based on AOAC (2019) 999.10 and 2011.14. LOD = 0.01 mg/kg. LOQ = 0.03 mg/kg.

[f] Method based on AOAC (2019) 999.10 and 2011.14. LOD = 0.02 mg/kg. LOQ = 0.03 mg/kg.

[g] Method based on US EPA, February 2007, Method 7473, Mercury Analyzer. LOD = 0.004 mg/kg. LOQ = 0.01 mg/kg.

[h] Method based on AOAC (2019) 999.10 and 2011.14. LOD = 0.10 mg/kg. LOQ = 0.70 mg/kg.

[i] LOD = 10 CFU/g. In accordance with ISO 4833-1:2013, the presence of 1 to 3 colonies should be reported as <40 CFU/g.

[j] LOD = 100 CFU/g (surface plating).

[k] Qualitative test to confirm "absent in 100 g" by testing 10 samples of 10 g each and totalling the results.

[l] Qualitative test to confirm "absent in 10 g."

[m] Qualitative test to confirm "absent in 25 g."

[n] LOD = 10 CFU/g.

[o] LOQ = 0.0001563 EU/mg.

B.2.6 Analytical Method for Detection

Kyowa uses an in-house HPLC method which has been confirmed to be suitable for the detection of 2'-FL (HPLC-PAD). Detailed information on this method is provided in Appendix J **[CONFIDENTIAL AND PROPRIETARY]**. Kyowa notes that this HPLC-PAD method would be appropriate for use in the detection of 2'-FL in infant formula products to which it is added.

B.2.7 Information on the Proposed Food Label

As Kyowa is not seeking to change the uses or use level of 2'-FL in infant formula products, no change to the labelling of these products is expected. Kyowa's 2'-FL shall be identified on infant formula product labels as "2'-fucosyllactose," and the labeling of infant formula products containing Kyowa's 2'-FL will continue to be compliant with Standard 2.9.1 of the Code.

B.3 Information Related to the Safety of the Nutritive Substance

The safety of 2'-FL under the currently permitted conditions of use in Australia and New Zealand (*i.e.,* as an ingredient in infant formula products) has been assessed by FSANZ previously. Kyowa does not seek to amend the permitted conditions of use of 2'-FL.

To identify publications relevant to the safety of 2'-FL published since previous applications to FSANZ for 2'-FL produced by fermentation using a genetically modified production organism (Applications A1155, A1190, A1233, A1251, and A1265; covering the period from 2017 to 2022), a comprehensive search of the published scientific literature was conducted in October 2023 according to the literature search strategy described in Appendix L. Studies of 2'-FL published since 2021 are summarized in Sections B.3.2.1 and B.3.2.2. As detailed below, no newly published studies were identified that indicated the potential for allergic, toxic, or adverse health effects related to consumption of 2'-FL in adults, children, or full-term infants.[5]

B.3.1 Information on the Toxicokinetics and Metabolism of the Nutritive Substance and, if Necessary, its Degradation Products and Major Metabolites

FSANZ has evaluated the metabolic fate of 2'-FL in previous applications. Given the identical chemical nature of Kyowa's 2'-FL to the 2'-FL that occurs naturally in human milk and to previously evaluated 2'-FL ingredients, Kyowa's 2'-FL will have the same metabolic fate and toxicokinetic profile as 2'-FL ingredients previously evaluated by FSANZ (Applications A1155, A1190, A1233, A1265, A1277). Briefly, 2'-FL is expected to pass through the upper gastrointestinal tract intact, and to be metabolized by the colonic microbiota into short-chain fatty acids, with less than 1% of an oral dose of 2'-FL absorbed into the systemic circulation.

B.3.2 Information from Studies in Animals or Humans that is Relevant to the Toxicity of the Nutritive Substance and, if Necessary, its Degradation Products and Major Metabolites

The safety of 2'-FL under the currently permitted conditions of use in Australia and New Zealand has been assessed by FSANZ previously. Kyowa does not seek to amend the currently permitted conditions of use of 2'-FL.

[5] Studies pertaining to administration of 2'-FL in pre-term infants were excluded as these infants are not representative of the target population for 2'-FL–containing infant formula under the currently permitted conditions of use.

To identify publications relevant to the safety of 2'-FL published since the most recent FSANZ approvals for 2'-FL (Applications A1233 and A1265; covering the period from 2017 to 2022), a comprehensive search of the published scientific literature was conducted in October 2023 according to the literature search strategy described in Appendix L. Studies of 2'-FL published since 2021 are summarized in Sections B.3.2.1 and B.3.2.2. As detailed below, no newly published studies were identified that indicated the potential for allergic, toxic, or adverse health effects related to consumption of 2'-FL in adults, children, or full-term infants.[6]

B.3.2.1 Toxicity Studies of 2'-Fucosyllactose

Two animal studies of 2'-FL published since June 2021 were identified in the literature; these studies are summarized in Table B.3.2.1-1. Although these studies were conducted primarily to evaluate the effects of 2'-FL on growth and development in neonatal piglets and rats, no compound-related adverse effects were reported with respect to the measured parameters (Daniels *et al.,* 2022; Wang *et al.,* 2022). In accordance with animal studies of 2'-FL previously evaluated by FSANZ, the results of these studies support the safety of 2'-FL under the currently permitted conditions of use.

Table B.3.2.1-1 Summary of Subchronic and Chronic Toxicity Studies Conducted with 2'-Fucosyllactose

Species	Duration	Test Article and Dose	Outcome Parameters	Conclusions on Safety	Results Relevant to GI Function and Tolerance	Reference
Piglets, 2 days old (strain NR) 12 to 15 M/group	32 days	Test Article 2'-FL; 0 or 1.22 g/L Control Commercial milk replacer	bw gain, organ growth, formula intake, GI development, GI function	No compound-related adverse effects on bw, organ weight, formula intake, intestinal structure, or intestinal function.	SS increase in ileal crypt depth (p=0.04) and ileal sucrase activity (p=0.011).	Daniels *et al.* (2022)
Rat (Sprague-Dawley) 4 to 21 days of age 13 to 14 M/group	17 days	Test Article 2'-FL (1.2 g/L) Control Basal rat milk substitute	bw, bowel movements, food intake, oral glucose tolerance, body composition, organ weight, fecal microbiome	No compound-related adverse effects on measured parameters.	No adverse effects on bowel movements, organ weights, or fecal microbiome.	Wang *et al.* (2022)

2'-FL = 2'-fucosyllactose; bw = body weight; GI = gastrointestinal; M = males; NR = not reported; SS = statistically significant.

B.3.2.2 Human Studies of 2'-Fucosyllactose

Eleven human studies of 2'-FL that were published since 2021 were identified in the literature; these studies are summarized in Table B.3.2.2-1, below. Of the 11 human studies on 2'-FL published since June 2021, 8 were conducted in full-term infant populations. Six of these studies included healthy infants (Parschat *et al.,* 2021; Alliet *et al.,* 2022; Bauer *et al.,* 2021; Lasekan *et al.,* 2022; Wallingford *et al.,* 2022; Jochum *et al.,* 2023), and 2 studies included otherwise healthy full-term infants with cow's milk protein allergy (Gold *et al.,* 2022; Vandenplas *et al.,* 2022). In these studies, infants were administered formula (based on human milk, bovine milk, or amino acid-based formula for infants with cow's milk protein allergy) containing 2'-FL at concentrations of 1 to 3 g/L, alone or with other human milk oligosaccharides or *Limosilactobacillus reuteri*, for up to 12 months. No compound-related adverse effects were reported with respect to anthropometrics, symptoms of gastrointestinal (GI) tolerance, or other measured

[6] Studies pertaining to administration of 2'-FL in pre-term infants were excluded as these infants are not representative of the target population for 2'-FL–containing infant formula under the currently permitted conditions of use.

parameters, and the study authors unanimously concluded that 2'-FL is well tolerated and supports healthy growth and development in infants.

In a study including obese children 6 to 12 years of age, consumption of 4.5 g 2'-FL/day (alone or with LNnT) for 8 weeks had no adverse effects with respect to measures of gastrointestional (GI) inflammation, mucosal integrity and metabolism, or tolerance, and the authors concluded that the study products were "*safe and well tolerated*" (Fonvig *et al.,* 2021). In 2 studies in adults, subjects with diagnosed inflammatory bowel disease (IBD) or irritable bowel syndrome (IBS) consumed 4 or 8 g 2'-FL/day (alone or with lacto-*N*-tetraose [LNnT]) for 4 or 6 weeks (Iribarren *et al.,* 2021; Ryan *et al.,* 2021). Although the study objectives were related primarily to GI symptoms and tolerance, no compound-related adverse effects were reported, and the authors concluded that supplementation with 4 to 8 g 2'-FL/day would be appropriate and potentially beneficial for subjects with IBS or IBD.

No published reports of sensitization, case reports of allergic reactions, or allergenicity studies on 2'-FL were identified in a comprehensive and detailed search of the published scientific literature that was conducted in October 2023 to identify studies relevant to the safety of 2'-FL that were published since the most recent approvals of 2'-FL by FSANZ (Applications A1233 and A1265; covering the period from 2017 to 2022). See Appendix L for the literature search strategy and the publication titles identified.

Table B.3.2.2-1 Human Studies of 2'-Fucosyllactose

Study Population, Design, Country	Duration	Groups	Dose	Safety-Related Outcome Parameters	Findings	Conclusions on Safety	Reference
Studies in Infants (n=6)							
117 full-term infants 7 days to 2 months of age OL, MC Germany and Austria	8 weeks	Test group (ITT: n=51; PP: n=46) Exclusively formula-fed infants C (ITT: n=22; PP: n=22) Infants fed with test formula and breast milk Reference control (ITT: n=44; PP: n=38) Exclusively breast-fed infants	2'-FL (1 g/L) and LNnT (0.5 g/L)	Primary Anthropometry and GI tolerance Secondary Adverse events and formula satisfaction	• NSD in measures of growth and anthropometry • No adverse effects on measures of GI tolerance	The study authors concluded that the study results indicated *"robust effects for growth, safety, and tolerance in association with HMO-supplemented infant formulas."*	Jochum *et al.* (2023)
366 full-term, singleton infants, 0 to 14 days old MC, R, DB, C, P U.S.	4 months	Test group (ITT: n=128; PP: n=72) 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL C (ITT: n=126; PP: n=77) No HMOs Matrix HM Reference control group (ITT: n=102; PP: n=73) HM	3.0 g 2'-FL/L, 0.8 g 3-FL/L, 1.5 g LNT/L, 0.2 g 3'-SL/L, and 0.3 g 6'-SL/L	Primary bw gain from Study Days 14 to 19 (PP population only) Secondary Formula intake, stool consistency, stool frequency, anthropometrics, percent feedings with spit-up/vomit, AEs	• NSD in percent feedings with spit-up/vomit, AEs, or formula intake. • SS increase in bw gain in males in the test group compared to males in the reference control group from Study Days 84 to 119 (p=0.023) (PP only). • SS decrease in body length gain in test group compared to reference control group (P<0.001). • SS increase in stool frequency, average constipation, and average loose stools • SS decrease in MRSC in test group compared to C on Days 56, 84, and 119 (all P<0.038) (PP only), and at all timepoints (all P<0.036) (ITT only).	The study authors concluded that the *"EF containing five HMOs supported normal growth, GI tolerance and safe use in healthy term infants."*	Lasekan *et al.* (2022)

Table B.3.2.2-1 Human Studies of 2'-Fucosyllactose

Study Population, Design, Country	Duration	Groups	Dose	Safety-Related Outcome Parameters	Findings	Conclusions on Safety	Reference
					• SS decrease in average stool frequency per day and stool consistency in C and test group compared to the reference control group (p=0.004) (ITT only).		
255 singleton full-term infants, 0 to 14 days old MC, R, C, P Germany, Italy, Spain	16 weeks	Test group (ITT: n=113; PP: n=79) C (ITT: n=112; PP: n=81) Reference control group (ITT: n=116; PP: n=79)	2.99 g 2'-FL/L, 0.75 g 3-FL/L, 1.5 g LNT/L, 0.23 g 3'-SL/L, and 0.28 g 6'-SL/L	Primary Mean daily bw gain over 4-month period Secondary Stool consistency, stool frequency, anthropometrics, behavioural parameters, percent feedings with spit-up/vomit, AEs	• NSD in anthropometric data, formula intake, stool consistency, percent feedings with spit-up/vomit, or mean daily bw gain. • SS non-inferior mean daily bw gain between test group and C (P<0.001). • SS increase in stool frequency in test group and reference control group at Study Day 112 (p=0.0136). • SS increase in bw at Study Day 112 in test group compared to reference control group (p=0.0496). • SS increase in body length at Study Days 85 and 112 in test group compared to reference control group (p=0.014 and p=0.0019, respectively). • SS increase in incidence of genital fungal infections in test group compared to C (p=0.029).	The study authors concluded that *"5HMO-Mix at 5.75 g/L in infant formula is safe and well tolerated by healthy term infants during the first months of life."*	Parschat *et al.* (2021)

Table B.3.2.2-1 Human Studies of 2'-Fucosyllactose

Study Population, Design, Country	Duration	Groups	Dose	Safety-Related Outcome Parameters	Findings	Conclusions on Safety	Reference
176 healthy, full-term infants 0 to 28 days old DB, R, PC Honduras and U.S.	16 weeks	Test group (ITT: n=66; PP: n=47) Commercial formula + 2'-FL C (ITT: n=66; PP: n=41) Commercial formula Reference C (ITT: n=89; PP: n=79) Breast-fed	2'-FL (1 g/L)	Primary Growth and effects on fecal microbiome Secondary Adverse events	• No adverse effects on withdrawal rate or reporting of AE. • NSD on measures of growth. • No AE on composition of microbiome.	The study authors concluded that *"the addition of a physiologic level of 2'FL has no effect on growth or incidence of adverse effects of formula-fed infants, adding evidence of safe use of this HMO in the infant formula."*	Wallingford *et al.* (2022)
289 full-term infants, 0 to 14 days old R, DB, C Belgium and Italy	6 months	Test group (ITT: n=144; PP: n=99) *Limosilactobacillus reuteri* + 2'-FL C (ITT; n=145; PP: n=85) No HMOs Matrix BM-based formula Reference control group (ITT: n=60; PP: n=NA) HM	*L. reuteri* DSM 17938 (1×10^7 CFU/g) + 1.0 g/L 2'-FL	Primary bw gain from BL through 4 months of age Secondary Anthropometrics, stool characteristics, GI tolerance and associated behaviours, gut immunity faecal markers, gut health, parent-reported AEs, physician-confirmed AEs	• NSD in bw gain, anthropometric data, GI tolerance and associated behaviours, stool frequency, stool consistency, faecal biomarkers, percent feedings with spit-up/vomit, and AEs. • SS non-inferior bw gain between groups (P<0.001). • SS decrease in stool frequency in test and control group compared to reference control group (p=<0.05). • SS higher stool consistency scores in test group and C compared to reference control group (P<0.001)	The study authors concluded that *"L. reuteri-containing infant formula with 2'FL supports age-appropriate growth, is well-tolerated and may play a role in shifting the gut microbial pattern towards that of breastfed infants."*	Alliet *et al.* (2022)

Table B.3.2.2-1 Human Studies of 2'-Fucosyllactose

Study Population, Design, Country	Duration	Groups	Dose	Safety-Related Outcome Parameters	Findings	Conclusions on Safety	Reference
782 full-term infants, >7 to <21 days old MC, R, DB, C Country NR	6 months	Test group 1 (n=229; ITT: n=NR; PP: n=NR) 5 HMOs Test group 2 (n=227; ITT: n= NR; PP: n=NR) 5 HMOs C (n=230; ITT: n=NR; PP: n=NR) No HMOs Matrix BM-based formula Reference control group (n=96, non-randomised) HM	Test group 1 1.5 g HMOs/L (2'-FL, 2', 3-di-FL, LNnT, 3'-SL, and 6'-SL; individual levels NR) Test group 2 2.5 g HMOs/L (2'-FL, 2',3-di-FL, LNT, 3'-SL, and 6'-SL)	Primary bw gain from enrolment to 4 months of age Secondary Anthropometrics, stooling pattern, AEs, GI tolerance and associated behaviours	• NSD in IGSQ index scores, GI tolerance and associated behaviours, anthropometric data, stooling patterns, frequency of spit-up/vomit, and AEs between groups. • SS non-inferior bw gain between all groups (P-value NR).	The study authors concluded that *"infant formula supplemented with a unique blend of five HMOs supports age-appropriate growth and soft stooling pattern, is safe and well-tolerated."*	Bauer *et al.* (2021)
32 full-term infants (diagnosed with CMPA), 1 to 8 months old MC, SA Australia	12 months	Test group (ITT: n=32; PP: n=21) 2'-FL + LNnT Matrix AAF	1.0 g 2'-FL/L and 0.5 g LNnT/L	Primary bw gain from enrolment to 4 months of age Secondary Anthropometrics, CMPA symptoms, behavioural parameters, formula intake	• NSD in stool characteristics, AEs, formula intake, or anthropometric data. • SS decrease in CMPA symptoms, including fussing, percent feedings with spit-up, vomiting, and skin problems (p=0.009, p=0.039, p=0.0013, and p=0.059, respectively).	The study authors concluded that *"the study formula with two HMO achieved adequate growth, with some catch-up growth. The formula was tolerated well and had an excellent safety profile."*	Gold *et al.* (2022)

Table B.3.2.2-1 Human Studies of 2'-Fucosyllactose

Study Population, Design, Country	Duration	Groups	Dose	Safety-Related Outcome Parameters	Findings	Conclusions on Safety	Reference
194 infants, 0 to 6 months old (diagnosed with CMPA) MC, R Europe (41 sites), Singapore (3 sites)	12 months	Test group (ITT: n=97; PP: n=73) 2'-FL + LNnT C (ITT: n=97; PP: n=64) No HMOs	1.0 g 2'-FL/L and 0.5 g LNnT/L	Primary bw gain from enrolment to 4 months (PP population only) Secondary GI tolerance, anthropometric data, CMPA symptoms, incidence of infection, AEs, medication use from enrolment to 12 months of age (ITT population)	• NSD in bw gain, anthropometric data, CMPA symptoms, GI tolerance, AEs, or medication use. • SS non-inferior weight gain in the test group compared to C (p=0.0049). • SS increase in mean formula intake volumes in test group compared to C, at Month 6, 8, and 10 (p=0.009, p=0.006, and p=0.047, respectively). • SS decrease in URTI frequency from enrolment to Month 12 and ear infections from enrolment to Month 8, in test group compared to C (p=0.003 and p=0.045, respectively).	The study authors concluded *"HMO-supplemented formula supports normal growth in infants with CMPA and suggests a protective effect against respiratory and ear infections in the first year of life."*	Vandenplas *et al.* (2022)
Studies in Children and Adults (n=3)							
75 children (diagnosed with obesity), 6 to 12 years old R, DB, C, PRO Denmark	8 weeks	Test group 1 (ITT: n=25; PP: n=21) 2'-FL Test group 2 (ITT: n=25; PP: n=24) 2'-FL + LNnT C (ITT: n=25; PP: n=18) No HMOs	Test group 1 4.5 g 2'-FL/day Test group 2 4.5 g 2'-FL + LNnT/day (4:1 mix)	Primary No safety-related parameters measured Secondary Inflammatory biomarkers, gut barrier integrity, GI tolerance, faecal calprotectin concentration, GI mucosal metabolism, AEs	• NSD in calprotectin concentrations, AEs, gut barrier integrity biomarkers, and inflammatory biomarkers. • SS decrease in GSRS urgency score in test group 1 from BL to week 8 (p=0.013). • SS decrease in GSRS bloating scores at Week 4 between groups (p=0.042) (not considered clinically relevant).	The study authors concluded that *"both 2'-FL and the Mix beneficially modulate intestinal microbiota by increasing bifidobacteria. Furthermore, supplementation with either 2'-FL alone or a Mix is safe and well tolerated in children."*	Fonvig *et al.* (2021)

Table B.3.2.2-1 Human Studies of 2'-Fucosyllactose

Study Population, Design, Country	Duration	Groups	Dose	Safety-Related Outcome Parameters	Findings	Conclusions on Safety	Reference
58 adults (diagnosed with IBS), 19 to 73 years old P, R, DB, PC Sweden	4 weeks	Test group 1 (n=20; ITT: n=NR; PP: n=NR) 2'-FL + LNnT Test group 2 (n=19; ITT: n=NR; PP: n=NR) 2'-FL + LNnT C (n=19; ITT: n=NR; PP: n=NR) No HMOs Matrix HM	Test group 1 5.0 g 2'-FL + LNnT/day (4:1 mix) Test group 2 10.0 g 2'-FL + LNnT/day (4:1 mix)	Primary No safety-related parameters measured Secondary IBS symptom and psychological symptom severity	• NSD in IBS-SSS or HADS scores between any groups.	The study authors concluded that *"findings support the assertion that 2'-FL/LNnT supplementation modulate the intestinal microenvironment of patients with IBS, potentially related to health,"* and that *"2'-FL/LNnT supplementation might be a valuable strategy to improve the intestinal microenvironment in IBS patients."*	Iribarren *et al.* (2021)
12 adults, (diagnosed with IBS or ulcerative colitis), 21 to 75 years old PS U.S.	6 weeks	Test group (ITT: n=12) 2'-FL + micronutrients	4.0 g 2'-FL/day	Primary GI tolerance Secondary IBS symptoms (only IBS patients) and IBD symptoms (only IBD patients)	• SS increase in GIQLI total scores and GI symptoms (p=0.02 and p=0.022, respectively). • SS increase in IBDQ score in IBD population from BL to Week 6 (P<0.001). • NSD in DSFQ score from BL to Week 6.	The study authors concluded *"2'-FL-containing nutritional formula by adults with IBS or ulcerative colitis was associated with improvements in intra- and extra-intestinal symptoms, and bifidogenic and butyrogenic effects."*	Ryan *et al.* (2021)

Table B.3.2.2-1 Human Studies of 2'-Fucosyllactose

Study Population, Design, Country	Duration	Groups	Dose	Safety-Related Outcome Parameters	Findings	Conclusions on Safety	Reference

2'-FL = 2'-fucosyllactose; 2'3-di-FL = 2'3-di-fucosyllactose; 3-FL = 3-fucosyllactose; 3'-SL = 3'-sialyllactose; 6'-SL = 6'- sialyllactose; AAF = amino acid-based formula; AE = adverse event; BL = baseline; BM = bovine milk; bw = body weight; C = controlled; CFU = colony-forming units; CMPA = cow's-milk protein allergy; DB = double-blind; DSFQ = Digestive Symptom Frequency Questionnaire; GI = gastrointestinal; GIQLI = Gastrointestinal Quality of Life Index; GSRS = Gastrointestinal Symptom Rating Scale; HADS = Hospital Anxiety and Depression Scale; HM = human milk; HMO = human milk oligosaccharide; IBD = inflammatory bowel disease; IBDQ = Inflammatory Bowel Disease Questionnaire; IBS = irritable bowel syndrome; IBS-SSS = Irritable Bowel Syndrome–Symptom Severity Scale; IGSQ = Infant Gastrointestinal Symptom Questionnaire; ITT = intention-to-treat; LNnT = lacto-*N*-neotetraose; LNT = lacto-*N*-tetraose; MC = multi-centre; MRSC = mean rank stool consistency; NA = not applicable; NR = not reported; NSD = no significant difference; OL = open-label; P = parallel; PC = placebo-controlled; PP = per protocol; PRO = prospective; PS = pilot study; R = randomised; SA = single-arm; SS = statistically significant; U.S. = United States; URTI = upper respiratory tract infection.

B.3.3 Other Studies

Although 2'-FL is already permitted to be used as nutritive substance under the conditions specified in the Code, and Kyowa does not seek changes to the current approved uses, additional studies demonstrating the safety and suitability of Kyowa's 2'-FL ingredient for use in infant formula products have been conducted to satisfy the requirements of other jurisdictions. Summaries of these studies are provided below.

B.3.3.1 Genotoxicity

Bacterial Reverse Mutation Assay

The potential mutagenicity of Kyowa's 2'-FL ingredient was evaluated in a bacterial reverse mutation assay conducted in accordance with Good Laboratory Practice (GLP) (OECD, 1998) and OECD Test Guideline 471 (*Bacterial reverse mutation test*; OECD, 1997) (see Appendix M **[CONFIDENTIAL AND PROPRIETARY]** for study report). In this study, *Salmonella typhimurium* strains TA100, TA1535, TA98, and TA1537 and *E. coli* strain WP2 *uvrA* were incubated with Kyowa's 2'-FL (produced according to the same manufacturing method and using the same production organism but using an early-development preliminary fermentation media; Lot A; assay 92%[7]) at concentrations of 0 (water as negative control or positive controls[8]), 313, 625, 1,250, 2,500, or 5,000 µg/plate, in the presence or absence of metabolic activation. The assay was conducted according to the pre-incubation method, as described in Appendix M **[CONFIDENTIAL AND PROPRIETARY]**. No precipitation of the test material or increase in the number of revertant colonies were observed in the presence or absence of metabolic activation, and the study authors concluded that 2'-FL was not mutagenic under the conditions of this study.

In Vitro Mammalian Cell Micronucleus Test

The clastogenic and aneugenic potential of Kyowa's 2'-FL ingredient was evaluated in an *in vitro* mammalian cell micronucleus test conducted with Chinese hamster lung cells (CHL/IU cell line) according to OECD Test Guideline 487 (*In Vitro mammalian cell micronucleus test*; OECD, 2016a) and in compliance with the OECD principles of GLP (OECD, 1998). The full study report is provided in Appendix M **[CONFIDENTIAL AND PROPRIETARY].**

In a preliminary dose range finding test, neither cytotoxicity nor precipitation was observed at any of the concentrations tested (up to 2,000 µg/mL). Therefore, in the main tests, CHL/IU cells were exposed to 2'-FL (Lot H; dissolved in physiological saline) with and without metabolic activation (S9 mix) for 6 hours and without metabolic activation for 27 hours. 2'-FL was tested at concentrations of 500, 1,000, and 2,000 µg/mL.

A negative control of physiological saline (vehicle), and positive controls of Mitomycin C (MMC) at 0.1 µg/mL for short-term exposure (6 hours) or 0.05 µg/mL for long-term exposure (27 hours) in the absence of S9, or cyclophosphamide (CP) at 8 µg/mL for short-term exposure with metabolic activation were used to verify the results. A positive result for clastogenicity/aneugenicity was defined as a dose-dependent, statistically significant increase in the frequency of micronuclei compared to the negative controls, with the frequency being outside the distribution of the historical negative controls.

[7] Due to improvements in sample preparation and analysis, the purity value and water content of 2'-FL (Lot A) are more accurately represented in the Certificate of Analysis dated 08 February 2021 provided in Appendix F and copied into Appendix M (purity 92%; water content 5.0%) compared to the study report provided in Appendix M (purity 96.6%; water content 3.72%). An explanation of the reasons for these discrepancies is provided in Appendix M.

[8] Positive controls include: 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide; sodium azide; 2-methoxy-6-chloro-9-[3-(2-chloroethyl)aminopropylamino]acridine●2HCl; 2-aminoanthracene; and benzo[a]pyrene.

There was no significant increase in the frequency of micronuclei at any concentration of 2'-FL tested in the absence or presence of metabolic activation compared to the negative control and no evidence of a dose response. No change in colour or precipitation was observed at any concentration. In contrast, the positive controls induced statistically significant increases in the frequency of micronuclei, both in the presence and absence of metabolic activation, which demonstrated the sensitivity of the assay and metabolic activity of the S9 preparations. The micronucleus frequencies of the negative and positive controls were within the background range, confirming the validity of the test.

It was concluded that 2'-FL was neither clastogenic nor aneugenic and did not have micronucleus-inducing potential at concentrations up to 2,000 µg/mL in the absence or presence of metabolic activation.

In Vivo Micronucleus Test

The potential genotoxicity of Kyowa's 2'-FL ingredient (produced according to the same manufacturing method and using the same production organism but using an early-development preliminary fermentation media; Lot A; assay 92%[9]) was evaluated in an *in vivo* micronucleus study that was conducted in accordance with GLP and OECD Test Guideline 474 (*Mammalian erythrocyte micronucleus test*; OECD, 1998, 2016b) (see Appendix M **[CONFIDENTIAL AND PROPRIETARY]** for study report). In this study, male Slc:ICR mice (5/group) were administered oral doses of 0, 500, 1,000, or 2,000 mg 2'-FL/kg body weight. Water was used as a negative control, while mitomycin C was used as a positive control. Two doses were administered 24 hours apart, and bone marrow samples were collected 22 to 24 hours after the final administration. There were no deaths and no abnormalities in the general condition of the mice. No statistically significant differences in the frequency of micronucleated immature erythrocytes or the proportion of immature erythrocytes were reported. The study was considered to be properly conducted, as the frequency of the micronucleated immature erythrocytes in the negative and positive control groups were within the range of historical laboratory controls and there was a significant increase in micronucleated immature erythrocytes in the positive control group compared to the negative control group. The study authors concluded that 2'-FL does not induce chromosomal aberrations in mice.

[9] Due to improvements in sample preparation and analysis, the purity value and water content of 2'-FL (Lot A) are more accurately represented in the Certificate of Analysis dated 08 February 2021 provided in Appendix F and copied into Appendix M (purity 92%; water content 5.0%) compared to the study report provided in Appendix M (purity 96.6%; water content 3.72%). An explanation of the reasons for these discrepancies is provided in Appendix M.

B.3.3.2 Subchronic Toxicity

The potential toxicity of Kyowa's 2'-FL ingredient was evaluated in a 90-day toxicity study conducted in accordance with OECD Test Guideline 408 (*Repeated dose 90-day oral toxicity study in rodents*) and GLP (OECD, 1998, 2018). Details regarding the design, conduct, and results of this study are provided in Appendix M **[CONFIDENTIAL AND PROPRIETARY]**. In this study, 6-week-old Crl:CD(SD) rats (10/sex/group) were administered Kyowa's 2'-FL (produced according to the same manufacturing method and using the same production organism but using an early-development preliminary fermentation media; Lot A; assay 92%) in distilled water by gavage at doses of 0 (distilled water), 500, 1,000, or 2,000 mg/kg body weight/day. The animals were observed daily throughout the study period, with body weight, food consumption, and behavioural observations made regularly. A functional observational battery was conducted during Week 12 of the study period, while ophthalmology and urinalysis were conducted during Week 13. Hematology, clinical biochemistry, organ weight, gross necropsy, and histopathology examinations were conducted upon study termination. Several statistically significant differences were reported with respect to food consumption, functional observation, clinical biochemistry, organ weights, gross necropsy, and histopathology. However, due to small effect size, lack of dose-response relationship, and lack of correspondence between organ weight changes and gross or histopathological observations, these effects were considered by the study authors to be incidental and not toxicologically relevant. Details pertaining to study design, historical control data, methodology, and results are provided in Appendix M **[CONFIDENTIAL AND PROPRIETARY]**.

Overall, no mortality, abnormal clinical signs, or toxicologically relevant compound-related adverse effects on any measured parameters were reported, and the study authors determined the no-observed-adverse-effect level to be 2,000 mg/kg body weight/day, the highest dose tested.

B.3.3.3 Safety Assessment Reports Prepared by International Agencies of Other National Government Agencies

EFSA has published a positive opinion on the use of Kyowa's 2'-FL (produced using the same production organism and manufacturing process as those described herein) for use as a novel food ingredient in various foods and beverages, including infant formula at a use level of up to 1.2 g/L (EFSA, 20023).

The U.S. FDA had no questions in response to the GRAS status of Kyowa's 2'-FL (produced using the same production organism and manufacturing process as those described herein) for use in various foods and beverages, including infant formula at a use level of up to 2.4 g/L (GRN 1051).

B.4 Information on Dietary Intake of the Nutritive Substance

Kyowa is not requesting changes to the currently permitted uses or use levels of 2'-FL evaluated by FSANZ in Applications A1155, A1190, A1233, A1265, and A1277, and as specified in Schedule 29 of the Code (96 mg/100 kJ, equivalent to 2.4 g/L). Kyowa's 2'-FL is intended to be substitutional to other 2'-FL ingredients currently on the market in Australia and New Zealand. Since no changes in the uses or use levels of 2'-FL are sought by Kyowa, and changes in the current dietary intake of 2'-FL as a component of infant formula products are not expected, Kyowa considers that the remaining sections under Part D of Guideline 3.3.3 of the FSANZ Handbook are not necessary to include herein.

B.5 Information Related to the Nutritional Impact of a Nutritive Substance Other than Vitamins and Minerals

The purpose for the addition of Kyowa's 2'-FL to infant formula products is the same as those described in previous FSANZ approvals (Applications A1155, A1190, A1233, A1265, A1277) *i.e.,* to more closely

mimic the composition of human milk, to contribute to a healthy intestinal microbiota (bifidogenic effect), and to reduce the binding of pathogens (*C. jejuni*) to intestinal epithelial cells.

B.6 Information Related to Potential Impact on Consumer Understanding and Behaviour

Kyowa's 2'-FL is intended to be substitutional to other 2'-FL ingredients which have already been evaluated by FSANZ and are already on the market in Australia and New Zealand. Thus, no changes in consumer perception or behavioural responses to infant formula products containing Kyowa's 2'-FL are expected. Likewise, the addition of Kyowa's 2'-FL to infant formula products, in accordance with uses currently permitted in the Code, is not expected to adversely affect any population groups.

C. SPECIAL PURPOSE FOOD – INFANT FORMULA PRODUCTS (SECTION 3.6.2 OF THE HANDBOOK)

FSANZ has previously assessed the safety of 2'-FL for use as an ingredient in infant formula products (Applications A1155, A1190, A1233, A1251, A1265, A1277 (FSANZ 2019b, 2021, 2022a,b, 2023, 2024). Kyowa's 2'-FL is chemically identical to 2'-FL naturally occurring in human milk, and the 2'-FL ingredients previously approved by FSANZ. Kyowa is not seeking to change the uses or use levels of 2'-FL currently permitted in the Code. Therefore, there are no public health and safety concerns associated with Kyowa's proposed use of 2'-FL in infant formula products.

Kyowa is not seeking changes to the composition of infant formula products as currently specified in Standard 2.9.1 of the Code. The safety, nutritive purpose, and physiological benefits of the addition of 2'-FL to infant formula products has been assessed by FSANZ previously. The addition of Kyowa's 2'-FL to infant formula products is not expected to result in changes to the dietary intake of infant formula products, or to the labelling requirements of these products.

D. SUMMARY

The information presented above supports the conclusion that Kyowa's modified strain of *E. coli* W is safe and suitable for use in the production of 2'-FL intended for use in accordance with uses currently permitted (*i.e.,* as a nutritive substance in infant formula products under Schedule 29), and thus, Schedules 3, 26, and 29 of the Code should be amended accordingly. This conclusion is based on the following:

- The Code already permits the use of 2'-FL as a nutritive substance in infant formula products, and Kyowa is not seeking to alter the permitted use levels for 2'-FL.
- Kyowa's 2'-FL has been demonstrated to be chemically and structurally identical to 2'-FL isolated from human milk.
- Kyowa's specifications are comparable to other 2'-FL ingredients, produced synthetically or using other microbial sources, currently permitted for use in Australia and New Zealand, the EU, and the U.S.
- Kyowa's 2'-FL consistently meets the pre-established ingredient specifications.
- The stability of Kyowa's 2'-FL is supported by the structural and chemical stability of other 2'-FL ingredients authorized under Schedule 26-5 of the Code.
- The host organism, *E. coli* W, is well-characterized, has a long history of use in the manufacture of food ingredients, and is considered to be a safe strain for such uses.
- The modifications made to the host organism have been demonstrated to result in a production organism that is functionally and genotypically stable; evaluation of the production strain indicates no safety concerns resulting from the genetic modifications.
- Protein and DNA derived from Kyowa's manufacturing organism are absent in the final 2'-FL ingredient; the ingredient is therefore considered unlikely to pose allergenicity concerns.
- In previous assessments, FSANZ concluded that there is no evidence of a nutritional concern regarding the use of 2'-FL in infant formula products at concentrations occurring naturally in human milk (*i.e.,* up to 2.4 g/L). Kyowa has not identified information in a recent comprehensive search of the published literature that would contradict FSANZ's previous conclusions.
- Toxicity studies of Kyowa's 2'-FL demonstrate that the ingredient is not genotoxic and does not result in compound-related adverse effects upon 90-day administration to rats at doses up to 2,000 mg/kg body weight/day.

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