Application to amend the Australia New Zealand Food Standards Code to permit a new genetically modified source organism – *Escherichia coli* K-12 MG1655 INB-2FL_03 for the production of 2′-Fucosyllactose

[redacted]

2023-05-25

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List of Abbreviations

2′-FL	2′-Fucosyllactose / 2′-O-fucosyllactose
a1,2-FT	alpha-1,2-fucosyltransferase
AFNOR	Association française de normalisation
agp	glucose-1-phosphatase
ANVISA	Agência Nacional De Vigilância Sanitária
ATCC	American Type Culture Collection
Aus	Australia
BaSP	sucrose phosphorylase
CCI	Confidential Commercial Information
CFU	Colony forming units
CPI	Cell Performance Index
crl	RNA polymerase holoenzyme assembly factor gene
CscB	sucrose permease
DFL	Difucosyllactose
DM	Dry mass
DNA	Deoxyribonucleic acid
ECCB	Exclusive capturable commercial benefit
EFSA	European Food Safety Authority
EU	Endotoxin units
FastANI	Fast alignment-free computation of whole-genome average nucleotide identity
fcl	GDP-L-fucose synthase
FDA	Food and Drugs Administration
frk	fructokinase
FSANZ	Food Standards Australia New Zealand
GDP	Guanosine diphosphate
glpR	Glycerol-3-phosphate repressor gene
GILSP	Good Industrial Large-Scale Practice
gmd	GDP-mannose 4,6-dehydratase
GRAS	Generally Recognized as Safe
GMM	Genetically modified microorganism
GRN	GRAS Notification
HACCP	Hazard Analysis and Critical Control Points
HMO	Human milk oligosaccharides
ICP-MS	Inductively coupled plasma mass spectrometry
ISO	International Organization for Standardization
lacY	Lactose permease
lacZ	β-galactosidase
MARA	Ministry of Agriculture and Rural Affairs
manA	Mannose-6-phosphate isomerase
manB	Phosphomannomutase
manC	Mannose-1-phosphate guanylyltransferase
MOHW	Ministry of Health and Welfare
MTP	Micro-titer plate
NADP+	Nicotinamide-adenine-dinucleotide phosphate (oxidized form)
NEN	Royal Netherlands Standardization Institute
NLT	Not less than
NMR	Nuclear magnetic resonance
NMT	Not more than
NS	Not specified
NZ	New Zealand
OCI	Other confidential information
OECD	Organisation for Economic Co-operation and Development

Ph. Eur.	European Pharmacopoeia
P	Phosphate
PCR	Polymerase chain reaction
pfkA	6-Phosphofructokinase 1
pfkB	6-Phosphofructokinase 2
pgi	Glucose-6-phosphate isomerase
pgm	Phosphoglucomutase
RH	Relative humidity
RI	Refractive index
rfb-50	Mutation downstream of the rfb gene cluster (part of O-antigen synthesis)
RNA	Ribonucleic acid
rph-1	Frameshift mutation of rph (truncated ribonuclease PH)
TFDA	Taiwan Food and Drug Administration
TGA	Therapeutic Goods Administration
TU	Transcription unit
UDP	Uridine diphosphate
UPLC	Ultra-performance liquid chromatography
US	United States
U.S. EPA	United States Environmental Protection Agency
wcaJ	UDP-glucose:undecaprenyl-phosphate glucose-1-phosphate transferase
WGS	Whole Genome Sequencing
zwf	NADP+-dependent glucose-6-phosphate dehydrogenase.

List of Figures, Tables and Appendices

3.1.1 GENERAL REQUIREMENTS

B. Applicant details

(a) applicant name

Inbiose N.V. Belgium

(b) name of contact person

[redacted]

[redacted]

(e) email address

[redacted]

(f) nature of applicant's business

Supplier of food ingredients

(g) details of other individuals, companies or organisations associated with the application

[redacted]

C Purpose of the application

This application seeks an amendment to Schedule 26 of the Australia New Zealand Food Standards Code (the Code) to permit an alternative genetically modified source organism – *Escherichia coli* K-12 MG1655 INB-2FL_03 – for the production of the Human Milk Oligosaccharide (HMO) 2′-Fucosyllactose (2′-O-fucosyllactose, 2′-FL) by fermentation. The 2′-FL of Inbiose is chemically and structurally identical to the 2′-FL ingredients previously

assessed and found safe by FSANZ (please refer to the applications A1155[1], A1190[2] and A1233[3]).

Inbiose is not requesting any changes to the food categories and use levels of 2′-FL as permitted following FSANZ's assessments of A1155, A1190 and A1233. The intended use levels are consistent with the permitted levels of addition of 2′-FL in infant formula products in Schedule 29 (96 mg/100 kJ, equivalent to 2.4 g/L).

Inbiose therefore argue that most (if not all) of section 3.3.3 does not need to be addressed in this application because FSANZ has already assessed it for 2′-FL; and this application is only requesting a new source of 2′-FL to be permitted in the Code.

Hence, FSANZ's previous safety assessments of 2′-FL are relevant to this application. Inbiose has conducted a scientific literature review to ascertain whether any studies published after FSANZ's most recent risk assessment are relevant to the safety of 2′-FL. More information on this literature review is provided in section 3.3.3.C.2 and Appendixes 8.1 and 8.2 of this application.

D Justification for the application

Schedule S29-5[4] (*Infant formula products – substances permitted as nutritive substances*) permits the addition of 2′-O-fucosyllactose (2′-fucosyllactose), as a nutritive substance, to infant formula products, as a result of Applications A1155, A1190 and A1233.

Inbiose produces 2′-FL by microbial fermentation, using the non-pathogenic *E. coli* K-12 MG1655 INB-2FL_03 strain. Details about the production and production host is outlined in Part 3.3.2.

Inbiose is not requesting any changes to the permitted food categories and use levels of 2′-FL. The intended use levels are consistent with the permitted levels for 2′-FL in Schedule 29 (96 mg/100 kJ, equivalent to 2.4 g/L). The 2′-FL of Inbiose is chemically and structurally identical to the 2′-FL ingredients previously assessed by FSANZ. Hence, FSANZ's previous assessments of 2′-FL are relevant to the 2′-FL of Inbiose.

[1] https://www.foodstandards.gov.au/code/applications/Pages/A1155.aspx
[2] https://www.foodstandards.gov.au/code/applications/Pages/A1190.aspx
[3] https://www.foodstandards.gov.au/code/applications/Pages/A1233%20-2%E2%80%B2-FL-from-new-GM-source-for-infant-formula.aspx
[4] https://jade.io/j/?a=outline&id=803601

Inbiose has also conducted a comparison of the 2′-FL from *E. coli* K-12 MG1655 INB-2FL_03 with 2′-FL from human breast milk. The identity of Inbiose's 2′-FL has been confirmed by nuclear magnetic resonance (NMR), with a 2′-FL reference standard derived from human milk. Based on NMR, the Inbiose 2′-FL is structurally identical to the reference. All major signals in the 1H-NMR spectra of 2′-FL were identical among materials isolated from Inbiose's 2′-FL and reference, and identical to 1H-NMR spectra reported in the literature (Kjærulff, 2014; van Leeuwen *et al.*, 2014). The typical shifts of the anomeric protons/carbons and those of the methyl group of the fucose moiety further confirm the 2′-FL structure. See appendix 1 for a report on the NMR analysis.

FSANZ has already assessed and approved the use of 2′-FL as a nutritive substance in infant formula products and the 2′-FL of Inbiose is chemically identical to these approved products (with the same level or less impurities than the approved 2′-FL products, see Table 8 and 9). Inbiose concludes that FSANZ has already addressed the nutritive substance requirements relating to the use of 2′-FL in infant formula products. As Inbiose is only requesting a new permitted source of the nutritive substance, the majority of the information requested in section 3.3.3 of the Application Handbook is therefore considered by Inbiose not to be required for this application. Information on the technical properties of Inbiose's 2′-FL ingredient is included in section 3.3.3 of this application, addressing specific technical requirements in section 3.3.3 of the Application Handbook.

Inbiose's source organism is not listed in Schedule 26 as a permitted source of 2′-FL (produced using gene technology of microbial origin). Inbiose seeks to amend Schedule 26 to add *E. coli* K-12 MG1655 INB-2FL_03 as a permitted source organism for 2′-FL. The *E. coli* K-12 strain used as production host in application A1233 is very closely related to the MG1655 lineage of *E. coli* K-12 (Bachmann, 1972).

Three 2′-FL products are already approved by FSANZ (please refer to applications A1155, A1190, A1233 and A). Approval of the 2′-FL product in this application would increase the freedom of choice for the customers in Australia and New Zealand. Appendix 2 lists some products that are on the market in Australia containing approved 2′-FL. Hence, as companies already include 2′-FL in their food products, this could be considered as a justification that 2′-FL is an interesting ingredient for the market in Australia and New Zealand.

D.1 Regulatory impact information

D.1.1 Costs and benefits of the application

Costs for consumers: There are no costs to consumers from this application. Consumers may benefit from the greater availability of foods containing 2′-FL through the approval of an additional source organism.

Costs for food Industry: There are no costs to food manufacturers and suppliers from this application. Food manufacturers may benefit from the opportunity to source 2′-FL from an alternative supplier.

Costs for Government: There are no additional costs to governments from this application, beyond the normal costs of ensuring compliance with food laws.

D.1.2 Impact on international trade

This application will align food legislation in Australia and New Zealand with food legislation in the USA[5] and the European Union[6], which both permit the addition of 2′-FL produced using *E. coli* K-12 strain to food products. This has the potential to enhance international trade in respect of both the import and export of infant formula products containing added 2′-FL.

E Information to support the application

The application has addressed the FSANZ Application Handbook requirements. In particular, after pre-application discussions with FSANZ, sections 3.3.2.D and 3.3.2.E of the Handbook have been addressed to enable FSANZ to adequately assess Inbiose's production organism. Sections 3.3.3.A and 3.3.3.B of the Handbook have been addressed to enable FSANZ to adequately assess technical aspects of Inbiose's 2′-FL. In relation to the safety of Inbiose's 2′-FL, the application includes the outcomes of literature reviews conducted to ascertain whether any relevant new data has been published since FSANZ's most recent assessment of 2′-FL. Appendixes 8.1-8.2 include the detail of these literature reviews.

F Assessment procedure

[5] GRAS Notices GRN 749 and GRN 897 https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=GRASNotices
[6] https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32018R1023&from=EN

2′-FL is already approved in the Code. This application is limited to the approval a new source organism for its production, consequently, the General Procedure, level 1 or 2, is the appropriate procedure to be adopted in assessing this application.

G Confidential Commercial Information (CCI)

The application contains confidential commercial information (CCI) for which confidential treatment is requested. The following CCI has been developed with significant financial resources and investment of time by Inbiose N.V. Inbiose N.V. considers this information as commercially sensitive information that would incur significant negative commercial impact for Inbiose N.V. if competitors acquired access to the information. CCI will be discussed in separate appendices, and a summary will be included in the relevant section in this dossier. See the Cover Letter for justification of the claim for CCI. Inbiose N.V. requests that identified information in Appendixes 1 and 4 through to 7 be treated as CCI. Batch identification data in Appendix 3 is the only information that is requested to be treated as CCI in that document; and has been removed by Inbiose N.V. in a redacted public version. In addition, section 3.3.2 (processing aids) contains CCI developed with significant time and cost inputs from Inbiose. Public release of this CCI will adversely impact on the value of this information to Inbiose and may provide commercial advantage to Inbiose's competitors.

H Other confidential information

The application contains other confidential information (OCI) for which confidential treatment is requested. This includes information that may identify individuals.
Other confidential information is redacted in the public version of the application, Appendix 9 and Cover letter.

I Exclusive capturable commercial benefit (ECCB)

This application seeks exclusive permission for the use of the *E. coli* K-12 MG1655 INB-2FL_03 strain containing the gene for α-(1,2)-fucosyltransferase from *Helicobacter* sp. For the production of 2′-FL. Inbiose is seeking a period of exclusive permission of 15 months from the time of gazettal of the permission in Schedule 26. The brand name of the 2′-FL product of Inbiose will be 2′-FL-Inbiose. An exclusive permission in the Code will confer an ECCB on Inbiose.

In addition, the following questions from the Application Handbook have been addressed.

Why are you making this application? What are you hoping to get out its approval?	As indicated in Section 3.1.1-D, there is a profitable/viable market in Australia and New Zealand for the manufacturing of infant formula products both for use in Australia and New Zealand, as well as for export to other countries, specifically in South East Asia and China. Furthermore, there is an increasing public interest in high quality infant formula with a composition more similar to human milk. Australia and New Zealand manufacturers of infant formula are generally recognised by their higher quality products that are attractive to the Asian markets, as well as the local market. The conferring of an exclusive permission will allow Inbiose to market to manufacturers of infant formula in Australia and New Zealand and to establish early access to these markets relative to other competitors. This may result in greater returns on the investment that these infant formula manufacturers have committed to by developing the new formula products.
How will you benefit from the approval of your application?	If exclusive permission is granted for the 2′-FL of Inbiose, it provides Inbiose the opportunity to market to infant formula manufacturers in Australia and New Zealand, and gain access to a broad number of Asian markets, increasing competition within the Human Milk Oligosaccharides in Australia and New Zealand.
Who besides you, will benefit from the approval of your application? How and why will they benefit?	Inbiose's partners (i.e. infant nutrition manufacturers) will benefit from potentially higher sales and greater market shares (local and export markets) by manufacturing higher quality products.
If your application is approved, whose permission will be required before anyone can derive a benefit from that approval?	The applicant, Inbiose, is the producer and will provide the permission to its partners to market and sell 2'FL. The technology used to manufacture Inbiose's 2′-FL is patented by Inbiose. Therefore, a commercial agreement with Inbiose will be required to permit the supply, the use or import of Inbiose's 2′-FL into Australia and New Zealand.
Who holds the intellectual property in the subject matter of your application?	Inbiose holds the intellectual property rights for certain information relating to the 2′-FL of Inbiose, including key steps in the manufacturing process and the production strain that is presented in this application.

J International and other national standards

J.1 International standards

There are no Codex standards that expressly name the use of 2′-FL as an ingredient in foods. However, Codex Standard for Infant Formula and Formulas for Special Medical Purposes Intended for Infants (Codex Stan 72[7]), permits "other ingredients may be added in order to provide substances ordinarily found in human milk".

[7] https://www.fao.org/fao-who-codexalimentarius/sh-proxy/es/?lnk=1&url=https%253A%252F%252Fworkspace.fao.org%252Fsites%252Fcodex%252FStandards%252FCXS%2B72-1981%252FCXS_072e.pdf

J.2 Other national standards or regulations

In March 2022, Inbiose submitted a GRAS notice to FDA for approval in the US for the 2′-FL product manufactured with *E. coli* K-12 MG1655 INB-2FL_03 (GRAS Notice No 1091[8], see appendix 3). FDA has approved a 2′-FL product from Inbiose made with an earlier production host of *E. coli* K-12 MG1655, in GRN 897[9]. The specification of the latest submission is equivalent to the 2′-FL approved in GRN 897.

The specification mentioned in Table 8 matches the specification approved for microbially produced 2′-FL listed in the EU Novel Food Union List[10].

The following list provides examples of regulatory approvals already sought and granted internationally. This is a non-exhaustive list of approvals granted for dossiers with 2′-FL worldwide, only key approvals are mentioned (see Table 1).

Table 1. Non-exhaustive list of approvals granted for dossiers with 2′-FL worldwide.

Region	Approval	Approval date	Applicant
AusNZ / FSANZ	A1155[11] Amendment 198 of FSC[12]	20 December, 2019	Glycom A/S
	A1190[13]	8 November, 2021	Jennewein Biotechnologie, GmbH
	A1233[14]	6 May, 2022	FrieslandCampina Nederland B.V.
	A1251[15]	14 December, 2022	Nutricia Australia Pty Ltd and Chr. Hansen A/S
Aus / TGA	TGA approval in dietary supplement [16, 17]	1 March, 2021	BASF
US / FDA	GRN 546[18]	October 10, 2014	Glycom A/S
	GRN 650[19]	November 23, 2016	Glycom A/S

[8] https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=GRASNotices&id=1091&sort=GRN_No&order=DESC&startrow=1&type=basic&search=fucosyl

[9] https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=GRASNotices&id=897&sort=GRN_No&order=DESC&startrow=1&type=basic&search=897

[10] https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32018R1023&from=EN

[11] https://www.foodstandards.gov.au/co20 Dec 201§9de/applications/Pages/A1155.aspx

[12] https://www.foodstandards.gov.au/code/changes/gazette/Pages/Amendment-No.198---26-March-2021.aspx

[13] https://www.foodstandards.gov.au/code/applications/Pages/A1190.aspx

[14] https://www.foodstandards.gov.au/code/applications/Pages/A1233%20-2%E2%80%B2-FL-from-new-GM-source-for-infant-formula.aspx

[15] https://www.foodstandards.gov.au/code/applications/Pages/A1251----2%CA%B9-FL-combined-with-galacto-oligosaccharides-and-inulin-type-fructans-in-infant-formula-products.aspx

[16] Therapeutic Goods (Permissible Ingredients) Determination (No. 1) 2023 (legislation.gov.au)

[17] https://www.tga.gov.au/compositional-guideline/2-fucosyllactose

[18] https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=GRASNotices&id=546&sort=GRN_No&order=DESC&startrow=1&type=basic&search=fucosyl

[19] https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=GRASNotices&id=650&sort=GRN_No&order=DESC&startrow=1&type=basic&search=fucosyl

Region	Approval	Approval date	Applicant
	GRN 815[20]	September 11, 2020	Glycom A/S
	GRN 1034[21]	October 21, 2022	Glycom A/S
	GRN 852[22]	November 15, 2019	BASF SE
	GRN 735[23]	April 6, 2018	Glycosyn, LLC and Friesland Campina Domo B.V
	GRN 571[24, 25]	November 6, 2015	Jennewein Biotechnologie, GmbH
	GRN 929[26]	February 26, 2021	Jennewein Biotechnologie, GmbH
	GRN 1014[27]	July 15 2022	Chr. Hansen A/S
	GRN 749[28]	April 23, 2018	DuPont Nutrition & Health
	GRN 897[29]	June 12, 2020	DuPont Nutrition & Health
	GRN 932[30]	February 18, 2021	Advanced Protein Technologies
EU / European Commision /EFSA	Decision 2016/376[31]	March 16, 2016	Glycom A/S
	Regulation 2019/388[32]	March 11, 2019	Glycom A/S
	Regulation 2019/1979[33]	November 26, 2019	Glycom A/S
	Regulation 2021/50[34]	January 22, 2021	Glycom A/S
	Regulation 2017/2201[35]	November 27, 2017	Jennewein Biotechnologie, GmbH
	Regulation 2018/1023[36]	July 23, 2018	DuPont Nutrition & Biosciences
	Regulation 2018/1023[37]	July 23, 2018	Friesland Campina DOMO
	Regulation 2023/859[38]	April 26, 2023	Advanced Protein Technologies Corporation

[20] https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=GRASNotices&id=815&sort=GRN_No&order=DESC&startrow=1&type=basic&search=fucosyl

[21] https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=GRASNotices&id=1034&sort=GRN_No&order=DESC&startrow=1&type=basic&search=fucosyl

[22] https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=GRASNotices&id=852&sort=GRN_No&order=DESC&startrow=1&type=basic&search=fucosyl

[23] https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=GRASNotices&id=735&sort=GRN_No&order=DESC&startrow=1&type=basic&search=fucosyl

[24] https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=GRASNotices&id=571&sort=GRN_No&order=DESC&startrow=1&type=basic&search=fucosyl

[25] https://fda.report/media/142982/GRAS-Notice-GRN-929-2-Fucosyllactose.pdf

[26] https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=GRASNotices&id=929&sort=GRN_No&order=DESC&startrow=1&type=basic&search=fucosyl

[27] https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=GRASNotices&id=1014&sort=GRN_No&order=DESC&startrow=1&type=basic&search=1014

[28] https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=GRASNotices&id=749&sort=GRN_No&order=DESC&startrow=1&type=basic&search=749

[29] https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=GRASNotices&id=897&sort=GRN_No&order=DESC&startrow=1&type=basic&search=fucosyl

[30] https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=GRASNotices&id=932&sort=GRN_No&order=DESC&startrow=1&type=basic&search=fucosyl

[31] https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32016D0376&qid=1683874735848

[32] https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32019R0388&from=EN

[33] https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32019R1979&from=EN

[34] https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32021R0050&from=EN

[35] https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32017D2201&from=EN

[36] https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32018R1023&from=EN

[37] https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32018R1023&from=EN

[38] https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32023R0859

Region	Approval	Approval date	Applicant
Canada / Health Canada	Health Canada[39]	December 5, 2018	Jennewein Biotechnologie, GmbH
	Health Canada[40]	November 3, 2021	DuPont Nutrition & Biosciences
	Health Canada[41]	June 9, 2021	Abbott Nutrition/Chr. Hansen
Taiwan / TFDA	Wei Shi Zi No. 1101301211[42, 43]	June 16, 2021	Glycom A/S
	MOHW Food No 1091303448[44, 45]	December 16, 2020	Jennewein Biotechnologie, GmbH
China / MARA	Strain safety approval[46]	October 27, 2021	DSM
Brazil[47]/ ANVISA	RE 1020	April 17, 2019	Foodstaff S/C ltda / Glycom de Alimentos ltda
	RE 3427	Sept 3, 2020	Mead Johnson do Brasil / Jennewein Biotechnologie, GmbH
	RE 4409	October 29, 2020	Foodstaff S/C ltda / Glycom Manuafacturing Denmark
	RE 1351	March 31 2021	Danone Ltda/Jennewein Biotechnologie, GmbH
	RE 1547	April 14, 2021	Danisco Brasil LTDA / DuPont Nutrition & Health
	RE 4802	December 23, 2021	DSM Brasil SA/ Glycom A/S Denamrk

K Statutory declaration

A signed Statutory Declaration is provided in Appendix 9.

L Checklist

A complete checklist of the required information for submission of this application is provided in Appendix 10.

[39] https://www.canada.ca/en/health-canada/services/food-nutrition/genetically-modified-foods-other-novel-foods/approved-products/2-fucosyllactose-escherichia-coli-bl21/technical-summary.html

[40] https://www.canada.ca/en/health-canada/services/food-nutrition/genetically-modified-foods-other-novel-foods/approved-products/fucosyllactose-escherichia-coli/document.html

[41] Novel Food Information: 2'-fucosyllactose (2'-FL) from Escherichia coli BL21 (DE3) Strain #1242 - Canada.ca

[42] https://members.wto.org/crnattachments/2021/SPS/TPKM/21_4183_00_e.pdf

[43] https://members.wto.org/crnattachments/2023/SPS/TPKM/23_0976_01_e.pdf

[44] https://members.wto.org/crnattachments/2020/SPS/TPKM/20_7748_00_e.pdf

[45] https://members.wto.org/crnattachments/2023/SPS/TPKM/23_0976_00_e.pdf

[46] https://www.nutritioninsight.com/news/dsm-celebrates-chinas-pivotal-ruling-on-hmo-manufacturing-strains.html

[47] https://app.powerbi.com/view?r=eyJrIjoiNTA3ZDQxOGEtYzg0NC00NTI1LTg0MzYtOGEzMWU4MThlNjAwIiwidCI6ImI2N2FmMjNmLWMzZjMtNGQzNS04MGM3LWI3MDg1ZjVlZGQ4MSJ9

Structure of the application

In pre-application discussions, FSANZ advised Inbiose that given the nature previous assessments of 2′-FL and that this application is only requesting approval of a new source of 2′-FL, that the requirements of section 3.3.2 – Processing aids, specifically Part D and Part E, would be sufficient to address in the application (rather than addressing the section 3.5.1 – Foods produced using gene technology requirements). Section 3.3.2 of this application addresses these processing aid requirements relating to Inbiose's source organism.

Pre-application discussions also noted that the Application Handbook's section 3.6.2 – Special purpose foods requirements do not need to be addressed in the application.

Inbiose considered the requirements of section 3.3.3 – Substances used for a nutritive purpose had been addressed by FSANZ in previous approvals of 2′-FL. FSANZ requested that information on the purpose of the use of 2′-FL in infant formula products must be stated and that a review of the scientific literature published since FSANZ's most recent risk assessment should be conducted to capture any new studies relating to safety of 2′-FL. Inbiose has also included information on technical aspects of its 2′-FL ingredient, which addresses section 3.3.3.B of the Application Handbook. Section 3.3.3 of this application addresses these nutritive substances aspects of the Application Handbook.

3.3.2 PROCESSING AIDS

D Information related to safety of enzyme processing aid derived from a microorganism

D.1 Information on the source microorganism

D.1.1. Taxonomic information on the microorganism

The host organism is *Escherichia coli*, which is the same host organism as described in A1155, A1190 and A1233. The taxonomy of the species of Inbiose´s *E. Coli* strain is as shown in Table 2:

Table 2 The current taxonomic placement of *E. Coli* K-12 MG1655

Domain	Bacteria
Kingdom	Bacteria
Phylum	Proteobacteria
Class	Gamma-Proteobacteria
Order	*Enterobacteriales*
Family	*Enterobacteriaceae*
Genus	*Escherichia*
Species	*Escherichia coli*
Sub-species	*E. coli* K-12
Strain	MG1655

D.1.2. Information on the source organism used to manufacture 2′-FL

D.1.2.1. Information about E. Coli K-12 used in food production

S26—3 (Food produced using gene technology – permitted food produced using gene technology and conditions) permits the use of *E. coli* K-12 to produce some HMOs permitted to be used as a nutritive substance in infant formula, as a result of Applications A1155 and A1233.

The host strain *E. coli* K-12 is currently in use to produce 2′-FL in two different processes:

- one strain containing a gene for an alpha-1,2-fucosyltransferase from *Helicobacter pylori*, application A1155
- one strain containing an alpha-1,2-fucosyltransferase from *Bacteroides vulgatus*, application A1233.

The 2′-FL produced from these modified strains may only be used as a nutritive substance in infant formula and sold under a specific brand of GlyCare and Aequival®2'FL respectively, during the exclusive use period.

Another modified *E. coli* K-12, containing a beta-1,3-N-acetylglucosaminyltransferase gene from *Neisseria meningitides* and a beta-1,4-galactosyltransferase gene from *Helicobacter pylori*, is producing lacto-N-neotetraose, to be added to infant formula products in combination with 2′-FL, under the brand GlyCare, also during the exclusive use period, following application A1155.

E. coli K-12 strain is very closely related to the MG1655 lineage of *E. coli* K-12 (Bachmann, 1972).

D.1.2.2. Information about E. Coli strain MG1655

The host strain, *E. coli* K-12 strain MG1655, is available from both American Type Culture Collection (ATCC) and the Coli Genetic Stock Center as ATCC#700926 and CGSC#7740, respectively. *E. coli* strains proliferate *via* asexual reproduction. This strain is non recombinant, stable, and can easily be maintained as a homogeneous population under the usual laboratory and production conditions. This strain does not produce spores.

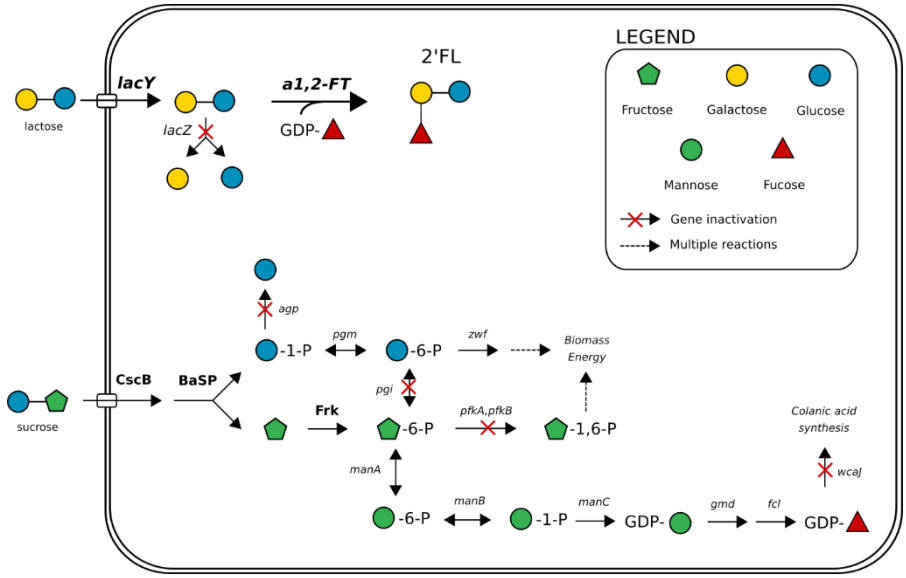
E. coli K-12 strain MG1655 is derived from the well-known *E. coli* K-12 strain via classical, non-recombinant genetics and cured of the temperate bacteriophage lambda and F plasmid by means of ultraviolet light and acridine orange, respectively. The genotype of the recipient microorganism is F-lambda-ilvG-rfb-50 rph-1, and the serotype is IRLH48:K- (Blattner *et al.*,

1997). Later additional mutations in commonly used stocks of *E. coli* K-12 strain MG1655 were identified and determined to cause loss of function of the *glpR* and *crl* genes, which are involved in glycerol 3-phosphate and RNA polymerase formation, respectively (Freddolino *et al.*, 2012). The complete genome of this strain has been sequenced (GenBank U000968[48]).

D.1.3. Method used to manufacture 2′-FL

The schematic overview of the 2′-FL biosynthetic pathway in INB-2FL_03 using sucrose as carbon source is provided in Figure 1. For more details on the manufacturing process of 2′-FL, please refer to the section 3.3.3.B.5 for details.

Figure 1 Schematic Overview of the 2′-FL Biosynthetic Pathway in INB-2FL_03 using Sucrose as Carbon Source



2′-FL = 2´-Fucosyllactose; a1,2-FT = alpha-1,2-fucosyltransferase; agp = glucose-1-phosphatase; BaSP = sucrose phosphorylase;CscB = sucrose permease; fcl = GDP-L-fucose synthase; Frk = fructokinase; GDP = guanosine diphosphate; gmd = GDP-mannose 4,6-dehydratase; lacY = lactose permease; lacZ = β-galactosidase; manA = mannose-6-phosphate isomerase; manB = phosphomannomutase; manC = mannose-1-phosphate guanylyltransferase; P = phosphate; pfkA = 6-phosphofructokinase 1; pfkB = 6-phosphofructokinase 2; pgi = glucose-6-phosphate isomerase; pgm = phosphoglucomutase; wcaJ = UDP-glucose:undecaprenyl-phosphate glucose-1-phosphate transferase; zwf = NADP+-dependent glucose-6-phosphate dehydrogenase.

D.2 Information on the pathogenicity and toxicity of the source microorganism

D.2.1. Source microorganism:

The United States Environmental Protection Agency conducted a risk assessment of *E. coli* K-12 under the Toxic Substances Control Act (U.S. EPA, 1997). This review concluded that "the use of *E. coli* K-12 under contained conditions in fermentation facilities will present a low

[48] https://www.ncbi.nlm.nih.gov/nuccore/545778205/.

risk of release of this microorganism to the environment and would not pose any significant ecological hazards, based on the following evidence:

- Wild-type *E. coli* is an inhabitant of the human colon;
- Studies have demonstrated that *E. coli* K-12 is a debilitated strain, defective in at least three cell wall characteristics that are important for colonization. As a result, *E. coli* K-12 is unable to colonize the human intestinal tract under normal conditions. Even in germ-free mice, *E. coli* K-12 is a poor colonizer;
- Experimental evidence has strongly suggested that indigenous intestinal microorganisms have a large competitive advantage over *E. coli* K-12 strains;
- *E. coli* K-12 lacks the ability to produce toxins that affect humans. There is no record in the literature of *E. coli* K-12 enterotoxin-induced disease in fermentation workers; and
- *E. coli* K-12 has a history of safe commercial use. Its derivative strains are currently used in many industrial applications, including the production of specialty substances L-aspartic, inosinic and adenylic acids, which the human body produces, and FDA-approved human drugs such as insulin and somatostatin."

Because *E. coli* K-12 is not considered a human or animal pathogen and is not toxicogenic, it falls into Biosafety Level 1 classification and meets the Organisation for Economic Co-operation and Development (OECD) Good Industrial Large-Scale Practice (GILSP) criteria (OECD, 1992). *E. coli* K-12 strain MG1655 has been classified Biosafety Level 1 by the ATCC[49].
Escherichia coli is the same species as the host organism; we have described the safety of this strain in greater detail above.

E. coli K-12 contains no known pathogenic genes (either colonization factors or toxin genes) and is a universally recognized as a safe commercial manufacturing host. *E. coli* K-12 is used globally in the commercial manufacturing of products ranging from amino acids and vitamins for foodstuff applications, to recombinant human proteins used in pharmaceutical applications, including protein products used as injectables. Pharmaceutical proteins expressed by *E. coli* include human insulin, growth hormones (somatostatin, somatotropin), immunomodulators (interferons, interleukins, tumor necrosis factor), growth factors (granulocyte colony-stimulating factor, epidermal growth factor), blood factors, coagulation inhibitors (tissue plasminogen activator, staphylokinase), and enzymes (Schiermann et al., 2015).

[49] https://www.atcc.org/Products/All/700926.aspx

There is also precedent for the safe ingestion of live microbial *E. coli* preparations. Molecular genetic differentiation and identification methods make it possible to unequivocally distinguish pathogenic *E. coli* variants from non-pathogenic strains. Mutaflor® is an example of a probiotic therapy in which the active ingredient consists of a viable non-pathogenic *E. coli* strain, *E. coli* Nissle 1917. It is used in Europe and Canada for inflammatory and chronic functional bowel diseases (Mutaflor® website, https://aralez.com/Portfolio/mutaflor/). In the US, Mutaflor® was considered to be a "medical food," however, FDA classified Mutaflor® as a "biologic," and the product is currently discontinued from the US market pending a final decision (Mutaflor® website: https://www.mutaflor.com/index.html). Probiotics are, by definition, living non-pathogenic micro-organisms that exert a positive effect on the host organisms when they enter the gastrointestinal tract in a viable condition in sufficiently large numbers (FAO/WHO, 2002). Mutaflor® has a 95-year record of being well tolerated and lacking adverse effects. In addition, non-pathogenic strains, unlike virulent *E. coli* variants, exhibit no harmful effects in toxicological studies in both conventional and germ-free animals (Schiermann et.al., 2015).

Considering the established safety of the host strain, the nature of the introduced genes, and the 2′-FL end product resulting from modification, no differences in pathogenicity are expected between *E. coli* K-12 and *E. coli* K-12 MG1655 and *E. coli* K-12 MG1655 INB-2FL_03.

D.3 Information on the genetic stability of the source organism

D.3.1. Stability of phenotype of the production strain INB-2FL_03

The stability of the INB-2FL_03 production strain was investigated by a six-day shake flask experiment followed by a growth experiment with inoculum withdrawn at day 6 of the shake flask experiment. Cell density, growth speed and 2′-FL synthesis served as phenotypic markers. The experimental details are described below.

D.3.1.1.Material and methods

D.3.1.1.1.Shake flask medium composition

Standard minimal shake flask medium consists of (final concentration) [redacted]

[redacted]

D.3.1.1.2.Serial shake flask experiment

At day 0, inoculation of [redacted] in parallel with strain INB-2FL_03 in [redacted] shake flask medium and start incubation at [redacted]. Every day at a fixed time point, 20 µl culture was used to inoculate another Erlenmeyer with [redacted] fresh shake flask medium (referred to as the seed flasks; the [redacted]. At each timepoint, [redacted] culture was used for the preparation of a cryovial [redacted] of each Erlenmeyer series, which was further used as inoculum for a growth experiment.

D.3.1.1.3.MTP growth experiment

To determine the maximum growth speed, an MTP growth experiment was performed where the reference (i.e. start cryovial) and the 3 cryovials sampled at day 6 were used as inoculum (performed in triplicate for all cryovials). The MTP experiment was performed in polystyrene 96-halfdeepwell MTPs (square wells, flat transparent polystyrene bottom) (System Duetz) with [redacted] shake flask medium. The incubation was done for [redacted] shaking incubator ([redacted]) and every [redacted] was measured in a [redacted] Afterwards, [redacted] data was used to calculate the maximum growth speed (µmax), and end samples at [redacted] were used to determine the 2′-FL titer with UPLC.

D.3.1.1.4.UPLC method

The 2′-FL concentration is determined by means of an [redacted] UPLC with RI detector [redacted] using an [redacted] set at 50°C. The eluent solution consisted of 25 mL ultrapure water mixed with 75 mL acetonitrile and 0.15 mL triethylamine. An isocratic flow was used at [redacted] The parameters of the detector were set as follows: [redacted] The outcome was analysed with the [redacted] and quantified using an ultrapure standard of 2′-FL.

D.3.1.2.Stability of the phenotype - results

The results shown in Figure 2 point towards no substantial difference in growth and productivity of the production strain over a period of 6 days [redacted] compared to the reference strain.

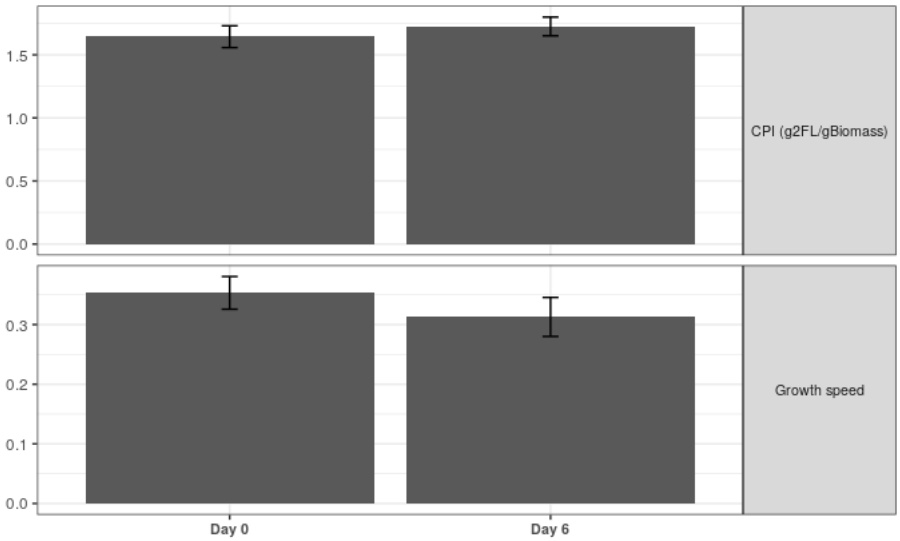


Figure 2 Phenotype data of strain INB-2FL_03, in a growth experiment with cryovials created at day 0 (0 generations) vs day 6 [redacted] of a serial shake flask experiment. Plotted on the y-axis is the measured 2'FL titer divided by the biomass at the end of the experiment (Cell Performance Index (CPI) (g2FL/gBiomass), upper facet) and the growth speed (lower facet).

D.3.2. Stability of the genotype of production strain INB-2FL_03

To investigate the stability of the genotype of strain INB-2FL_03, Whole Genome Sequencing (WGS) was performed on genomic DNA obtained from the biomass taken at the end of the serial shake flask experiment (day 6, [redacted]) and multiple fed-batch fermentations. The procedure for WGS is the same as described below. Results of WGS of the sample at the end of the process were compared with WGS results of an [redacted] of INB-2FL_03. In all samples, no additional mutations appeared in the genome or on the plasmid [redacted] of production strain INB-2FL_03. These analyses support the genetic stability of production strain INB-2FL_03 during the period of production.

D.3.2.1.Method for WGS analyses

WGS was conducted using the [redacted]

[redacted]

Assembly annotation was then performed by Prokka (1.13.3) (Seemann, 2014). The average coverage to the *E. coli* K-12 MG1655 reference genome U00096.3 is [redacted]

Taxonomical identification was performed with FastANI[50], [redacted]

E Additional information related to the safety of an enzyme processing aid derived from a genetically-modified microorganism

E.1 Information on the methods used in the genetic modification of the source microorganism

E.1.1. Method for gene deletions (knock-out) and insertions (knock-in) in the genome of INB-2FL_03

Several modifications, like gene knock-out, gene insertions, and the addition of a production plasmid, were performed on *E. coli* K-12 strain MG1655 to create a 2′-FL production strain and to obtain efficient biosynthesis of 2′-FL. A production strain, INB-2FL_03, has been developed, through which the safety was assessed.

The general method to introduce genetic modifications like gene deletions and gene knock-in into the genome of the production strain genome is based on the methods described in detail by Datsenko and Wanner, 2000; Snoeck et al., 2019. The method is briefly described below in

[50] https://github.com/ParBLiSS/FastANI

Figure 3. Every engineering step is verified by means of sequencing and polymerase chain reaction (PCR).

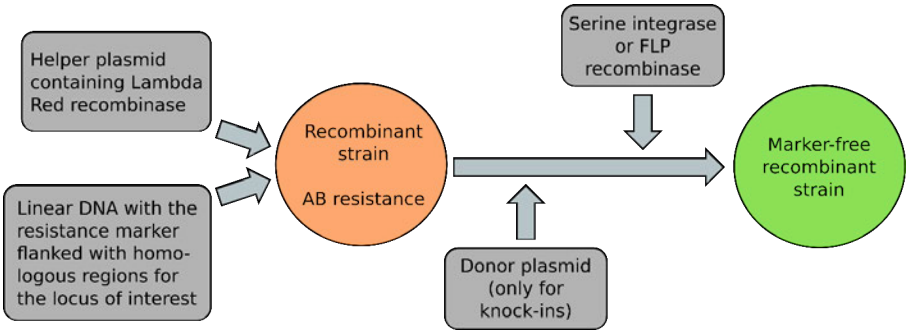
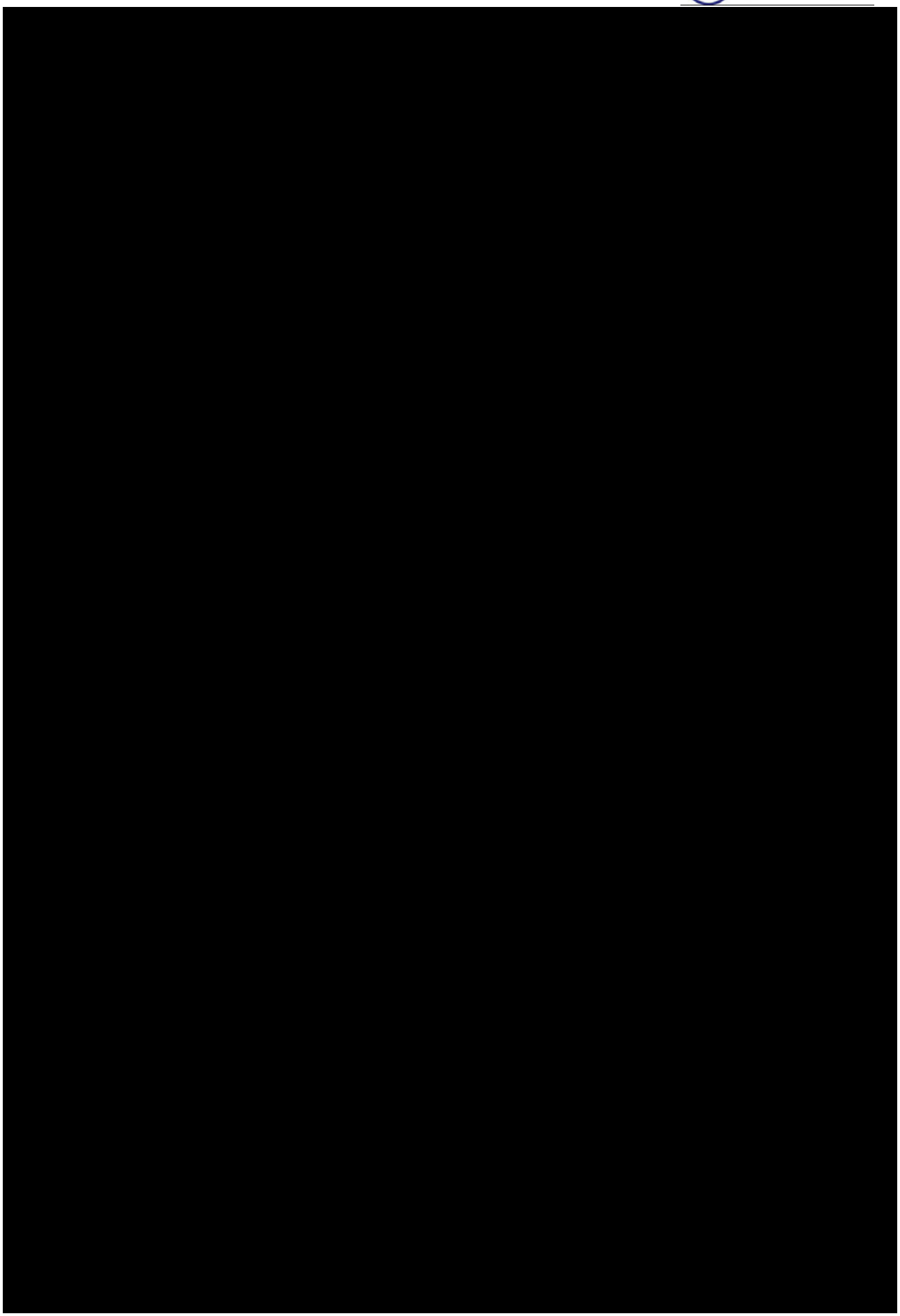


Figure 3 General scheme of the strain construction process.

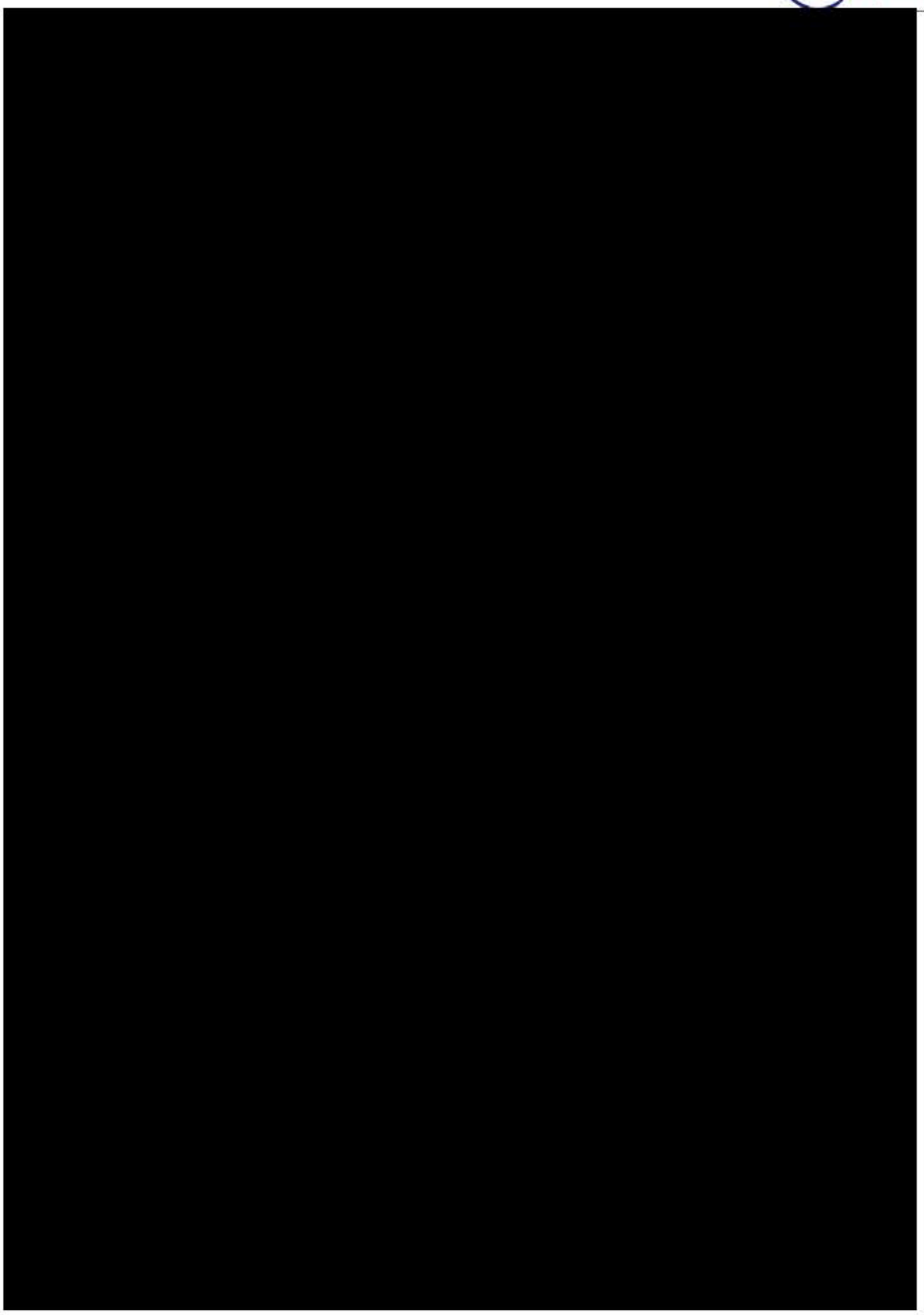
[redacted]



[redacted]

The final strain does not contain any trace of the helper plasmids nor from the antimicrobial resistance genes. [redacted]

After each modification, the previous modifications have been checked by [redacted] to ensure no other modifications have occurred during the engineering process. No additional modifications or chromosome re-arrangements have been observed, which was validated by WGS. In most cases, [redacted]



E.1.1.1.Designed sequences

[redacted]

An overview of all inserted DNA sequences is given in Table 3. For a more detailed list of mutations, see Appendix 5.

Table 3 Overview of synthetic genes and their TUs inserted on the plasmid [redacted] and on the genome of strain INB-2FL_03.

[redacted]

All heterologous genes introduced into INB-2FL_03 were produced by DNA synthesis and were based on well-known annotated genomes from the respective donor organism. As such, no PCR techniques were used, indicating that there is no risk of undesirable or unintended genes from the donor organism being introduced to the production host. If needed, the heterologous genes were codon-optimized using bio-informatic tools. Additionally, before and after introducing these heterologous genes into the genome of the production host organism, a full Sanger sequencing of the transcription units was performed to ensure their identity.

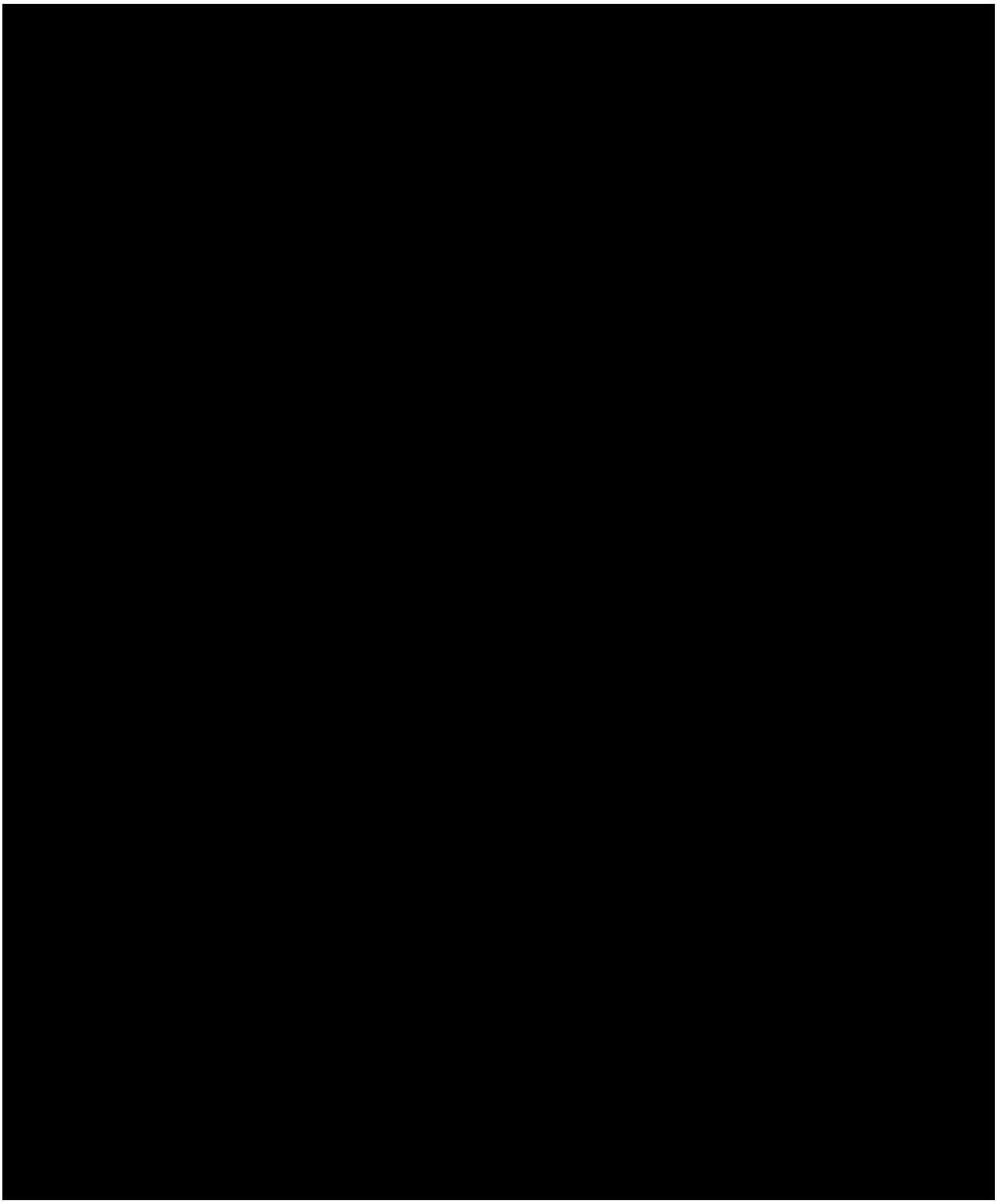
Table 4 Genetic Modification of the Production Organism (Gene Knock-ins)

Origin	Function
Escherichia coli	Lactose permease
Escherichia coli	Sucrose permease
Bifidobacterium adolescentis	Sucrose phosphorylase
Zymomonas mobilis	Fructokinase
Helicobacter sp.	α(1,2)-fucosyltransferase

Knock-out were performed to avoid breakdown of lactose, improve the flux towards guanosine diphosphate (GDP)-fucose, and avoid the production of unwanted metabolic by-products (see Table 5, section E.1.1.2). This strain was further modified to biosynthesize 2′-FL by the introduction of genes throughout the genome (see Table 4). In addition to the chromosomal modifications, a plasmid was also introduced in the production host INB-2FL_03 for overexpression of a fucosyltransferase gene from *Helicobacter sp.* No antibiotic resistance genes were present on the plasmid. The whole vector was synthesized *de novo* and is named [redacted] After strain construction, colony PCR, Sanger sequencing, and WGS checks were performed to verify all genetic modifications introduced in the 2′-FL production strain. Production strain INB-2FL_03 does not contain any antibiotic resistant marker on the plasmid or introduced inside its genome.

Assembled contigs of the production strain were compared to *E. coli* K-12 MG1655 (U00096.3) reference genome. A whole-genome average nucleotide identity (ANI) of >99.95% was obtained confirming that the production strain is *E. coli* K-12 MG1655.
For more information on the host source organism, please refer to the *section 3.3.2.(* 3-D.1.2.2; 3-D.2.1; 3-E.1.1).

E.1.1.1.1.Recombinant genes inserted in the genome of INB-2FL_03



[redacted]

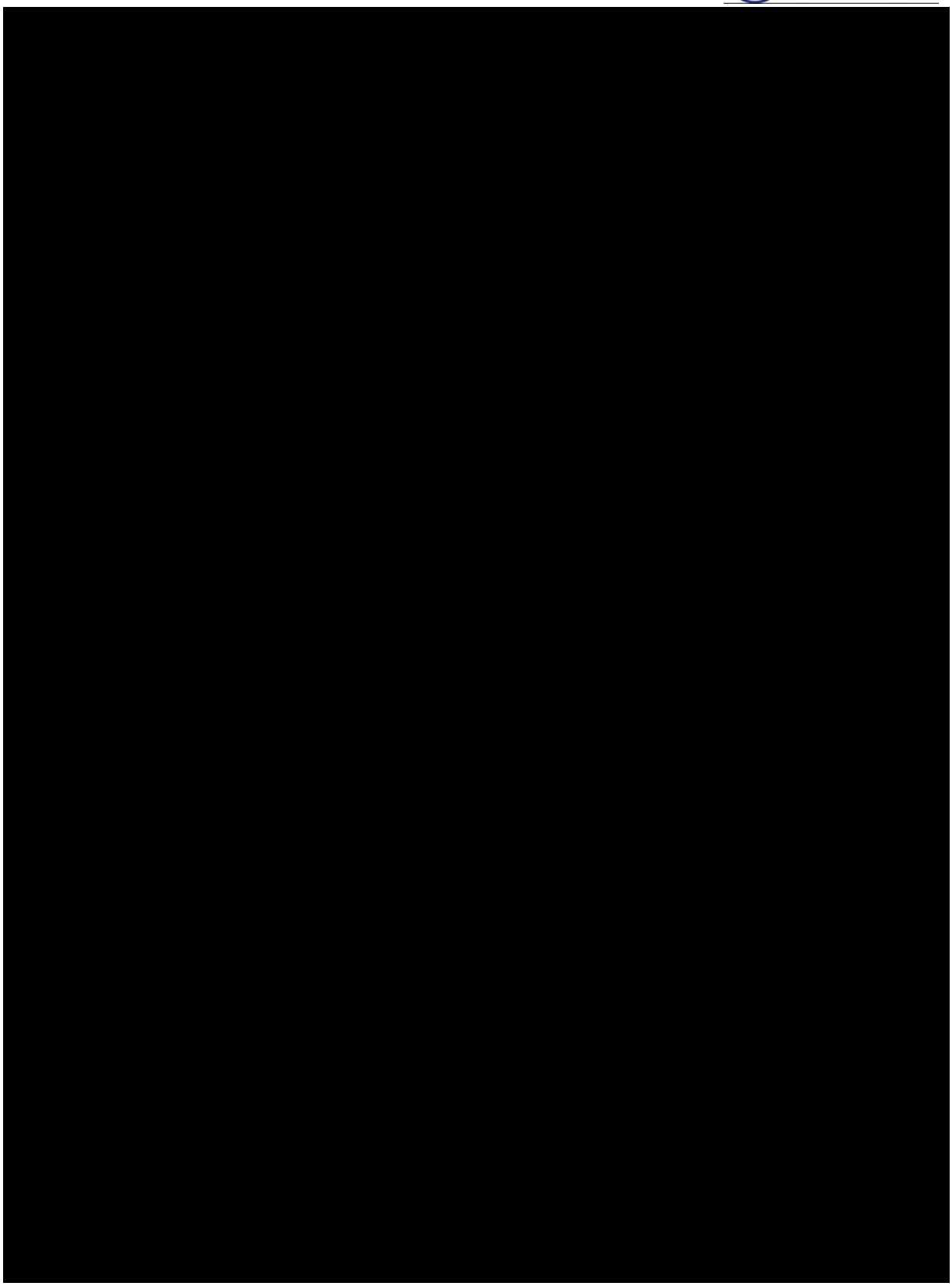
E.1.1.1.2. Genetic information regarding the plasmid [redacted]

[redacted]

E.1.1.2.Deleted sequences

To avoid by-production formation, the breakdown of lactose, and generally to increase the flux towards GDP-fucose (and thus also 2′-FL) production, several genes were deleted in the production strain INB-2FL_03. An overview of the deleted sequences with the explanation of the intended effect is given in Table 5. For a more detailed list of mutations, see Appendix 5.

Table 5 Overview of all genes that were deleted in production host INB-2FL_03 together with their intended effect.



E.1.1.3.Base pair substitutions and frameshift mutations

E.2. Genetic information regarding plasmid

Plasmid [redacted] contains the alpha-(1,2)-fucosyltransferase expression module to ensure 2′-FL production in production host INB-2FL_03.

To enable the selection of production strain INB-2FL_03 [redacted] the strain was made [redacted] More details on the [redacted] are provided in Table 6 and Figure 5.

Table 6 Overview of the [redacted] in production strain INB-2FL_03.

[redacted]

Figure 5 Structure of the plasmid [redacted]

E.3 Donor Organisms for the Introduced Genes

Please note that all genes are synthesized *in vitro*. We provide the following background on the "donor" organisms for the Food Directorate's consideration, but the organisms themselves are not used in the manufacture of 2′-FL.

The *Helicobacter* genus consists of microaerophilic gram-negative bacteria with a characteristic helical shape and flagella. They are part of the natural gastric ecology in both humans and animals and are well susceptible to antibiotics. The most important member of the genus in term of human health is *Helicobacter pylori*. It is a spiral-shaped microaerophilic gram-negative bacterium usually found in the human stomach. Although linked to chronic gastritis and gastric ulcers, up to 85% of people infected with *H. pylori* never experience symptoms or complications. It is found in about two-thirds of the world's population, and it may play an important role in the natural stomach ecology.

Bifidobacterium adolescentis is also a normal inhabitant of healthy human and animal intestinal tracts. Their presence in the gut has been associated with a healthy microbiota, and specific strains of Bifidobacteria are being used as food additives, such as dairy products (Gomes and Malcata, 1999). It is a gram-positive, nonmotile, often branched anaerobic bacterium.

Zymomonas mobilis is a rod shaped facultative anaerobic gram-negative bacterium that can be found in sugar rich plant saps. This organism has been well characterized for its usage in producing lignocellulosic biofuels (Carreón-Rodríguez et al., 2019; Yang et al., 2019) as well as levan (Silbir et al., 2014).

E.4. Stability of the genotype of the plasmid

The stability of the genotype of the plasmid [redacted] is covered by the stability of the production strain. Please refer to section D.3.2.

3.3.3 SUBSTANCES USED FOR A NUTRITIVE PURPOSE

Inbiose intends to sell 2′-FL for use as a nutritive substance in infant formula products and is not requesting any amendments to the current permissions for 2′-FL in the Code.

As noted in section 3.1.1.C above, Inbiose is not requesting any changes to the food categories and use levels of 2′-FL as permitted in A1155, A1190 and A1233. The intended use levels are consistent with the permitted levels for 2′-FL in Schedule 29 (96 mg/100 kJ, equivalent to 2.4 g/L). Hence, this application is not requesting any changes to these permitted levels. Inbiose therefore argue that most of section 3.3.3 does not need to be addressed because FSANZ has already assessed it for 2′-FL; and this application is only requesting a new permitted source of 2′-FL to be permitted in the Code. Is

A.1 Information on the purpose of the use of a nutritive substance

The purpose of adding Inbiose's 2′-FL to infant formula products is consistent with the previously assessed purposes in Applications A1155, A1190 and A1233. The addition of 2′-FL to infant formula products brings the composition of these products closer to the composition of human milk, particularly in relation to levels of human milk oligosaccharides. FSANZ has also assessed beneficial roles related to the addition of 2′-FL to infant formula products, including contributing to a *Bifidobacterium*-enriched micbrobiota more similar to breastfed infants (than in those fed unsupplemented formula); and competitive inhibition by 2′-FL of binding of *Campylobacter jejuni* to intestinal epithelial cells.

B Technical information on the use of the nutritive substance

Complete technical information about the 2′-FL and the production strain *E. coli* K-12 MG1655 INB-2FL_03 was submitted to FDA by Inbiose in March 2022 (FDA has assigned the notice with a file number: GRN 001091). The submission to FDA is therefore included in this submission as appendix 3. Moreover, Inbiose´s production strain, INB-2FL_03 is derived from the same parental strains that were previously assessed as part of GRNs 749 and 897, and subsequently the safety of the final products were confirmed. The technical information

added below under sections 3.3.3.B.1 to B.6, is extracted from this submitted GRAS Notification.

B.1 Information to enable identification of the nutritive substance

B.1.1 Chemical identity of 2′-FL

2′-FL is a trisaccharide/f fucosylated oligosaccharide that is naturally occurring in human breast milk.

Chemical name: α-L-fucopyranosyl-(1-2)-β-D-galactopyranosyl-(1-4)-D-glucopyranose. Synonyms: 2´-O-fucosyllactose; 2´-O-L-fucosyl-D-lactose; fucosyl-α-1,2-galactosyl-β-1,4-glucose; Fuc α-(1-2)-Gal-β-(1-4)-Glc; 2′-FL.

Chemical formula: C18H32O15

Molecular weight: 488.44 daltons

CAS Number: 41263-94-9

B.2 Information on the chemical and physical properties of the nutritive substance

B.2.1 Chemical structure of purified 2′-FL

2′-FL is composed of L-fucose, D-galactose, and D-glucose (Figure 6). The monosaccharide L-fucose is linked to the disaccharide D-lactose by an α-(1-2) bond.

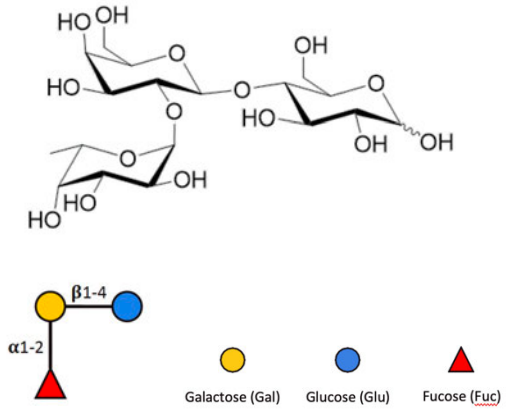


Figure 6 Chemical structure of 2′-FL

B.3 Information on the impurity profile

The primary constituent of the ingredient is 2′-FL (≥ 94 %), with minor concentrations (typically below 3% each) of related sugars, including lactose and difucosyllactose. Analytical results for three nonconsecutive lots of 2′-FL are outlined in Table 7.

Table 7 Compositional analysis of three nonconsecutive lots of 2′-FL

Parameter	**Lot number**		
	[redacted]		
Identification			
Appearance (color)	White	White	White
Appearance (form)	Powder	Powder	Powder
Appearance in solution	Clear, colorless to slightly yellow	Clear, colorless to slightly yellow	Clear, colorless to slightly yellow
pH (20°C, 10% solution)	6.8	5.5	5.3
Carbohydrates, water free (%DM)			
2′-FL	97.5	97.3	98.4
Lactose	2.1	2.3	1.0
Difucosyllactose (DFL)	0.13	0.13	0.13
Sum of other carbohydrates[a]	0.30	0.29	0.51
Chemical Analysis			
Water content, volumetric (% w/w)	3.5	4.3	4.2
Protein content (µg/g)	<25	<25	<25
Total ash (%)	<0.1	<0.1	<0.1
Endotoxin (E.U./g)	230	62.2	<50
Heavy Metals			
Arsenic (mg/kg)	<0.01	<0.01	<0.01
Cadmium (mg/kg)	<0.005	<0.005	<0.005
Lead (mg/kg)	<0.01	<0.01	<0.01
Mercury (mg/kg)	<0.01	<0.01	<0.01
Standard plate count (CFU/g)	30	<10	<10
Yeast (CFU/g)	<10	<10	<10
Mold (CFU/g)	<10	<10	<10
Coliform / *Enterobacteriaceae* (/10g)	Absent	Absent	Absent
Salmonella spp. (/25g)	Absent	Absent	Absent
Cronobacter (Enterobacter) sakazakii (/25g)	Absent	Absent	Absent
Listeria monocytogenes (/25g)	Absent	Absent	Absent
Bacillus cereus (CFU/g)	<10	<10	<10

2′-FL = 2′-fucosyllactose; CFU = colony forming units; DM = dry matter; EU = endotoxin units; GRN = Generally Recognized as Safe Notice

a - Sum of other carbohydrates, such as 3-fucosyllactose, 2-fucosyl-D-lactulose, fucosyl-galactose, glucose/galactose, fucose, sorbitol/galactitol, mannitol, and trihexose

B.4. Stability 2′-FL

The stability of Inbiose's 2′-FL is supported by the real-time and accelerated stability studies summarized in A1155, as well as in GRNs 735 and 749 (FDA 2018a; FDA 2018b). The compositional similarities between Inbiose's 2′-FL and other 2′-FL preparations indicate that the stability of the ingredients will be similar.

Additionally, the stability of 2′-FL has been tested in studies conducted under some of the intended conditions of use, which further supports the stability of 2′-FL as an ingredient in food and beverages matrices, which were included in GRN 546 (FDA 2015a).

The bulk stability of crystalline, chemically synthesized as well as produced from microbial fermentation, Glycom's 2′-FL ingredient was evaluated in a 60-month real-time test and a 6-month accelerated test. In the real-time study [25°C, 60% relative humidity (RH)], no significant change was observed in the synthetically produced 2′-FL content or microbiological parameters of the stored sample and only a minor increase in water content (remaining within acceptable defined product specifications) was measured at the 60-month time point (please refer to A1155). Similarly, the crystalline Glycom's 2´-FL produced by fermentation was stable up to 18 months (interim results). Results from the 6-month accelerated stability study (40°C, 75% RH) also indicate that 2′-FL does not undergo significant degradation under the described storage conditions. No unknown degradation products were measured following HPLC analysis of the 2′-FL following accelerated storage (please refer to A1155).

As described in Section 7 and Appendix C of GRN 749, the shelf life of DuPont's 2′-FL was assessed *via* a 6-month accelerated stability study (40°C, 75% RH). The results of this study indicated no significant changes in the evaluated carbohydrate content (*i.e.* 2′-FL, difucosyllactose, lactose, and other unspecified carbohydrates), moisture content, and microbiological parameters (*i.e.*, standard plate count, yeast and mold, coliform/*Enterobacteriaceae*, *Salmonella* spp., and *Cronobacter sakazakii*) in 3 representative batches following storage for up to 6 months. A minor degree of degradation was reported in the purity of one sample and the moisture content slightly increased over the test period due to the hygroscopic nature of the ingredient. No other changes were noted (FDA 2018b).

As described in Section E.2 of GRN 735, a range of chemical and microbiological specification parameters was tested, along with the overall purity of Friesland Campina's 2′-FL ingredient, in a 6-month accelerated storage (40°C, 75% RH) and an ongoing 36-month real-time study (25°C, 60% RH). The results from the accelerated stability study indicated no changes in the appearance of the ingredient or the evaluated chemical (*i.e.*, 2′-FL, lactose,

allo-lactose, glucose, ash, and water content) and microbiological parameters. The 6-month interim results from the real-time study confirmed that 2′-FL is stable when stored at ambient room temperatures (FDA 2018a).

The stability of 2′-FL has also been assessed under the intended conditions of use. The 2′-FL ingredient produced by Glycom *via* chemical synthesis (compositionally comparable to 2′-FL produced from fermentation) was assessed in powdered infant formula, as described in Section II.D.2 of GRN 546 (FDA 2015a).

Glycom's 2′-FL added to a powdered infant formula supplemented with other human-identical milk oligosaccharides (*i.e.*, lacto-*N*-neotetraose), containing a range of other ingredients (*i.e.*, salts, carbohydrates, and proteins), was observed to be stable for up to 18 months of storage at various temperatures (4°C, 20°C, 30°C, and 37°C). The results from additional stability testing of Glycom's 2′-FL in yogurts, ready-to-drink flavored milk, and citrus fruit beverages also indicated that 2′-FL was stable in a range of different products, with no loss in 2′-FL content following typical processing and storage conditions,for up to 28 days post-processing (FDA 2015a).

The application A1190 similarly reflects on stability tests that have been conducted and reported earlier in GRN 571 filing (FDA 2015b).

Overall, the results demonstrate that 2′-FL is not significantly degraded when stored under the tested conditions and is anticipated to be stable in most food matrices.

B.5 Manufacturing process

Commercial production of 2′-FL will be executed at manufacturing sites that comply with Good Manufacturing Practice (GMP) or Global Food Safety Initiative (GFSI) certifications. A Hazard Analysis and Critical Control Points (HACCP) plan will be implemented that ensures the identification, evaluation and control of possible food safety hazards.

The manufacture of 2′-FL is largely comparable to the production processes previously evaluated for other HMOs produced by microbial fermentation, involving construction of a production organism engineered to synthesize human milk oligosaccharides from lactose, with large-scale fermentation and downstream processing to isolate the human milk oligosaccharide. All additives, processing aids, and food contact articles used during manufacturing are food grade.

In summary, the manufacturing method for 2′-FL entails a fermentation process with the production host. This host produces 2′-FL through the utilization of a carbon source

(sucrose), combined with lactose in a minimal medium. The 2′-FL product is released into the medium. The remaining intracellular 2′-FL is released after pasteurization. The broth is then subjected to downstream purification and concentration processes to isolate 2′-FL, to remove impurities originating from fermentation (*e.g.*, minerals, substrates, proteins, and other cellular matter) followed by drying (see Figures 7 and 8 below).

In the first step of the purification process, biomass is removed together with cell components and large molecules (DNA, protein, and lipopolysaccharides). After removal of larger particles, the color is removed using activated charcoal. Subsequently, the salts present in the medium are removed, which are cations (*e.g.*, magnesium, calcium, and ammonium) and anions (*e.g.*, phosphate and sulfate), which are minerals used for growth of the microorganism. Leftover water is removed from the product mainly through evaporation and the product is filtered again to ensure the microbial specification before drying.

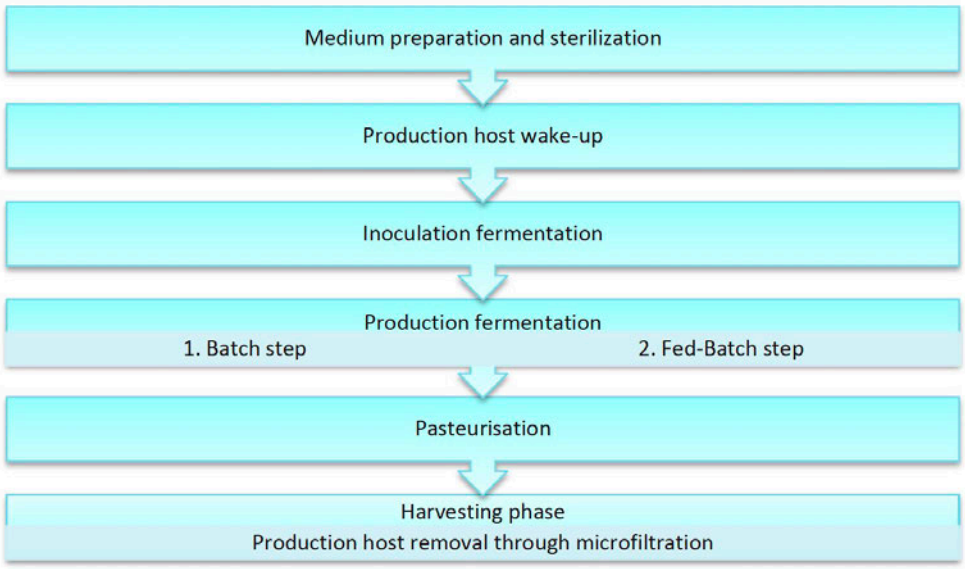


Figure 7: Fermentation process

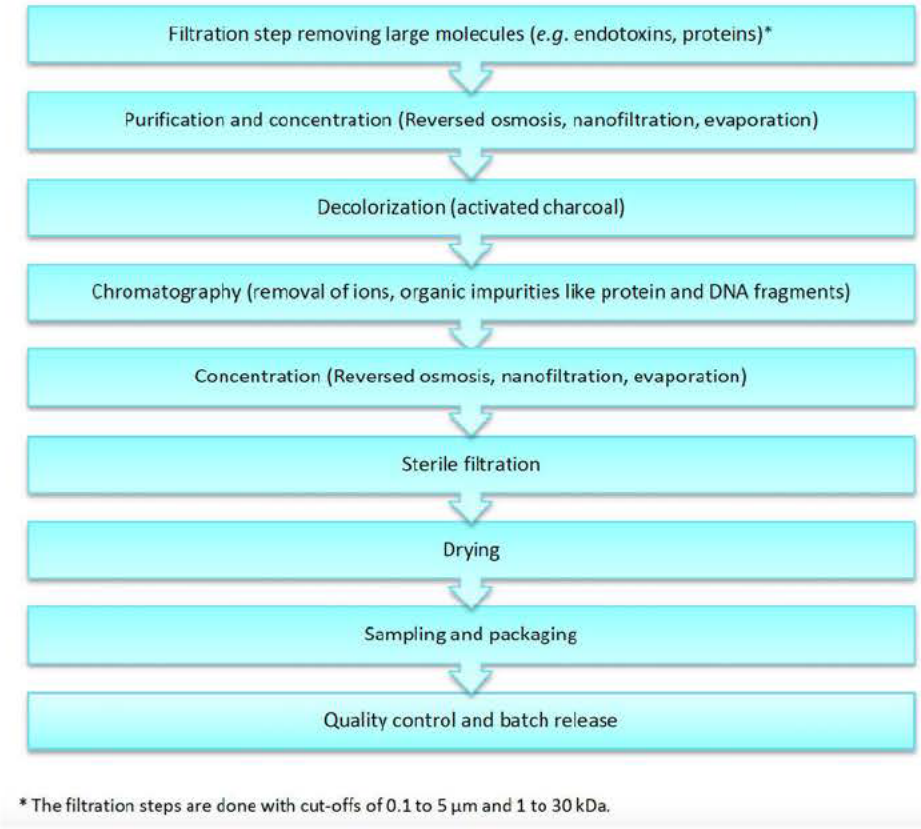


Figure 8 Purification process

B.6 Specification for identity and purity

To ensure consistent product quality and based on the compositional analysis as explained in Table 7, Inbiose has established a set of specifications for 2′-FL, which includes the acceptability criteria for purity of 2′-FL and the presence of other carbohydrates, chemical parameters, heavy metals, and microbial contaminants, and confirms the absence of the genetically modified production strain and any related endotoxins. The specifications proposed for 2′-FL are presented in Table 8. All parameters are determined using compendial or validated methods.

Table 8 Product Specification for 2′-FL of Inbiose

Parameter	Specification	Method of Analysis
Identification		
Appearance (Color)	White to off white	Visual
Appearance (Form)	Powder	Visual
Appearance in solution	Clear, colorless to slightly yellow	Visual
Identity (2′-FL)	Conform to reference standard, 2′-FL derived from human milk	NMR
Chemical Specifications		
Moisture (w/w)	NMT 5.0%	Karl-Fischer, volumetric
pH (20°C, 10% solution)	3.0 to 7.5	Eurofins' internal method, potentiometry
Protein	NMT 100 µg/g	Roti®Nanoquant
Ash	NMT 0.5%	NEN 6810

Endotoxins	NMT 10 EU/mg	Ph. Eur. 2.6.14
Carbohydrates (% DM)		
2′-FL	NLT 94%	UPLC-RI
D-Lactose	NMT 5.0%	UPLC-RI
Difucosyl-D-lactose	NMT 5.0%	UPLC-RI
Heavy Metals		
Arsenic	NMT 0.2 mg/kg	ICP-MS
Cadmium	NMT 0.1 mg/kg	ICP-MS
Lead	NMT 0.02 mg/kg	ICP-MS
Mercury	NMT 0.5 mg/kg	ICP-MS
Microbiological Contaminants		
Total aerobic mesophilic plate count	NMT 1,000 CFU/g	ISO 4833
Yeast	NMT 100 CFU/g	ISO 7954
Mold	NMT 100 CFU/g	ISO 7954
Enterobacteriaceae	Absent in 10 g	ISO 21528-1
Salmonella spp.	Absent in 25 g	ISO 6579-1
Cronobacter spp.	Absent in 25 g	ISO/TS 22964
Listeria monocytogenes	Absent in 25 g	AFNOR EGS 38/05-03/17
Bacillus cereus	NMT 50 CFU/g	ISO 7932

2′-FL = 2´-fucosyllactose; AFNOR = Association Française de Normalisation; CFU = colony forming units; DM = dry matter; EU = endotoxin units; EGS = Eurofins GeneScan; GRN = Generally Recognized as Safe Notice; ICP-MS = inductively coupled plasma mass spectrometry; ISO = International Organization for Standardization; NEN = Royal Netherlands Standardization Institute; NLT = not less than; NMR = nuclear magnetic resonance; NMT = not more than; Ph. Eur. = European Pharmacopoeia; UPLC-RI = ultra-high performance liquid chromatography coupled with refractive index detector.

The level of impurities in the 2′-FL product of Inbiose is the same or lower as compared to the already approved 2′-FL products in applications A1155, A1190 and A1233. For ease of reference, table 9 lists the specifications of the 2′-FL of Inbiose and the 2′-FL products in A1155, A1190/A1251 and A1233. In addition, the specification of Inbiose´s 2′-FL is compared with the specification of 2′-FL in the Code S3-40 sourced from *Escherichia coli* K-12 containing the gene for alpha-1,2-fucosyltransferase from either *Helicobacter pylori or Bacteroides vulgatus*.

Table 9 Comparison of specification for different 2′-FL products

Parameter	**Inbiose**	**A1155**	**A1190 / A1251**	**A1233**	**Specifications in the Code (S3—40)**[51,52]
Identification					
Appearance (Color)	White to off white	White to off white	White to ivory	White to off white	White to off white
Appearance (Form)	Powder	Powder	Powder	Powder	Powder
Chemical Specifications					
Moisture (w/w)	NMT 5.0%	NMT 5%	NMT 9%	NMT 5%	NMT 9%
Ash (w/w)	NMT 0.5%	1.5%	NMT 0.5%	NMT 0.2%	NMT 2%
pH	3.0 to 7.5 (20°C, 10% solution)	3.2-5.0	NS	3.0 to 7.5 (10% solution)	3.0-7.5 (20°C, 5% solution)
Residual proteins	NMT 100 µg/g	NMT 0.01%	NMT 100 µg/g	NMT 0.01%	NMT 0.01%
acetic acid	NS	NMT 1%	NS	NS	NMT 1.0%

[51] https://www.foodstandards.gov.au/code/changes/gazette/Documents/Gazette%20205.pdf
[52] Australia New Zealand Food Standards Code—Amendment No. 209 - 2022-gs2810 - New Zealand Gazette

Endotoxins	NMT 10 EU/mg	NMT 10 EU/mg	NMT 300 EU/g	NMT 10 EU/mg	NMT 10 EU/mg
Carbohydrates					
2′-FL	NLT 94%	NLT 94%	NLT 90%	NLT 90%	NLT 83%
D-Lactose	NMT 5.0%	NMT 3.0%	NMT 5.0%	NMT 3.0%	NMT 10.0%
Difucosyl-D-lactose	NMT 5.0%	NMT 1.0%	NMT 5.0%	NS	NMT 5.0%
L-Fucose	NS	NMT 1.0%	NMT 3.0%	NMT 2.0%	NMT 2.0%
2′-fucosyl-D-lactulose	NS	NMT 1.0%	NS	NS	NMT 1.5%
sum of saccharides*	NS	NLT 96%	NS	NS	NLT 90%
Microbiological Contaminants					
Total aerobic mesophilic plate count	NMT 1,000 CFU/g	NMT 500 CFU/g	NMT 10,000 CFU/g	NMT 3,000 CFU/g	NMT 3,000 CFU/g
Yeast	NMT 100 CFU/g	NMT 10 CFU/g	NMT 100 CFU/g	NMT 10 CFU/g	NMT 100 CFU/g
Mold	NMT 100 CFU/g	NMT 10 CFU/g	NMT 100 CFU/g	NMT 10 CFU/g	NMT 100 CFU/g

NLT = not less than; NMT = not more than; NS = Not specified; *2′-FL, D-lactose, L-fucose, difucosyl-D-lactose, 2′-fucosyl-D-lactulose

B.7 Analytical method for detection

Inbiose has an extensive practical knowledge on the analysis of human milk oligosaccharides, including 2′-FL. The Ultra-High Performance Liquid Chromatography coupled with Refractive Index detector (UPLC-RI) method was developed and validated internally to analyze Inbiose's 2′-FL product. The method is described in Appendix 6.
As concluded in appendix 3, 2′-FL is stable for four days in all tested conditions and is also stable for three freeze and thaw cycles.

B.8 Absence of residual DNA

To ensure the absence of residual DNA of the production organism, PCR tests were performed on 3 batches of INB-2FL_03. A short subsequence of the inserted α-(1,2)-fucosyltransferase gene (derived from *Helicobacter* sp.) on the plasmid and a subsequence of the sucrose phosphorylase gene (derived from *Bifidobacterium adolescentis*) on the genome were targeted to check for residual DNA in the product. For every batch, the analysis was performed in triplicate together with 3 types of positive controls and 1 negative control. The analysis of all batches of 2′-FL showed no detectable levels of residual DNA in the final product. The limit of detection for the PCR method is <1 ng DNA per gram 2′-FL, as recommended in European Food Safety Authority (EFSA) guideline (EFSA, 2018). For more details see appendix 7.
As can be seen in tables 1 and 2 there was no *Enterobacteriaceae* detected in a 10 g sample. As *E. coli* is part of the Order *Enterobacteriaceae* this shows that no GMM *E. coli* production host is present in 10 g sample.

C Information related to the safety of the nutritive substance

C.1 Information on the toxicokinetics and metabolism of the nutritive substance and, if necessary, its degradation products and major metabolites

Inbiose's 2′-FL produced by microbial fermentation is structurally identical to the 2′-FL found in human milk and will be physiologically equivalent in terms of absorption, distribution, metabolism, and excretion. Therefore, the metabolism of Inbiose´s 2′-FL, when added to infant formula, is expected to be identical to those of other HMOs naturally present in human breast milk.

The metabolism of HMOs, including 2′-FL, has been previously described in detail (A1155, A1190 and A1233). The applicant conducted a literature search to identify any new published studies relevant to toxicokinetics and metabolism of 2′-FL. The literature search was performed in PubMed starting as of 12 August 2021. No relevant toxicokinetic data was identified. Below, the applicant has summarized the information on the toxicokinetic profile of 2´-FL. In addition, newly identified studies focused on HMO-modulatory effects on gut microbiota and metabolite's profiles upon the HMO digestion. The outcomes of some most recent studies were briefly summarised (Natividad et al., 2022; Walsh et al., 2022; Bajic et al., 2023; Lindner et al.; 2023).

HMOs including 2′-FL are resistant to enzymatic hydrolysis and are therefore not significantly digested in the upper gastrointestinal tract (Brand-Miller et al., 1998; Engfer et al., 2000; Rudloff and Kunz, 2012; EFSA, 2019). Only minor structural changes of the HMOs were observed after in vitro digestion of HMOs using artificial gastric fluid (porcine intestinal brush border membranes within the physiologic range of incubation time, pH, and enzyme activity) (Gnoth et al., 2000). As a result, intact 2′-FL can reach the large intestine, where it is partially metabolized by microbiota into short-chain fatty acids (Salli et al., 2019; Van den Abbeele et al., 2021). The effects of HMOs on gastrointestinal bacterial growth are bacterial strain- and HMO structure-dependent. Different growth patterns were observed for different bacterial strains when exposed to the same HMOs *in vitro* (Cheng et al., 2021; Van den Abbeele et al., 2021; Walsh et al., 2022). In addition, the metabolic end-products of HMOs are dependent on strain combination and HMO substrate (Walsh et al., 2022 Lindner et al.; 2023). The interpersonal and age-dependent differences in microbiota may also affect HMO metabolite profile (Bajic et al., 2023).
Bifidobacteriaceae were shown to be a major group of bacteria involved in the fermentation of 2′-FL (Bunesova et al., 2016; Van den Abbeele et al., 2021; Natividad et al., 2022; Walsh et al., 2022; Lindner et al., 2023; Bajic et al., 2023). In addition the HMO supplementation

promoted increase in diversity of the different bifidobacterial communities (Natividad et al., 2022). 2′-FL was showed to have a bifidogenic effects in young children and adults, thus 2′-FL effect on gut microbiota was age-independent (Bajic et al., 2023).
The supplementation of the 2′-FL alone or in combination with LNnT or 5 other HMOs (LNnT, difucosyllactose, lacto-N-tetraose, 3´- and 6´-sialyllactose) increased acetate, butyrate, and propionate production, as well as SCFAs using ex/in vitro assays (baby M-SHIME® and the intestinal epithelium Caco-2/HT29-MTX co-culture, respectively). Significant increases of these metabolites were also observed in other *ex vivo* studies conducted by Lindner et al. (2023) and Bajic et al. (2023). In addition, other *Bifidobacterium*-mediated metabolites were reported, including aromatic lactic acids (indole-3-lactic acid, 3-phenlyllactic acid), 2-hydroxyisocaproic acid (HICA), 3-hydroxybutyric acid, the -aminobutyric acid (GABA) and melatonin in both children´s and adults gut microbial samples (Bajic et al., 2023).

A large portion of undigested 2′-FL (ranging from 40 to 97%) was shown to be excreted in the feces (Chaturvedi et al., 2001; Coppa et al., 2001). Only a small fraction of neutral HMOs including 2′-FL was suggested to be transported transcellularly by receptor-mediated transcytosis, and/or by paracellular flux (Gnoth et al., 2001). Indeed, less than 1% of ingested 2′-FL was found to be systematically available in breast-fed infants or infants fed with formula supplemented with 2′-FL (Goehring et al., 2014; Marriage et al., 2015). A small fraction of absorbed HMOs was excreted unchanged or only slightly metabolized in the urine of breast-fed infants, at levels that correlate with their dietary intake from breast milk (Rudloff et al., 2012; Goehring et al., 2014).

C.2 Newly Identified Human Clinical and Animal Studies of 2′-FL

The applicant conducted a literature search to identify any new published studies relevant to safety/tolerance of 2′-FL product. The applied search term was “2′-fucosyllactose” OR “2′-O-Fucosyllactose’’. For the purpose of this application, the literature search was performed in PubMed starting as of 12 August 2021 to include only studies which were not yet evaluated by FSANZ.

In total, ten new human clinical studies and two animal studies with 2′-FL, or HMO mixtures containing 2′-FL, were identified (see Appendix 8.1 and Appendix 8.2, respectively).

No evidence of toxicity related to the oral consumption of 2′-FL was reported in the additional identified studies. Therefore, none of the identified studies summarized in the sections below are expected to affect the overall conclusion of safety of 2′-FL product.

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