ORIGINAL SUBMISSION

Nestlé Nutrition U.S.

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#620

Nestlé Nutrition

GRN000620

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OFFICE OF
FOOD ADDITIVE SAFETY

December 22, 2015

Dr. Paulette Gaynor
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Dear Dr. Gaynor:

Re: GRAS Exemption Claim for Galacto-oligosaccharides

In accordance with proposed 21 CFR §170.36 [Notice of a claim for exemption based on a Generally Recognized as Safe (GRAS) determination] published in the *Federal Register* [62 FR 18938 (17 April 1997)], I am submitting one hard copy and one electronic copy (on CD), as the notifier [Nestlé Nutrition, 12 Vreeland Road, Florham Park, NJ 07932], a Notice of the determination, on the basis of scientific procedures, that galacto-oligosaccharides, produced by Nestlé Nutrition, as defined in the enclosed documents, are GRAS under specific conditions of use in non-exempt term infant formula (*i.e.,* infants 0 to 12 months of age), and therefore, is exempt from the premarket approval requirements of the *Federal, Food, Drug and Cosmetic Act*. Information setting forth the basis for the GRAS determination, which includes detailed information on the notified substance and a summary of the basis for the GRAS determination, as well as a consensus opinion of an independent panel of experts in support of the safety of Nestlé galacto-oligosaccharides under the intended conditions of use, also are enclosed for review by the agency.

The enclosed electronic files for the Notice entitled, "GRAS Exemption Claim for Galacto-oligosaccharides" were scanned for viruses prior to submission and is thus certified as being virus-free using McAfee VirusScan 8.8.

Should you have any questions or concerns regarding this GRAS Notice, please do not hesitate to contact me at any point during the review process so that we may provide a response in a timely manner.

Sincerely,

(b) (6)

Cheryl Callen
Director, Regulatory Affairs
Nestlé Infant Nutrition

Nestlé Nutrition U.S.

12 Vreeland Road • Box 697
Florham Park, New Jersey 07932-0697

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Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
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5100 Paint Branch Parkway
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Sincerely,

(b) (6)

Cheryl Callen
Director, Regulatory Affairs
Nestlé Infant Nutrition

GRAS Exemption Claim for Galacto-oligosaccharides

Submitted to:

Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition (CFSAN)
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD
U.S.A. 20740-3835

Submitted by:

Nestlé Nutrition
12 Vreeland Road
Florham Park, NJ
07932

December 22, 2015

GRAS Exemption Claim for Galacto-oligosaccharides

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Annex A Expert Panel Consensus Statement Concerning the Generally Recognized as Safe (GRAS) Determination of Nestlé Galacto-oligosaccharides (Nestlé GOS) for Use as an Ingredient in Term Infant Formula and Toddler Formula

Figures and Tables

I. GRAS EXEMPTION CLAIM

I.A Claim of Exemption from the Requirement for Premarket Approval Pursuant to Proposed 21 CFR §170.36(c)(1) [62 FR 18938 (17 April 1997)]

Nestlé Nutrition (Nestlé) hereby claims that the use of galacto-oligosaccharides (GOS) in non-exempt term infant formula (*i.e.*, infants 0 to 12 months of age) and toddler formula, as described in Section I.D below, is exempt from the requirement of premarket approval of the *Federal Food, Drug, and Cosmetic Act* because we have determined that such uses are Generally Recognized as Safe (GRAS).

Signed,

(b) (6)

December 22, 2015

Date

Cheryl Callen
Director, Regulatory Affairs
Nestlé Infant Nutrition
Cheryl.Callen@us.nestle.com

I.B Name and Address of Notifier

Nestlé Nutrition
12 Vreeland Road
Florham Park, NJ 07932

I.C Common Name of the Notified Substance

The common name of the substance that is the subject of this GRAS Notification is galacto-oligosaccharides (GOS).

I.D Conditions of Intended Use in Food

I.D.1 Foods in which the Substance is to be Used

The substance is intended for use as a food ingredient for addition to non-exempt term infant formula and toddler formula at a use-level providing up to 7.8 g of galacto-oligosaccharides per L of the reconstituted or ready-to-drink formula.

I.D.2 Purpose for Which Substance is Used

GOS ingredients are intended for addition to term infant formula and toddler formula as a dietary source of non-digestible oligosaccharides. The addition of GOS to infant formula is generally

recognized as a safe and suitable alternative to human milk oligosaccharides that are present in high concentrations in human breast milk (Oozeer *et al*., 2013).

I.D.3 Description of the Population Expected to Consume the Substance

Nestlé GOS is expected to be consumed under the intended conditions of use by term infants and toddlers who may reasonably be expected to consume non-exempt term infant formula and/or toddler formula products.

I.E Basis for the GRAS Determination

Pursuant to 21 CFR § 170.30 of the *Code of Federal Regulations* (CFR) (U.S. FDA, 2015), Nestlé GOS, as described herein, has been determined by Nestlé to be GRAS through scientific procedures.

I.F Availability of Information

The data and information that serve as the basis for this GRAS Notification will be sent to the United States (U.S.) Food and Drug Administration (FDA) upon request, or will be available for review and copying at reasonable times at the offices of: Nestlé Nutrition, 12 Vreeland Road, Box 697, Florham Park, NJ 07932-0697. Attn: Cheryl Callen, Director, Regulatory Affairs, Cheryl.Callen@us.nestle.com.

Should the FDA have any questions or additional information requests regarding this notification, Nestlé will supply these data and information.

II. DETAILED INFORMATION ABOUT THE IDENTITY OF THE NOTIFIED SUBSTANCE

II.A Identity

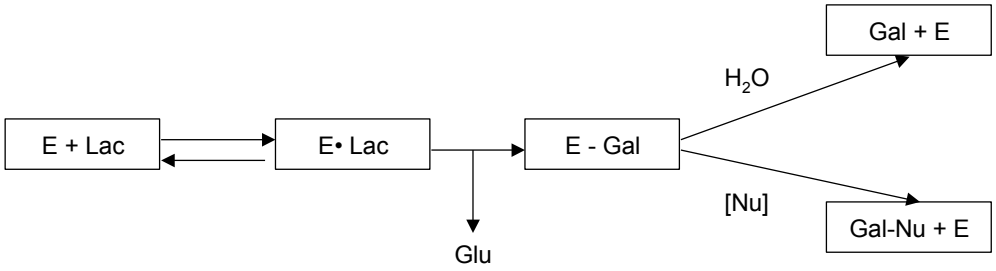
Common or Usual Name	Galacto-oligosaccharides; GOS
Synonyms	Trans-galacto-oligosaccharides; oligogalactosyl-lactose; oligogalactose; β-galactooligosaccharides
Trade Name	To be filed

There is no globally-adopted definition of GOS. Food Standards Australia New Zealand (FSANZ) has defined GOS as follows: "*… The term 'galacto-oligosaccharides' (sometimes referred to as oligogalactosyl-lactose) is used consistently to describe those substances comprised of between two and eight saccharide units with one of these units being a terminal glucose and the remaining saccharide units being galactose*." (FSANZ, 2008). The European Commission Scientific Committee on Food (SCF) has described GOS in the following manner: "*Oligogalactose is produced from lactose with the help of a bacterial β-galactosidase, it contains*

one molecule of glucose and typically between 1 and 7 molecules of galactose." (SCF, 2001a,b).

GOS preparations previously determined to be GRAS, and that have been the subject of pre-market notification to the FDA (*i.e.*, GRN 236, 285, 286, 334, 484, 489, 495, 518) (U.S. FDA, 2008, 2009a,b, 2010, 2014a-d), are all produced in a similar manner. In general, a *beta*-galactosidase enzyme preparation derived from a safe and suitable microbial source is utilized to convert lactose into a GOS mixture containing low-molecular weight neutral oligosaccharides of varying chain length and minimal branching, usually between 2 to 8 sugar moieties (Figure II.A-1). The sugar molecules in these GOS preparations, glucose and galactose, are connected by *beta* linkages in a combination of 1→2, 1→3, 1→4, or 1→6 anomeric configurations. The linkage combinations in the final GOS product depend upon the *beta*-galactosidase enzyme preparation and manufacturing conditions employed; enzymes from different source organisms exhibit biases towards certain linkage configurations and molecular weight distributions (Torres *et al.*, 2010). As such, the use of different *beta*-galactosidase enzyme preparations and product specific manufacturing conditions (*i.e.,* time, temperature, pH) result in GOS preparations exhibiting manufacturer-specific profiles (Torres *et al*., 2010).

Figure II.A-1 Production of GOS from Lactose by *beta*-Galactosidase



Nestlé GOS is manufactured by a contract manufacturer for Nestlé, to Nestlé specifications, using a *beta*-galactosidase enzyme preparation derived from *Aspergillus oryzae* that generates oligomers with a bias towards *beta*-1,4 and *beta*-1,6 linkages. The contract manufacturer is audited on a periodic basis by Nestlé for compliance with Nestlé's quality standards for food manufacturing. The ingredient is manufactured as a powder with a minimum oligosaccharide content of ≥46% on a dry weight basis. Based on the raw materials, production methods, and available compositional analyses, GOS synthesized by the Nestlé manufacturing process produces a product that is consistent with the available global definitions of GOS discussed

above and other sources of GOS that have been determined to be GRAS for use in infant formula.

II.B Method of Manufacture

II.B.1 Raw Materials and Processing-aids

All raw materials and processing-aids used to manufacture Nestlé GOS, as described herein, are food grade ingredients[1] permitted by U.S. regulation, or have been previously determined to be GRAS for their respective uses (Table II.B-1).

Table II.B-1 Raw Materials and Processing-Aids

Material	Purpose	Regulatory Status
Demineralized whey permeate	Source of lactose for GOS synthesis	GRAS (U.S. FDA, 2014e)
beta-Galactosidase from *Aspergillus oryzae*	Processing-aid	GRAS (U.S. FDA, 2002; 2014b). FDA Partial List of Enzyme Preparations Used in Food (U.S. FDA, 2013a)
Potassium hydroxide	Processing-aid (for adjusting pH)	Under 21CFR184.1631 potassium hydroxide is affirmed as GRAS where the ingredient is permitted for use in food with no limitation other than current Good Manufacturing Practice (U.S. FDA, 2015)

GRAS = Generally Recognized as Safe

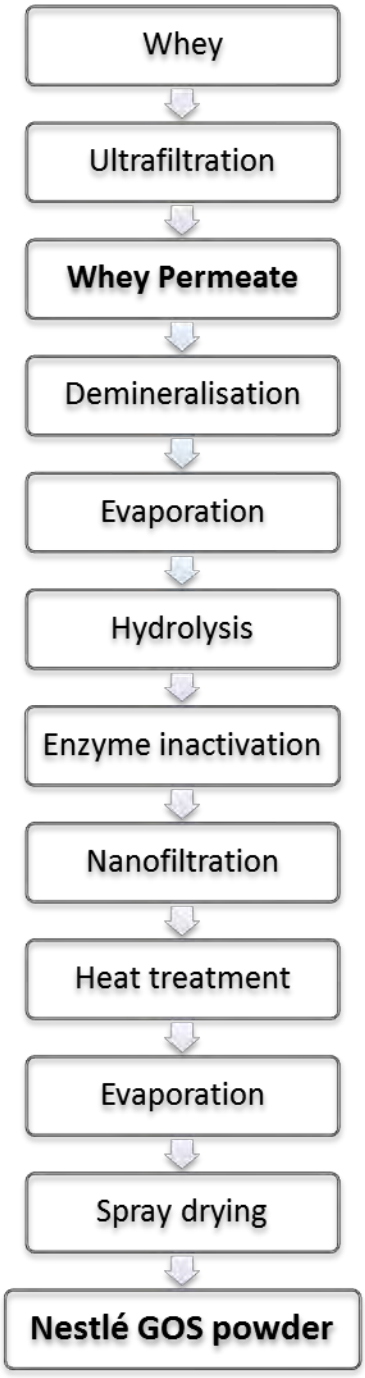
II.B.2 Description of Manufacturing

Nestlé GOS is manufactured by a contract manufacturer in accordance with Nestlé's quality standards. A brief overview of the manufacturing methods is described below. Demineralized sweet whey permeate is used as a food grade source of lactose for the synthesis of Nestlé GOS. The partially demineralized whey permeate containing lactose is concentrated by evaporation to 50% total dry matter and then incubated with a *beta*-galactosidase enzyme derived from *A. oryzae* to catalyze the hydrolysis of lactose into glucose and galactose. This is followed by the polymerization of galactose to generate a characteristic mixture of GOS with a degree of polymerization typically ranging from 2 to 5. Upon completion of hydrolysis and oligomerization, the enzyme is denatured and inactivated by heat treatment and the solution is subjected to a membrane nano-filtration step to reduce the mineral content, part of the lactose, and much of the free glucose and galactose generated during hydrolysis. Removal of the monosaccharides by nano-filtration is necessary to enable efficient spray-drying of the material. The ingredient is then heat-treated to ensure microbial stability, further concentrated by evaporation, and finally spray-dried to produce a powdered ingredient.

[1] Compliant with the specifications set forth in the Food Chemicals or equivalent international food or pharmacopeia standard (*e.g.*, JECFA, CODEX, EP).

The manufacturing process described for the production of Nestlé GOS (Figure II.B-1) is consistent with those described for other GOS preparations that have been previously determined to be GRAS, with the addition of a nano-filtration step to remove the monosaccharides to enable drying of the final product. Product specifications for Nestlé GOS and corresponding batch analyses are presented below in Section II.C.

Figure II.B-1 Schematic Overview of the Manufacturing Process for Nestlé GOS



II.C. Specifications and Product Analysis

II.C.1 Specifications

Food grade chemical and microbiological specifications for Nestlé GOS are presented in Table II.C-1.

Table II.C-1 Specifications for Nestlé GOS

Specification Parameter	Specification	Method
Dry matter (DM)	≥96 %	AS-INC-012
Total moisture (Karl-Fisher)	Max 5.5%	Nestlé LI-08.055
Protein (N x 6.38) on DM	Max 4.47	Nestlé LI-00.556 & Nestlé LI-00.561
Total oligosaccharides/GOS (on DM) Sialyllactose (g/100 g on DM)	Min 46% Min 0.2	Nestlé LI-00.590 (Austin *et al.,* 2014) / AOAC 2001.02 LI-08.007
Total Nitrogen on DM	Max 0.7%	Nestlé LI-00.556
Ash content on DM	Max 4%	Nestlé LI-00.565
Lactose on DM	20 – 40%	Nestlé LI-00.593
Glucose on DM	Max 10%	Nestlé LI-00.593
Galactose on DM	Max 5%	Nestlé LI-00.593
Nitrite	Max 2 mg/kg	ISO 14673-2: 2004
Nitrate	Max 50 mg/kg	ISO 14673-2: 2004
pH (10% solution)	5 - 6	Nestlé LI-00.908
Sodium (mg/100 g on DM)	≤50	AOAC 2011.14
Potassium (mg/100 g on DM)	1,200 – 2,100	AOAC 2011.14
Chloride (mg/100 g on DM)	≤100	Nestlé LI-00.580
Calcium (mg/100 g on DM)	≤100	AOAC 2011.14
Phosphorus (mg/100 g on DM)	150 – 350	AOAC 2011.14
Magnesium (mg/100 g on DM)	≤100	AOAC 2011.14
Manganese (mg/kg on DM)	≤0.2	AOAC 2011.14
Iron (mg/kg on DM)	≤5	AOAC 2011.14
Copper (mg/kg on DM)	≤2.5	AOAC 2011.14
Microbial Specifications[a]		
Aerobic mesophilic microorganisms (per g)	10,000	ISO 4833
Aerobic mesophilic spores (per g)	500	80°C, 10 min – Nestlé LI-00.718
Enterobacteriaceae (per g)	10	ISO 21528 Incubation temperature 37°C
Salmonella sp. (per 25 g)	Negative	ISO 6579

DM = dry matter; ISO = International Organization for Standardization (Betts RP, Oscroft CA, Baylis CL (2004). *A Code of Practice for Microbiology Laboratories Handling Food, Drink and Associated Samples, 3rd revised edition*. Gloucestershire, UK: Campden & Chorleywood Food Research Association).

[a] Nestlé GOS is intended for exclusive use in Nestlé infant formula products manufactured using wet blending techniques and is always added to the formula prior to thermal processing. Accordingly, extended microbial specifications for *Cronobacter sakazakii* and other microbial pathogens are not necessary and infant formula to which Neslté GOS has been added would therefore be compliant with the microbial requirements for infant formula as defined under 21 CFR 106.55 (U.S. FDA, 2015).

II.C.2 Product Analysis

Batch Analyses of Nestlé GOS

Batch analyses of 4 non-consecutive lots of Nestlé GOS demonstrating the manufacture of a consistent product in compliance with the product specifications defined above are presented in Table II.C-2.

Table II.C-2 Summary of the Batch Analysis for 4 Lots of Nestlé GOS

Parameter	Specification	Batch No.			
		341741	**341778**	**347230**	**291604**
Total water (%)	Max 5.5	2.87	3.41	3.07	2.92
Dry matter (DM) (%)	≥96	97.5	97.0	97.3	97.8
pH (10% solution)	5 to 6	5.72	5.68	5.66	5.68
Total nitrogen (% DM)	Max 0.7	0.27	0.28	0.27	0.28
Protein (N x 6.38)	Max 4.47	1.73	1.81	1.72	1.77
Ash (% DM)	Max 4	3.75	3.54	3.30	3.05
Galactose (% DM)	Max 5	4.50	5.09	3.26	3.93
Glucose (% DM)	Max 10	9.02	10.1	6.74	8.27
Lactose (% DM)	20 – 40	27.8	27.2	31.8	30.5
Total oligosaccharides/GOS (% DM)	Min 46	51.1	49.1	52.4	48.0
Sialyllactose (g/100 g on DM)	Min 0.2	0.25	0.24	0.25	0.23
Sodium (mg/100 g DM)	≤50	41.0	28.9	26.7	31.0
Potassium (mg/100 g DM)	1,200 – 2,100	1,624	1,610	1,426	1,368
Chloride (mg/100 g DM)	≤100	<3	<3	<3	<5
Calcium (mg/100 g DM)	≤100	49.2	55.7	52.4	48.6
Phosphorus (mg/100 g DM)	150 – 350	290	295	242	253
Magnesium (mg/100 g DM)	≤100	36.5	37.7	36.8	33.1
Manganese (mg/100 g DM)	≤0.2	<0.01	0.01	0.01	<0.01
Iron (mg/100 g DM)	≤5	<0.1	<0.1	<0.1	<0.1
Copper (mg/100 g DM)	≤2.5	0.10	0.10	0.10	0.10
Nitrate (mg/kg)	Max 50	10.3	12.4	13.4	13.4
Nitrite (mg/kg)	Max 2	<RL	<RL	<RL	<RL
Citric acid (%)	NS	2.40	2.33	2.65	2.30
Heavy Metals					
Lead (ppm)	NS	NM	ND (<0.0046)	ND (<0.0046)	ND (<0.0046)
Microbiological					
Aerobic mesophilic microorganisms (per g)	10,000	<15	<20	<20	<20
Aerobic spores (per g)	500	<10	<10	10	10
Enterobacteriaceae (per g)	10	NE	NE	NE	NE
Salmonella sp. (per 25 g)	Negative	NE	NE	NE	NE

DM = dry matter; ND = not detectable; NE = negative; NM, not measured; NS = no specification; RL = reporting limit

Microbiological Analysis

Additional microbial testing has been conducted on 3 non-consecutive lots of Nestlé GOS to verify the absence of specific microbial contaminates including *Cronobacter sakazakii*, *Bacillus cereus*, and *Listeria monocytogenes* (Table II.C-3). Nestlé GOS is intended for use in wet blending applications and is always added at a step in the formula process prior to thermal processing; infant formula to which Nestlé GOS has been added would therefore be compliant with the microbial requirements for infant formula as defined under 21 CFR 106.55 (U.S. FDA, 2015).

Table II.C-3 Summary of Additional Microbial Testing for 3 Lots of Nestlé GOS

Parameter	Batch No.		
	341778	**347230**	**291604**
Cronobacter sakazakii (per 100 g)	NE	NE	NE
Bacillus cereus (CFU/g)	<10	<10	70
Listeria monocytogenes (per 25 g)	NE	NE	NE
Escherichia coli (per g)	NE	NE	NE

CFU = colony forming units; NE = negative

II.C.3 Comparison of Nestlé GOS with Other GRAS Sources of GOS

Oligosaccharide Composition

All GOS ingredients on the market have slightly different oligosaccharide profiles; however, trisaccharides with *beta*-1,3, *beta*-1,4, and *beta*-1,6 linkage configurations typically dominate the oligomer distribution in most GOS preparations (Kimura *et al.,* 1995; Coulier *et al.,* 2009; Hernandez-Hernandez *et al.,* 2012). As shown in Table II.C-4, the oligosaccharide linkage composition and degree of polymerization of Nestlé GOS is chemically representative of GOS preparations that have previously been determined to be GRAS (*e.g.,* Vivinal GOS). Vivinal GOS was chosen for comparison as it was the first GOS preparation determined to be GRAS and therefore has been used in many of the GOS safety studies conducted to date.

Nestlé's GOS contains small amounts of sialyllactose (3'-sialyllactose and 6'-sialyllactose) originating from cow's milk whey permeate. These oligosaccharides are naturally present in cows' milk and are found in human milk and colostrum from lactating women. Concentrations of 54 to 600 mg/L milk of 3'-sialyllactose and approximately 29 to 1770 mg/L of 6'-sialyllactose have been measured in human milk samples depending on the period of lactation (Thurl *et al.,* 2010; Radzanowski *et al.,* 2013; Galeotti *et al.,* 2014; Sakaguchi *et al.,* 2014). These milk oligosaccharides are not created in the Nestlé GOS production process, but originate from the cow's milk whey permeate starting material and would result in small amounts of 3'-sialyllactose

(≈ 50 mg/L)[2] and 6'-sialyllactose (≈ 13 mg/L)[2] in infant formula containing Nestlé GOS. Infants have been exposed to sialyllactose oligosaccharides through both human breast milk and from the historical use of cow's milk based formula containing demineralized whey. Dietary intakes of sialyllactose from the use of Nestlé GOS in infant formula are therefore within levels that have an established history of safe consumption among breastfed infants.

Table II.C-4 Typical Oligosaccharide Composition for Nestlé GOS and Vivinal GOS

Parameter[a]	Nestlé GOS	Vivinal GOS (Friesland Foods Domo)
GOS Chain Length Ratios		
DP2	1.7	2.4
DP3	2.8	2.2
DP4	1.0	1.0
DP5	0.03	0.4
Average DP	2.88	2.89
Final Composition on a Dry Matter Basis		
Total oligosaccharides/GOS (%)	50	57
Sialyllactose (g/100 g DM)	0.24	NM
Linkage Analysis		
T-Glc (mol %)	ND	11.1
1,4-Glc (mol %)	13.2	10.3
1,6-Glc (mol %)	4.3	5.8
T-Gal (mol %)	19.2	25.9
1,4-Gal (mol %)	2.7	11
1,3-Gal (mol %)	3.5	0.9
1,6-Gal (mol %)	8.9	0.8
1,3,6-Gal (mol %)	0.4	ND
1,4,6-Gal (mol %)	ND	ND

DP = degree of polymerization; Gal = galactose; Glc = glucose; GOS = galacto-oligosaccharides; ND = not detected; NM = not measured

[a] All analyses were performed by Nestlé analytical laboratories, except linkage analysis, performed at University of Oslo following the protocol of Pettolino *et al.* (2012).

Protein and Mineral Composition

As demineralized whey permeate is used as the starting material for the manufacture of Nestlé GOS, residual quantities of proteins and minerals are present in the ingredient at higher quantities than other GOS preparations produced from crystalline lactose. The protein content of Nestlé GOS is typically <2% and is expected to be comprised of small proteins and peptide fragments of whey protein. As intact and partially hydrolyzed whey protein based infant formulas have a long-history of safe use globally, the use of sweet whey permeate as a raw

[2] Total content was estimated by summing the typical content of the standard formula with the contribution from the addition of Nestlé GOS.

material for GOS synthesis will not introduce new milk proteins to the infant diet that do not already have a history of safe consumption. Based on a use-level of 7.8 g/L of Nestlé GOS, an infant consuming 1 L of infant formula would be expected to consume approximately 160 mg of whey protein. This quantity of protein is nutritionally insignificant and would not affect the nutritional composition of infant formulas prepared with Nestlé GOS.

Mineral content is defined within the product specifications so that inclusion rates of minerals into the finished formula can be adjusted as necessary to meet the regulatory requirements of Section 350a of the U.S. Federal Food, Drug, and Cosmetic Act (U.S. FDA, 2013b). The mineral contribution of Nestlé GOS to the finished infant formula is presented in Table II.C-5, and is based upon the average mineral content of the 4 batches presented in Table II.C-2. For example, Nestlé GOS is predominantly characterized by levels of potassium that are present at concentrations of 1.2 to 2.1 g/100 g dry matter. Assuming a use-level of 7.8 g/L, a 167 mL serving of infant formula providing approximately 100 kcal would contain a minimum of 15.6 mg of potassium. Under Section 350a of the U.S. Federal Food, Drug, and Cosmetic Act, infant formula must contain between 80 to 200 mg of potassium per 100 kcal of infant formula (U.S. FDA, 2013b). The amount of potassium in the final Nestlé GOS product is therefore not of nutritional or toxicological concern.

Table II.C-5 Nutrient (Mineral) Contribution of Nestlé GOS to Infant Formula

Nutrient	Nutrient Value Provided by a Use-Level of 7.8 g/L Nestlé GOS (per 100 kcal)[a]	Nutrient Requirements for Infant Formula under §350a of the FFDCA (per 100 kcal)
Sodium (mg)	0.4	20.0 – 60.0
Potassium (mg)	19.6	80.0 – 200.0
Chloride (mg)	≤0.07	55.0 – 150.0
Calcium (mg)	≤0.7	50.0
Phosphorous (mg)	3.5	25.0
Magnesium (mg)	0.5	6.0
Manganese (µg)	≤0.0001	5.0
Iron (mg)	≤0.001	0.15
Copper (µg)	0.001	60.0

FFDCA = Federal Food, Drug, and Cosmetic Act; GOS = galacto-oligosaccharides
[a] Values calculated based upon the average of the results listed in Table II.C-2 and the assumption that 167 mL of infant formula provides approximately 100 kcal.

II.D Stability

The stability of Nestlé GOS following storage was evaluated under two temperature and humidity scenarios, using samples of Nestlé GOS powder packaged in 25 kg bags and individually sealed aluminum bags. Total moisture content (as analyzed by the Karl Fisher method), powder wettability (at 40°C), and water activity (at 25°C) were evaluated in samples obtained at baseline and at 3-month intervals for up to 18 months, at temperatures of either

25°C (humidity not controlled) or 30°C (70% relative humidity). Lactose, glucose, galactose, and total oligosaccharide content were evaluated at baseline and after 12 months of storage at 25°C. Representative results obtained for Nestlé GOS powder stored in 25 kg bags are summarized in Table II.D-1. Results obtained from the sealed aluminum bags were comparable and are not presented herein. Under both temperature/humidity scenarios, Nestlé GOS powder was reported to be unchanged with respect to total moisture content, powder wettability, and water activity over 18 months. The lactose, glucose, galactose, and total oligosaccharide content of Nestlé GOS powder did not change over 12 months of storage at 25°C (humidity not controlled). Overall, the data demonstrate that Nestlé GOS is stable for at least 1 year when stored at 25°C (humidity not controlled) or 30°C (70% relative humidity). In-life storage tests up to 24 months are on-going, and it is anticipated that the shelf-life of the ingredient will be 2 years.

Table II.D-1 Storage Stability of Nestlé GOS Powder

Parameter	Specification	Baseline	3 months	6 months	9 months	12 months	18 months
Stability at 25°C (Humidity Not Controlled)							
Total water (Karl Fisher method) (%)	-	3.25	3.15	3.04	3.20	3.22	3.25
Wettability at 40°C (seconds)	-	3	3	3	3	3	3
Water activity at 25°C	-	0.163	-	0.172	-	0.228	0.190
Lactose, including di-GOS (g/100 g)	-	41.8	-	-	-	42.6	-
Glucose (g/100 g)	≤10	7.98	-	-	-	8.71	-
Galactose (g/100 g)	≤5.0	3.82	-	-	-	4.10	-
Total oligosaccharides (g/100 g)	Min 46	46.4	-	-	-	46.6	-
Stability at 30°C, 70% Relative Humidity							
Total water (Karl Fisher method)	-	3.25	-	3.36	3.63	3.74	4.04
Wettability at 40°C (seconds)	-	3	3	3	3	3	3
Water activity at 25°C	-	0.163	-	0.197	-	0.225	0.224

- = not established/data not obtained; GOS = galacto-oligosaccharides

III. SELF-LIMITING LEVELS OF USE

Self-limiting use-levels are not known.

IV. DETAILED SUMMARY OF THE BASIS FOR NESTLÉ'S GRAS DETERMINATION

The first GRAS determination notified to the FDA for the use of GOS in infant formula was submitted by Friesland Foods Domo in 2007 for their ingredient Vivinal GOS (GRN 236, U.S. FDA, 2008). Since this GRAS determination, the totality of publically available data and information pertinent to the safety of GOS for use in infant formula has been the subject of several systematic and comprehensive reviews by various qualified scientific experts, including the FDA (U.S. FDA, 2009b, 2010, 2014b,c). International regulatory bodies, including the European Commission SCF and FSANZ, also have issued opinions supporting the safe use of GOS as an ingredient in infant formula alone, or in combination with fructo-oligosaccharides (FOS) at a use-level of up to 8.0 g/L (SCF, 2003; FSANZ, 2008).

Based on information presented herein, it has been determined that Nestlé GOS is chemically and compositionally representative of other GOS ingredients that have been determined to be GRAS for use in infant formula (*e.g.*, Vivinal GOS). Based on the expected physiological/toxicological equivalence of Nestlé GOS to other GRAS GOS preparations (*e.g.*, Vivinal GOS) produced from lactose by enzymatic synthesis, publically available data and information establishing General Recognition of Safety of GOS preparations, including toxicological studies in animals and safety and tolerance reports in healthy adults and infants, are therefore incorporated by reference to previous GRAS determinations (U.S. FDA, 2008, 2009a,b, 2010, 2014a-d). Since the most recent GRAS determinations were notified to the FDA in 2014, an updated comprehensive search of the publically available scientific literature was conducted to identify new information relevant to the safety of GOS published only in years 2014 and 2015. The following databases were accessed: Medline, ToxFile, AGRICOLA, AGRIS, BIOSIS Toxline, FOODLINE: Science, CAB Abstracts, BIOSIS Previews, FSTA (Food Science and Technology Abstracts), NTIS (National Technical Information Service), EMBASE, and Adis Clinical Trial Insight. A summary of the historical basis for General Recognition of Safety and newly identified studies relevant to GOS safety are provided below.

IV.A Probable Consumption

IV.A.1 Estimated Dietary Consumption of Nestlé GOS from Intended Food Uses

Nestlé GOS is intended for use as a food ingredient in non-exempt term infant formula (0 to 12 months) and toddler formula at concentrations up to 7.8 grams of GOS per liter in the reconstituted or ready-to-drink product. Nestlé GOS dietary intakes among infant consumers of Nestlé GOS (at a use-level of 7.8 g/L) may increase by a small margin of up to 8.3% compared to existing GOS preparations that have previously been determined to be GRAS for use in infant formula at a use-level of 7.2 g/L (see Table IV.A-1).

The dietary intake of GOS among infant formula consumers has been previously estimated based on a use-level of 7.2 g/L and using dietary survey data as described previously in GRN 236, 286, and 334 (U.S. FDA, 2008, 2009b, 2010). Food codes representative of each proposed food use (*i.e.,* infant formula and follow-on formula) were selected from the National Center for Health Statistics' 2003-2004 National Health and Nutrition Examination Survey (NHANES) (CDC, 2006; USDA 2009) to estimate the intake of GOS. Based on a 100% market share and assumption that GOS would be included in all infant formulas sold in the U.S., it was determined that approximately 80% of the infant population in the U.S. would be GOS consumers (202 actual users ages 0 to 6 months, 138 users aged 7 to 12 months). Toddlers were considered separately, aged 1 to 2 years, and were found to represent only 3.7% of users (19 actual users). The summary of the estimated dietary intake of GOS from infant formula and follow-on formula in the U.S. by infants and toddlers, as described in GRN 286, is provided in Table IV.A-1 (U.S. FDA, 2009b).

Table IV.A-1 Estimated Daily Intake of GOS from Current and Proposed Uses in Infant and Follow-on Formula in the U.S. by Population Group (2003-2004 NHANES Data)[a]

Population Group	Age Group (Years)	% Users	Actual # of Total Users	All-Person Consumption		All-Users Consumption	
				Mean (g)	90th Percentile (g)	Mean (g)	90th Percentile (g)
Estimated daily consumption of GOS from GRAS use of GOS at 7.2 g/L (GRN 286, U.S. FDA, 2009b)							
Infants	0 to 6 months	80.8	202	4.8	8.5	5.9	8.5
Infants	7 to 12 months	81.2	138	4.5	7.6	5.2	7.9
Toddlers	1 to 2	3.7	19	*0.1	N/A	*2.8	*6.6
Estimated daily consumption of GOS from proposed use of Nestlé GOS at 7.8 g/L							
Infants	0 to 6 months	80.8	202	5.2	9.2	6.4	9.2
Infants	7 to 12 months	81.2	138	4.9	8.2	5.6	8.6
Toddlers	1 to 2	3.7	19	*0.1	N/A	*3.0	*7.1

GOS galacto-oligosaccharides; N/A = Not available, due to small number of users in this age group
* Due to the small sample size data may be statistically unreliable.
[a] Table adapted from GRN 286 (U.S. FDA, 2009b).

Under the conditions of intended use of Nestlé GOS in term infant and toddler formula at a concentration of 7.8 g/L, dietary intakes may increase by up to 8.3% (Table IV.A-1). However, Nestlé's proposed use-level of Nestlé GOS at 7.8 g/L remains within the 8 g/L level permitted for addition to infant formula in other countries such as Australia and New Zealand and China (FSANZ, 2008; Ministry of Health of the PRC, 2011), and therefore will not change overall dietary intakes in American infants relative to levels that have an established history of safe use globally. In addition, infants consuming human milk are exposed to oligosaccharides at levels higher than 7.8 g/L. For example, human milk oligosaccharide concentrations of 25 and 12 g/L have been reported in human colostrum and mature milk samples, respectively (Kunz *et al*., 1999, 2000).

IV.B Safety Data for Galacto-oligosaccharides

IV.B.1 Metabolic Fate and Toxicity

The metabolism of GOS has been previously described in detail and the related physiological effects of GOS consumption on gastrointestinal physiology have been well characterized (U.S. FDA, 2008, 2009a, 2010). Briefly, it is generally recognized that with the exception of lactose, which is hydrolyzed by small intestinal brush border lactases, *beta*-linked sugars are not digested by human pancreatic or intestinal enzymes. GOS are not absorbed and are transported intact to the large intestine where they are subjected to fermentation by the indigenous microbiota. Although *in vitro* studies have reported slight differences in the efficiency by which particular bacterial species metabolize GOS (Ishikawa *et al.*, 1995; German *et al.*, 2008), they are ultimately hydrolyzed to glucose and galactose, which are subsequently metabolized by the anaerobic microflora by the Embden-Meyerhof-Parnas pathway resulting in the production of short chain fatty acids, CO_2 and H_2 gas (common and innocuous dietary metabolites) (Miller and Wolin, 1996; Suarez *et al.*, 1999; Smiricky-Tjardes *et al.*, 2003). These products of microbial fermentation, short-chain fatty acids in particular, result in the reduced pH and osmotic effects within the colon that are characteristic of GOS consumption.

IV.B.2 Toxicological Studies

GOS preparations, produced from lactose by enzymatic synthesis, have consistently been reported to be without evidence of toxicity in rodent studies (Table IV.B-1). Anthony *et al*. (2006) reported a no-observed-adverse-effect level (NOAEL) of 2,250 mg/kg body weight, the highest dose tested, for repeated-dose gavage administration of GOS (Vivinal GOS) to male and female Sprague-Dawley rats for 90-days. A NOAEL of 2,000 mg/kg body weight, the highest dose tested, was determined by Kobayashi *et al*. (2009) for Sprague-Dawley rats administered GOS (Oligomate GOS) *via* gavage for 90-days. Friesland Foods Domo has cited findings from an unpublished subchronic feeding study supporting a NOAEL of 6,900 mg/kg body weight following dietary administration of GOS (Vivinal GOS) to Wistar rats for 90 days (GRN 236, U.S. FDA, 2008). Toxicity studies published since the last GRAS determination (GRN 518, U.S. FDA, 2014d) include a neonatal rodent toxicity study conducted in juvenile rats and a one-generation reproductive and developmental toxicity study (Kobayashi *et al*., 2014a,b). These studies were reviewed by Nestlé and are discussed in brief below.

In the study by Kobayashi *et al*. (2014a), juvenile Sprague-Dawley rats were administered GOS by gavage for 42 days starting on post-natal day 4. This study was conducted under current Good Laboratory Practice (cGLP). GOS consumption was reported to have no effect on the development of the animals and did not affect general condition, hematology, blood chemistry, or the outcome of any functional examinations. No abnormalities in any of the groups were observed during the macroscopic examination, assessment of organ weights, or histopathology of the reproductive organs. The NOAEL for Oligomate GOS in juvenile Sprague-Dawley rats

was 2,000 mg/kg/day (Kobayashi *et al*., 2014a). In all studies described above, the NOAELs were the highest doses tested. GOS related effects reported in these studies (*e.g.,* transient diarrhea, increased cecal weights) are well established physiological effects that are consistent with the transport of resistant sugars/carbohydrates to the colon and are widely recognized as not being toxicologically relevant to humans (WHO, 1987).

Table IV.B-1 Summary of GOS Toxicity Studies in Rodents

Species (Age)	Route and Dose (mg/kg/day bw)	Study Design	Duration (days)	NOAEL (mg/kg bw)	Reference
Repeat-Dose Studies					
Sprague-Dawley rat (10♂/10♀; 6 weeks)	Gavage: 1,125 or 2,250 (Vivinal GOS)	OECD 408	90	2,250*	Anthony *et al.* (2006)
Wistar rat (10♂/10♀; 6 weeks)	Dietary: 1,600 – 6,900 (Vivinal GOS)	OECD 408	90	6,900*	GRN 236, U.S. FDA (2008)
Sprague-Dawley rat (10♂/10♀; 6 weeks)	Gavage: 500, 1,000, or 2,000 (Oligomate GOS)	MoHW‡	90	2,000*	Kobayashi *et al.* (2009)
Neonatal Sprague-Dawley Rat (10♂/10♀; PND 4)	Gavage: 500, 1,000, or 2,000 (Oligomate GOS)	-	45	2,000*	Kobayashi *et al.* (2014a)
Wistar Rat (10♂10/♀; 7 weeks)	Gavage: 500, 1,000, or 2,000 (Nestlé GOS)	OECD 407	30	2,000*	Penard (2015)
Developmental and Reproductive Studies					
Mice (BALB/c) Pregnant females (8 weeks) Pups (weaning)	Diet: GOS, 1620 + inulin, 400 (GOS: Laiterie de Montaigu) Same diet as dams	-	Gestation to weaning 48, post-weaning	No toxicity observed in dams or pups	Desbuards *et al.* (2012)
Sprague-Dawley Rat (24♂; 5 weeks) (24♀; 12 weeks)	Gavage:500, 1,000, or 2,000 (Oligomate GOS)	OECD 415	~ 90	2,000*	Kobayashi *et al.* (2014b)

bw = body weight; GOS = galacto-oligosaccharides; PND = postnatal day
*Highest dose tested; ‡Ministry of Health and Welfare, Japan, Ordinance No. 21; 26 March 1997; and in accordance with 'the Guidelines for Designation of Food Additives and for Revision of Standards for Use of Food Additives' (Environmental Health Bureau, Ministry of Health and Welfare, Japan, Notification No. 29; 22 March 1996.

Kobayashi *et al.* (2014b) evaluated the developmental and reproductive effects of Yakult Oligomate GOS in male and female parental rats, pregnant females, and their offspring. Male and female Sprague-Dawley rats (24 per sex per group) were administered GOS by gavage at doses of 0, 500, 1,000, or 2,000 mg/kg/day as follows: males were dosed 10 weeks prior to mating and 3 weeks thereafter; females were dosed 2 weeks before mating and GOS administration continued through pregnancy to day 20 of lactation. GOS consumption did not produce any toxicological effects on male or female parental animals and did not adversely affect reproduction/development from premating, copulation, implantation, or maintenance of pregnancy. The offspring were unaffected by the maternal consumption of GOS. No effects were observed on the number of live births, sex ratio, and external observation at the time of birth, body weight, pup survival, or external differentiation during lactation. The NOAEL for

reproductive function of male and female parent animals was 2,000 mg Oligomate GOS per kg/day, the highest dose tested (Kobayashi *et al*., 2014b).

Nestlé notes that among all studies described above, the NOAEL determinations have represented the highest permissible doses tested. Among the available toxicity studies identified in the literature, there are no reported findings to suggest that the use of GOS in infant formula would be unsafe or unsuitable.

Studies Conducted with Nestlé GOS

To corroborate available published findings from toxicity studies on GOS, the subacute toxicity of Nestlé GOS was evaluated following daily gavage administration to male and female Wistar rats (10 per sex per group) for 30 consecutive days at doses of 0, 500, 1,000, or 2,000 mg/kg/day (Penard, 2015). Half of the control and high-dose groups were followed for a 2-week recovery period to evaluate the regression of any toxic signs. The study was conducted under cGLP and in accordance with OECD guideline 407. Morbidity/mortality checks were performed at least twice daily, clinical observations were performed once daily, and a full clinical examination was performed weekly. Individual body weights were recorded weekly and food consumption was measured weekly for each cage of animals (5 rats per cage). Ophthalmological examinations were performed pretest and on Day 27 and clinical laboratory determinations were obtained on Day 30. All animals were euthanized at the end of the treatment period or after a treatment-free period of 2 weeks and necropsied. In accordance with the OECD 407 guidelines organs were weighed and organ/tissue samples were fixed and preserved at necropsy for all animals. Selected organs/tissues from the control and high-dose groups were examined histopathologically.

There were no deaths, no relevant clinical signs, and no test item-related ophthalmological findings reported during the study. There was no variation in body weight or food consumption between groups. At study termination, there were no relevant changes reported in hematology, coagulation, serum clinical chemistry or urine parameters between groups. GOS consumption did not cause any significant organ weight, macroscopic, or histopathological changes. Under the defined experimental conditions, the oral administration of Nestlé GOS for 30 days in the Wistar rat at doses of 500, 1,000, and 2,000 mg/kg/day were well-tolerated clinically and histologically and did not induce any treatment-related effects. The NOAEL for Nestlé GOS was determined by the study authors, and by Nestlé, to be 2,000 mg/kg/day, the highest dose tested.

IV.B.2.1 Other Animal Studies

Additional studies identified during the updated literature search, and relevant to the GRAS assessment, were subject to critical and comprehensive reviews by Nestlé. Overall, data presented in these studies are consistent with the published literature demonstrating that GOS

is well-tolerated and without reported toxicological effects in experimental feeding studies. In experimental animal studies evaluating oral administration of GOS no findings have been reported to suggest that the current or proposed use of GOS in infant formula would be unsafe or unsuitable. These studies are summarized in brief below.

Two additional studies were identified during the updated literature investigating the effects of GOS consumption on the development of allergic asthma in mice (Hogenkamp *et al.,* 2015; Verheijden *et al.*, 2015). Although specific safety endpoints were not investigated in these studies, the results contribute to the abundance of literature supporting the safe consumption of GOS. In the first study, allergic asthma was induced in male BALB/c mice by intranasal sensitization and re-challenge with 1 µg and 10 µg of house dust mite, respectively. Mice were fed a diet containing 1% GOS (daily dose not reported) starting 2 weeks before sensitization and continued for an additional 2 weeks. GOS consumption was found to result in a significant inhibition of airway hyper-responsiveness induced by allergic asthma and prevented the induction of airway eosinophilia as well as cytokine and chemokine elevations in the lung (Verheijden *et al.*, 2015). In the second study, GOS was administered in combination with FOS (9:1) to female BALB/c mice in the diet (3% GOS/FOS) prior to mating with C57BL/6 males. GOS/FOS administration was continued through pregnancy to delivery. Allergic asthma was induced in male offspring at 6 weeks of age by injection of aluminum hydroxide combined with ovalbumin. Offspring from the GOS/FOS-treated dams showed significantly reduced symptoms of allergic asthma, as measured by acute allergic skin response after intradermal challenge with ovalbumin and airway challenges with nebulized ovalbumin. The authors concluded that supplementation of pregnant dams with GOS/FOS significantly decreased allergic symptoms in the offspring (Hogenkamp *et al.,* 2015).

The effects of GOS consumption on the intestinal microbiota was recently investigated in suckling Sprague-Dawley rat pups (6 males, 2 females per group) (Morel *et al.,* 2015). From Postnatal Day 5 to 14 suckling rat pups were administered GOS mixed with long-chain fructan (lcF) daily by gavage (9:1; 2.25 g GOS per kg/day body weight) and then weaned to a regular diet on Day 21. GOS/lcF consumption altered the composition of the intestinal microbiota at Day 14 by increasing the number of *Bifidobacteria* and decreasing the *Firmicutes* levels. On Day 131, the microbiota composition tended to revert to levels observed in the controls. No safety or tolerance endpoints were assessed in this study.

IV.B.2.2 Genotoxicity Studies

It is well established that GOS are comprised of common nutrients (*e.g.*, oligosaccharides galactose, lactose, glucose, minerals) and therefore do not contain substances or potential impurities that are of genotoxic concern. The genotoxicity of GOS has been evaluated in several studies including the bacterial reverse mutation assay, mammalian chromosomal aberration test, and *in vivo* micronucleus assay in mice and the outcome for all investigations has consistently demonstrated that GOS are not genotoxic (Table IV.B-2). The *in vitro* and *in*

vivo mutagenicity/genotoxicity of Nestlé GOS also has been evaluated and findings are summarized below. New studies identified in the literature relevant to the genotoxic potential of GOS are also discussed.

Table IV.B-2 Summary of GOS Genotoxicity Studies

Test	Concentration	Metabolic Activation	Result	Reference
***In vitro* Assays**				
Bacterial reverse mutation (*S.* Typhimurium & *E. Coli*)	312.5 – 5,000 µg/plate (Oligomate GOS)	± S9	Negative	Kobayashi *et al.* (2009)
Mammalian chromosomal aberration (CHL/IU)	1,250, 2,500, or 5,000 µg/mL (Oligomate GOS)	± S9	Negative	
Bacterial reverse mutation (*S.* Typhimurium & *E. Coli*)	492 - 5,000 µg/plate (Nestlé GOS)	± S9	Negative	Verspeek-Rip (2015)
Micronucleus assay (peripheral human lymphocytes)	512, 1,600, or 5,000 µg/mL (Nestlé GOS)	± S9	Negative	Verbaan (2015)
***In vivo* Assays**				
Micronucleus, mouse (CD-1)	Gavage; 500, 1,000, or 2,000 mg/kg bw (Oligomate GOS)	N/A	Negative	Kobayashi *et al.* (2009)
Comet assay, rat (SD)	Gavage; 500, 1,000 or 2,000 mg/kg/day bw (Oligomate GOS)	N/A	Negative	Narumi *et al.* (2014)

bw = body weight; GOS = galacto-oligosaccharides; N/A = not applicable

Studies Conducted with Nestlé GOS

The mutagenic activity of Nestlé GOS was evaluated in the *S. Typhimurium* (TA1535, TA1537, TA98, and TA100) and *E. Coli* (WP_2uvrA) reverse mutation assays according to OECD guideline no. 471 (Verspeek-Rip, 2015). The tests were conducted in two independent experiments, in the presence and absence of a metabolizing system (rat liver S9-mix induced by Aroclor 1254). Nestlé GOS was tested over a concentration range of 492 to 5,000 µg/plate in triplicate and no significant increase in the number of revertant colonies was reported in either assay.

Nestlé GOS was also evaluated for clastogenic and aneugenic potential in an *in vitro* micronucleus assay using cultured peripheral human lymphocytes according to OECD guideline no. 487 (Verbaan, 2015). Lymphocytes were prepared by collecting whole blood samples from healthy male subjects into heparin-coated tubes and the blood cells were then cultured in the presence of mitogen phytohaemagglutinin to generate stimulated lymphocytes. The stimulated lymphocytes were incubated with Nestlé GOS in two independent experiments. In the first test, stimulated lymphocytes were exposed to 512, 1,600, or 5,000 µg GOS/mL in the culture medium for 3 hours, both in the presence and absence of a metabolizing system (phenobarbital and β-naphthoflavone-induced rat liver S9-mix). Following exposure to GOS, the lymphocytes

were cultured for 27 hours to allow chromosome or spindle damage to induce micronuclei formation in interphase cells. No significant increase was reported in the GOS-treated cells with respect to the number of mono- and bi-nucleated cells with micronuclei. In the second assay, stimulated lymphocytes were exposed to GOS at the same concentrations but for a longer time period, 24 hours, only in the absence of metabolic activation. Following exposure to GOS, the lymphocytes were cultured for 24 hours and as was seen in the first test, GOS was not found to significantly increase the number of mono- and bi-nucleated cells with micronuclei. The results from these micronucleus assays indicate that Nestlé GOS is not clastogenic or aneugenic in human lymphocytes.

Other Studies

Narumi *et al*. (2014) evaluated the mutagenic potential of Oligomate GOS in the *in vivo* comet assay. Male Sprague-Dawley rats (5 per group) were orally administered 0, 500, 1,000, or 2,000 mg/kg of GOS 3 times over the course of 2 days. DNA was isolated from the stomach, colon, and peripheral blood and analyzed by the comet assay to measure the frequency of DNA strand breaks. No significant difference in the percentage of DNA in the comet tail between the treated and control groups was reported. The results of the comet assay were negative, supporting the conclusion that GOS is not genotoxic.

Based on available published studies characterizing the toxicity of various GOS preparations, and corroborating findings obtained in the *in vitro* Ames assay and micronucleus assays conducted with Nestlé GOS, it can be concluded that Nestlé GOS is non-genotoxic.

IV.B.3 Human Studies

Clinical studies evaluating the safety of GOS consumption in adults and infants have assessed a number of biological endpoints, including the effects of GOS on gastrointestinal physiology, fecal microflora, the immune system, and tolerance (U.S. FDA, 2008, 2009a,b, 2010, 2014a-d). A number of desirable physiological effects have been documented in infants consuming GOS-supplemented formula, including elevated levels of *Bifidobacterium* in the gastrointestinal tract, higher short-chain fatty acid concentrations in the stool leading to decreased pH, and improved stool consistency approaching that of breastfed infants (Veereman-Wauters, 2005; Roberfroid *et al.*, 2010; Oozeer *et al.,* 2013).

Based on Nestlé's review of previous GRAS determinations (U.S. FDA, 2008, 2009a,b, 2010, 2014a-d), other comprehensive safety evaluations and published reviews by qualified experts (*e.g*., FSANZ, 2008; Oozeer *et al.,* 2013), Nestlé has concluded that findings from available clinical studies have equivocally demonstrated that GOS are safe to consume and well-tolerated in infants.

The totality of the published literature investigating the safety of GOS administration in infants has been the subject of multiple comprehensive reviews during previous GRAS determinations

(U.S. FDA, 2008, 2009a,b, 2010, 2014a-d). These GRAS determinations have consistently concluded that that GOS preparations, synthesized from lactose using food grade *beta*-galactosidase enzyme preparations, are GRAS for use in infant formula at a use-level of 7.2 g/L. This use-level was based on early pivotal studies by Schmelzle *et al.* (2003) and Moro *et al.* (2006) evaluating the co-administration of GOS (7.2 g/L) and FOS (0.8 g/L) in combination in a 9:1 ratio at a use-level of 8 g/L. Based on the established similarities in the metabolic fate of GOS and FOS, Nestlé has concluded that the addition of GOS to infant formula at a use-level of 7.8 g/L would also be GRAS. This conclusion is consistent with the maximum use-level of 8 g GOS/L currently permitted for addition to infant formula in Australia and New Zealand and China (FSANZ, 2008; Ministry of Health of the PRC, 2011). This conclusion is further corroborated by the absence of adverse effects on growth reported in product specific studies evaluating the safety of Nestlé GOS in healthy term infants administered infant formula containing Nestlé GOS alone at a use-level 10 g/L, or when administered in conjunction with probiotic microorganisms at a use-level of 8 g/L. Nestlé GOS also was reported to be safe and well-tolerated when administered to healthy term infants or infants from HIV positive mothers. The aforementioned studies were conducted in infants born from mothers recruited from European or South African populations, and used infant formula products representative of infant formula preparations that have formed the basis of previous GRAS determinations. A summary of these studies is presented below.

IV.B.3.1 Studies of Nestlé GOS in Infants

Meli *et al*. (2014) evaluated the effects of consuming infant formula containing Nestlé GOS in a double-blind parallel group placebo controlled study in 281 infants conducted in Italy. Infants (≤14 days old; 2,500 to 4,500 g birth weight; gestational age ≥37 weeks) were randomized to 1 of 3 groups: 1) control infant formula (n=84); 2) infant formula supplemented with Nestlé GOS (10 g/L) (n=99); or 3) infant formula supplemented with Nestlé GOS (10 g/L) + probiotics [*Bifidobacterium longum* ATCC BAA-999 (Bl999) and *Lactobacillus rhamnosus* CGMCC 1.3724 (LPR) each at 2 x 10^7 CFUs per gram] (n=98). All treatments were provided for 6 months. A breastfed reference control group was also included in the study (n=30). Follow-up visits were conducted at 14 days, 1, 2, 3, 4, 5, 6, and 12 months. The primary outcome was mean weight gain in grams per day between 14 days and 4 months (112 days) of age. The study also included general measures of tolerance, clinical chemistry and hematology, safety analyses, as well as observation of fecal characteristics. The study was adequately powered to support the safety measures of growth in accordance with the requirements of the American Academy of Pediatrics (AAP)[3] (AAP, 1988).

[3] Authors' reported that "*Although the primary analysis in the present study included slightly fewer infants than the estimated number needed from the sample size calculation, it is unlikely that the addition of 1 more infant in the control group and 2 more infants in the IF-BMOS group would change the results of the analysis in a meaningful way*."

No differences in growth, hematology and blood biochemical analyses, adverse events, or clinical safety indices were reported between groups. Infants consuming infant formula supplemented with Nestlé GOS (prebiotic), with and without probiotics, had more frequent and less hard stools compared to the control group. The authors reported a statistically significant reduced likelihood of investigator-diagnosed colic in the control group relative to the prebiotic group (OR = 0.38; 95% CI 0.18, 0.81; p = 0.01). These findings were recorded despite the fact that no statistically significant differences in caregivers' records of flatulence, spitting up, vomiting, duration of crying, fussing, episodes of colic, or illness were reported. An explanation for this discrepancy was not discussed, and the authors suggested that the observed increase in colic in the prebiotic group may be related to the higher GOS use-level of 10 g/L, which is somewhat higher than the concentrations of GOS used previously in infant studies that typically have ranged from 4 to 8 g/L. Improved fecal consistency was observed for select fecal measures in association with prebiotic consumption: infants fed the control formula were more likely to have harder stools than infants consuming Nestlé GOS [odds ratio (OR) = 5.06; 95% CI: 1.33 to 19.32; p = 0.0003] or Nestlé GOS + probiotics (OR = 6.55; 95% CI: 1.49 to 28.78; p = 0.0001) formulae. Interpretation of the findings of investigator reported colic by the study investigators was complicated by limitations in the study design[4], and therefore no conclusions on an association between treatment and investigator reported colic could be established; however, Nestlé noted that the study was adequately powered to establish safety (*i.e.*, anthropometric growth indices). Tolerance issues with GOS have not been reported in other published infant studies, including studies where infants were provided up to 9 g GOS/L (Mihatsch *et al.*, 2006). In addition, findings from subsequent studies evaluating lower use-levels of Nestlé GOS (8 g/L GOS) have not reported findings suggestive that administration of Nestlé GOS to term infants is associated with poor tolerance. These studies are summarized below.

A study recently accepted for publication enrolled 115 healthy term infants (≤14 days old; 2,500 to ≤4,500 g birth weight; gestational age ≥37 to ≤42 weeks) in a controlled, randomized, double-blind nutritional intervention trial in France and Poland to assess the effects of administering infant formula supplemented with Nestlé GOS combined with a probiotic (Simeoni *et al.,* 2015). Infants received either starter infant formula as the control (n=37) or the same formula supplemented with Nestlé GOS (8 g/L in reconstituted formula) and probiotic (*B. lactis* strain CNCM I-3446, $1x10^7$ CFU/g of powder formula) (n=39) for a 12-week feeding period.

[4] Caregiver-reported colic was not significantly different between infants administered infant formula with prebiotic (Nestlé GOS) *versus* control; however, caregivers were asked to record episodes of colic (defined as bouts of intense, inconsolable crying with painful facial expressions and pulling up of the legs) for only 3 days prior to each visit at 14 days and at ages 1, 2, 3, 4, 6 and 12 months. The 3-day collection period may not have been sufficient to identify episodes of colic. The investigator-reported definition of colic required the occurrence of colic for 3 or more hours per day and for at least 3 days per week for at least 1 week. It is not clear how this information could have been collected by the investigator, given that it was not collected as such in the caregiver diaries. Nestlé also notes that the investigator definition of colic used by the authors would not be defined as colic under the Wessel Criteria whereby colic is defined as unexplained paroxysmal bouts of fussing and crying that lasted for >3 hours a day, for >3 days a week, for >3 weeks (Garrison and Christakis, 2000).

Infants from mothers who elected to breastfeed were included as a breastfed reference control group (n=39). Consumption of the test formula containing Nestlé GOS and probiotic was well-tolerated by all infants and the test and control formula groups showed no difference in "spitting up", vomiting, crying, colic, flatulence, or irritability. Anthropometric measurements (weight, height, head circumference) were found to be similar between all 3 groups (Nestlé GOS + probiotic, control, and breastfed reference) over the 12-week treatment period. Stool characteristics in the Nestlé GOS + probiotic group were found to be more similar to the breastfed reference group compared to the control formula group. For example, throughout the trial the proportion of yellowish *versus* greenish stools was equivalent between the Nestlé GOS + probiotic and breastfed reference groups, but not the control group, and stool consistency was also more similar to the breastfed reference (more liquid stools). The stool pH measured in the Nestlé GOS + probiotic and breastfed reference groups at 6 weeks was similar and significantly lower than the stool pH of the control group. The composition of the fecal microbiota was evaluated at baseline, Week 6 and Week 12 and it was found that the diversity and composition of microbiota in the Nestlé GOS + probiotic group became closer to those of breastfed infants. Furthermore, a statistically significant increase in the *Bifidobacterium*-dominated fecal microbiota titer was reported in the Nestlé GOS group relative to the control group at 6 and 12 weeks. Although the numbers of *Bifidobacteria* were increased in all 3 groups, it was most prominent in the Nestlé GOS + probiotic and breastfed reference groups, and least prominent in the control group. This study demonstrates that the use of Nestlé GOS combined with probiotic *B. lactis* is well-tolerated, supports similar growth compared to control formula, produces effects on fecal characteristics that are comparable to those observed in breastfed infants, and overall corroborates the safety of GOS.

The consumption of Nestlé GOS + probiotic (*B. lactis* strain CNCM I-3446, $1x10^7$ CFU/g powder formula) was evaluated in an unpublished controlled, double-blind randomized, multicenter clinical study in a group of 421 infants (≤3 days old; 2,500 to ≤4,500 g birth weight; gestational age ≥37 to ≤42 weeks) delivered by normal birth or caesarean section from HIV positive mothers in South Africa (Cooper*,* 2014). In total, 207 infants received infant formula containing Nestlé GOS (8 g/L) + probiotic and 214 infants received control formula. Infants received their respective formula for a duration of 6 months and then were transferred to a standard follow-on formula for a 6-month observation period. The primary efficacy objective was to evaluate fecal *Bifidobacteria* levels at 10 days and the primary safety objective was to evaluate growth (measured as mean weight gain in grams per day) between 10 days and 4 months of age. Secondary safety related outcomes included anthropometric indices of growth (weight, length, head circumference and corresponding z-scores, body composition, arm circumference, and subscapular and triceps skin folds), digestive tolerance, fecal IgA, blood levels of hepatitis B-specific IgG, HIV status, standard clinical chemistry parameters, and frequency of morbidity episodes/adverse events (including infections, other diseases, and medications).

The mean body weight gain of infants in the Nestlé GOS + probiotic group was not inferior to the growth of the control infants, based upon the non-inferiority margin of -3 g/day recommended by the AAP, irrespective of the mode of delivery (caesarean section *versus* vaginal birth). Fecal levels of *Bifidobacterium* on Day 10 were significantly elevated in infants delivered by caesarean section consuming Nestlé GOS + probiotic formula compared to control. In the vaginally delivered infants, *Bifidobacterium* counts were not different between the test group and control group at 10 days. At 4 weeks and 12 weeks, adjusted mean fecal *Bifidobacteria* counts were significantly higher in the Nestlé GOS + probiotic group compared to control, regardless of the mode of delivery.

Overall, the test formula containing Nestlé GOS with probiotic was well-tolerated and no adverse changes in safety measures, clinical chemistry, or gastrointestinal tolerance monitoring endpoints were reported. Statistically significant improvements in stool characteristics were reported in the Nestlé GOS + probiotic group, including lower fecal pH and lower adjusted mean percentage of days with hard stools. No significant differences were reported between the Nestlé GOS + probiotic and control groups with respect to the percentage of days with unusual stool odor, flatulence, spitting up, vomiting, crying, fussing, or colic. No increased incidence of adverse events (*e.g.,* colic) among infants fed formula containing Nestlé GOS with probiotic compared to control formula was reported during the 12-month study period. Findings from this study corroborate the safety of Nestlé GOS.

A second unpublished study was a prospective, controlled, double-blind, multicenter (Netherlands, Germany, France) trial conducted in 413 infants (≤13 days old; 2,500 to ≤4,500 g birth weight; gestational age ≥37 to ≤42 weeks) born by normal birth or caesarean section (Hascoet, 2015). Infants were randomized to 1 of 2 groups: 1) control infant formula (n=207); 2) Nestlé GOS (8 g/L) + probiotic (*B. lactis* strain CNCM I-3446, $1x10^7$ CFU/g) (n=206). A third group of infants from mothers electing to breast feed (n=63) were used as a reference control group. Test formula was provided for 6 months followed by standard follow up formula for another 6 months. Primary endpoints were mean event rates of gastrointestinal infection (diarrhea) and all infections with fever over 6 months and 12 months. Secondary outcomes included morbidity/adverse events over 12 months, anthropometric indices of growth (weight, length, head circumference, arm circumference, triceps and subscapular skin folds), digestive tolerance (recorded by parents/caregivers), and evaluation of efficacy related stool parameters and stool and salivary immune markers.

Formula containing Nestlé GOS + probiotic was well-tolerated throughout the duration of the study. Incidence rates for diarrhea as well as all infections with fever were not significantly different between the Nestlé GOS + probiotic and control groups at 6 or 12 months. The growth of infants consuming formula supplemented with Nestlé GOS + probiotic was not inferior to the control formula group, based upon the non-inferiority margin of -3 g/day recommended by the AAP. No adverse effects on the anthropometric indices growth were reported over the

12 months. Although the body mass index was slightly smaller at 2 months in the Nestlé GOS + probiotic group compared to the control, no difference between the breastfed reference and Nestlé GOS + probiotic groups was reported. Incidences of adverse events were comparable among the 3 groups (the Nestlé GOS + probiotic, control, and breastfed reference), with very low incidences of serious adverse events reported in all groups. In terms of digestive tolerance, infants in the Nestlé GOS + probiotic group experienced increased daily number of stools, improved stool color (*e.g.,* higher proportion of yellow *versus* green stools), improved stool consistency (*e.g.,* more soft and liquid *versus* hard stools) compared to the control in the first 3 months, and were more similar to the breastfed reference group. No differences were found between the Nestlé GOS + probiotic and control groups over 12 months with respect to the daily number of episodes of vomiting, spitting up, or colic and the restlessness distribution also did not differ. Although a statistically significant difference in crying time frequency (no answer; <1 hour; 1 to 3 hours; >3 hours) was reported at 3 months in the group receiving Nestlé GOS + probiotic (1%, 75%, 23%, 1%) relative to control (3%, 86%, 11%, 1%) (p=0.01), the frequency of crying time categories was comparable to that in the breastfed reference group (0%, 73%, 24%, 4%) and no differences in crying behavior were reported at any other time point (*i.e.,* months 1, 2, 6, 9, and 12). Improvements in stool characteristics were reported in the Nestlé GOS + probiotic group compared to control, including lowered stool pH at 3 and 6 months (comparable to that of breastfed infants), increased populations of *Bifidobacterium* and *Lactobacilli*, reduced numbers of *Clostridium*/*Eubacterium* at 3 and 6 months, and increased fecal immune markers sIgA at 3 and 6 months and *alpha*-1 antitrypsin at 3 months. This study demonstrates that the use of Nestlé GOS combined with probiotic supports normal infant growth, produces effects on fecal characteristics that are comparable to those observed in breastfed infants, and corroborates the safety of Nestlé GOS.

IV.B.3.2 Studies in Infants with Other GOS Preparations

In addition to the study by Meli *et al.* (2014), 9 other clinical studies investigating the effects of GOS-supplemented formula in infants and children were identified in the updated literature search that are summarized below in Table IV.B-3. Infant formula was supplemented with GOS alone in 3 of the new studies, one of which was a preliminary pilot investigation in premature infants where GOS was administered at increasing doses (2.5 to 20 g/L) over 5 weeks (Underwood *et al*., 2014). The other 2 studies with GOS alone were randomized, double-blind, controlled, multicenter trials where GOS was provided in the formula at a concentration of 4 g/L and/or 8 g/L (Giovannini *et al*., 2014; Williams *et al.*, 2014). No adverse events related to GOS consumption were reported and GOS was well-tolerated. The study by Giovannini *et al*. (2014) reported a lower risk rate of infantile colic in the GOS-supplemented group (4 g/L).

A number of investigations conducted with infant formula or growing up milk supplemented with GOS in combination with FOS were also identified as recent additions to the scientific literature (Armanian *et al*., 2014; Chatchatee *et al.,* 2014; da Costa Ribeiro *et al*., 2015; Dasopoulou *et*

al., 2015; Lee *et al*., 2015). In the majority of studies, GOS and FOS were supplemented at the standard 9:1 ratio, providing between 4 to 12 g/L of GOS in infant formula or growing up milk. No safety concerns were raised in any of the studies. The study by da Costa Ribeiro *et al.* (2015) reported that colic occurred less frequently in the test formula group receiving 4 g/L GOS combined with FOS compared to the mixed feeding group (breastfed and formula fed). The experimental details and measured outcomes of these new studies are summarized in Table IV.B-3 below.

IV.B.3.3 Adults

The survey of the recently published literature identified 2 new clinical studies on GOS consumption in adults. In the first study, the effect of GOS consumption on the gut microbiota of healthy adults (18 to 40 years old) receiving amoxicillin treatment was evaluated (Ladirat *et al*., 2014). Healthy adults (n=12) received a combined treatment of amoxicillin (375 mg) and GOS (2.5 g Vivinal GOS) 3 times per day for 5 consecutive days. GOS consumption (2.5 g three times daily) was continued for an additional 7 days post-amoxicillin therapy. The control group received maltodextrin in combination with amoxicillin. Subjects recorded compliance to the treatment, defecation frequency, stool consistency, gastrointestinal discomfort, and adverse events in a study diary. One subject in the amoxicillin-GOS treatment group was withdrawn from the study after Day 5 due to diarrhea. No other significant differences in adverse gastrointestinal events were reported between the groups. Due to the exploratory nature of this study, low numbers of subjects, and lack of appropriate comparator groups (*e.g*., GOS alone), it is unclear if the reported case of diarrhea was due to the GOS, amoxicillin, or the combination of the two.

The second study in adults investigated the effect of GOS consumption on the secretion of stress hormone cortisol. Healthy adults (22 males, 23 females; aged 18 to 45 years) received oral Bimuno GOS powder supplements with breakfast (5.5 g/day) for 3 weeks and subjects were instructed to adhere to a regular diet and to avoid consuming dietary supplements or special diets. GOS supplementation was found to significantly decrease the salivary cortisol awakening response compared to placebo and also decreased attentional vigilance to negative *versus* positive information in a dot-probe task. No safety or tolerance measures were reported (Schmidt *et al.,* 2015).

Table IV.B-3 Recent Studies of GOS Consumption in Infants – Updated Literature Search January 2014 to December 2015

Reference and Study Design	Subjects	Dose	Duration	Results	
Studies Conducted with GOS Alone					
Giovannini *et al*. (2014) Randomized, double-blind, controlled, parallel-group, multicenter	362 infants (term; enrolled by DOL 15; gestational age from 37 to 42 weeks; birth weight ≥2,500 g; 181 M, 181 F; healthy)	***Test Formula:*** 1. Formula + GOS (4 g/L) (Vivinal GOS) ***Control Formula:*** 1. Standard formula 2. Breastfed reference	4 months	Adverse event reporting	Risk rates of adverse events comparable between all groups. Infantile colic showed lower risk rate in GOS group.
				Growth	All groups showed appropriate physical growth throughout the study period.
				Fecal microflora	Significantly lower count of *Clostridium* and higher count of *Bifidobacterium* found in GOS group compared to control, specifically in infants with colic.
				Stool effects	GOS group presented normal and soft stools in the majority of episodes (89%). Decreased stool frequency in GOS group. Lower risk rate of watery stools in GOS group.
Meli *et al*. (2014) Randomized, double-blind, controlled, parallel-group, single-center	281 infants (term; enrolled by DOL 14; gestational age ≥37 weeks; birth weight 2,500 - 4,500 g; 161 M, 120 F; healthy)	***Test Formulas:*** 1. Formula + Nestlé GOS (10 g/L) 2. Formula + Nestlé GOS (10 g/L) + probiotics ***Control Formula:*** 1. Standard formula 2. Breastfed reference	6 months	Adverse event reporting	Caregiver reports found no significant differences between flatulence, vomiting, spitting up, crying, fussing, and colic. Incidence of investigator-diagnosed colic was lower in control group compared to GOS alone group.
				Growth	Weight, length, and head circumference gain per day were equivalent between all groups.
				Fecal microflora	Clostridia detected in lower percentage of stool samples from both GOS groups compared to control. *Bifidobacteria* and *lactobacilli* counts higher in both GOS groups compared to control.
				Stool effects	Daily stool frequency was significantly higher in both GOS groups compared to control, and stools were also softer.

Table IV.B-3 Recent Studies of GOS Consumption in Infants – Updated Literature Search January 2014 to December 2015					
Reference and Study Design	**Subjects**	**Dose**	**Duration**	**Results**	
Underwood *et al*. (2014)	27 infants (preterm, gestational age < 33 weeks; birth weight <1,500 g, 14 M and 13 F)	***Test Formula A:*** 1. Formula + GOS (2.5 to 20 g/L over 5 weeks) 2. Formula + donor human milk (HMO) ***Test Formula B:*** 3. Mother's milk + donor human milk (HMO) 4. Mother's milk + bovine powdered fortifier	A: 5 weeks B: 6 weeks	Adverse event reporting	2 infants in GOS group (GOS syrup) developed blood streaked stools with mild abdominal distension (no diarrhea, vomiting, or abnormal findings on radiographs or blood tests). Bloody stools resolved quickly after stopping the GOS syrup supplementation (both infants were removed from the study). Test GOS syrup was switched to GOS powder for remaining 4 subjects in GOS group. No infants with vomiting, abdominal distension, constipation, diarrhea, bloody stools, or NEC in group fed GOS powder.
				Fecal microflora	Some infants in GOS group showed increases in *Bifidobacteria* and decreases in *γ-Proteobacteria* with increasing GOS dose (not significant for entire group). Increase in relative abundance of *Clostridia* with increasing GOS dose.
Williams *et al.* (2014) Randomized, double-blind, controlled, multicenter	175 infants (term; enrolled by DOL 8; gestational age from 37 to 42 weeks; birth weight ≥2,490 g; 90 M, 85 F; healthy)	***Test Formula:*** 1. GOS-supplemented formula (4 g/L) 2. GOS-supplemented formula (8 g/L) ***Control Formula:*** Standard formula (Breastfed reference, or human milk bottle-fed)	4 months	Adverse event reporting	No significant differences among groups in proportion of subjects with specific adverse events. Significantly higher proportion of respiratory tract infections in GOS8 groups compared to GOS4 and control.
				Growth	No significant differences in mean weight or length among groups. No significant differences among groups in mean gains in weight or head circumference.
				Stool effects	Stool consistency for infants fed GOS formula was more similar to that of human milk-fed infants. Significantly higher percentage of watery stools in GOS8 group compared to control up to DOL 14.
				GI tolerance	Average number of stools in all formula groups significantly lower than human milk-fed group up to DOL 56. Percentage of feedings with spit up and/or vomiting within 1 hour after feeding was significantly higher in GOS8 group compared to human milk-fed group up to DOL 14.

Table IV.B-3 Recent Studies of GOS Consumption in Infants – Updated Literature Search January 2014 to December 2015

Reference and Study Design	Subjects	Dose	Duration	Results	
Studies Conducted with GOS Combined with FOS					
Armanian *et al.* (2014) Randomized, controlled, single-center	75 infants (preterm; gestational age ≤34 weeks; birth weight ≤1,500 g)	***Test Formula:*** Breast-milk with GOS/FOS added (9:1; 6.4 – 12.9 g/L GOS) ***Control Formula:*** Breast-milk only (Breastfed reference)	Up to 80 days	Adverse event reporting	Weight loss, constipation, and incidence of diarrhea were not different between groups. Significantly decreased incidence in GOS/FOS group of NEC, stay in hospital, and time to establish total milk intake. Trend towards lower neonatal sepsis in GOS/FOS group.
				Growth	Average body weights at DOL 30 marginally higher in GOS/FOS group.
Chatchatee *et al.* (2014) Randomized, double-blind, controlled, parallel, multicenter	767 children (children; 11 – 29 months old; birth weight <1,500 g; 435 M, 332 F; healthy)	***Test Formula:*** GUM with GOS/FOS (Danone Research) (9:1; 12 g/L GOS) and n-3 LCPUFAs (19.2 mg/100 mL) ***Control Formula:*** GUM alone (Cow's milk reference)	4-week run-in + 52 weeks	Adverse event reporting	Mild GI symptoms occurred equally between groups. Approximately half of adverse events reported were related to the respiratory system.
				Growth	Subjects in supplement arm weighed significantly less at baseline, but over the course of the study, there was no difference found in change in weight and height between groups.
				Infections	Decreased risk of developing at least 1 infection in supplement group. Trend towards a reduction in the total number of infections in the supplement group.
da Costa Ribeiro *et al*. (2015) Randomized, double-blind, controlled, parallel-group, multicenter, prospective	272 infants (term; 2 weeks to 4 months old; birth weight 2,500 g to 4,500 g; 139 M, 133 F; healthy from HIV+ mothers)	***Test Formula:*** Test formula – 2.1 g protein/100 kcal, GOS/FOS (9:1; 4 g/L GOS) ***Control Formula:*** Standard formula – 2.6 g protein/100 kcal, no prebiotics (Breastfed reference &	4 months 12-month follow-up	Adverse event reporting	Frequency of adverse events was not significantly different between treated and control groups. Colic occurred less frequently in the test formula group compared to the mixed feeding group.
				Growth	Mean daily weight gain was not significantly different between any of the groups up to 4 months. Mean weight was higher for test formula *vs*. breastfed reference at 12 months.
				Stool effects	No difference between all groups in stool frequency. Frequency of liquid/watery stools tended to be lower in test formula group *vs*. breastfed reference.

Table IV.B-3 Recent Studies of GOS Consumption in Infants – Updated Literature Search January 2014 to December 2015					
Reference and Study Design	**Subjects**	**Dose**	**Duration**	**Results**	
		mixed- diet reference)			Stools were softer in more liquid in test formula compared to control group.
				GI effects	Frequency of spitting up and vomiting were not significantly different between all groups. Flatulence occurred slightly more frequently in the test formula group than control or breastfed reference.
Dasopoulou *et al*. (2015) Randomized, double-blind, controlled, single-center	167 infants (preterm; gestational age median 34 weeks; healthy)	***Test Formula:*** Preterm formula with GOS/FOS (9:1; 7.2 g/L GOS) ***Control:*** Standard preterm formula	Between DOL 1 – 16	Adverse event reporting	GOS/FOS formula well-tolerated by all preterms. Incidences of NEC stage I and infections was similar between groups.
				Growth	Mean increase in weight was significantly greater for control *vs*. GOS/FOS group.
				Stool and GI effects	Gastric residue was less frequent in GOS/FOS group on Day 1 and 3, but remained similar to control for Days 4-16. Stool frequency and proportion of neonates with softer stools was similar between groups. Number of vomits and regurgitation episodes not significantly different between groups.
				Gut peptides	Degree of increase in motilin levels was significantly greater in GOS/FOS group. Gastrin levels did not change in either group.
Lee *et al*. (2015) Randomized, double-blind, controlled, parallel-group, single-center, prospective	123 infants (term; gestational age from 37 to 42 weeks; birth weight ≥2,500 and 4,500 g; 58 M, 65 F; healthy)	***Test Formula:*** Formula with GOS/FOS (5.5/0.36 g/L) and *L. reuteri* (10^8 CFU/day) ***Control:*** Formula with *L. reuteri* (10^8 CFU/day)	4 months	Adverse event reporting	Slightly more infants in GOS/FOS group had GI system disorders and skin and appendages disorders but not statistically significant. None of the adverse events reported were related to study formulas.
				Growth	No significant difference in mean weight, length, head circumference, and BMI between groups.
				Stool and GI effects	Liquid stools occurred more frequently in GOS/FOS group. All other parameters for digestive tolerance were not different between groups.
				Stool bacteria	Mean *Bifidobacterium* counts were about half a log higher in GOS/FOS group and made up larger portion of stool bacterial population (83.8 *vs*. 39%).

Table IV.B-3 Recent Studies of GOS Consumption in Infants – Updated Literature Search January 2014 to December 2015					
Reference and Study Design	**Subjects**	**Dose**	**Duration**	**Results**	
Study Conducted with GOS Combined with Polydextrose					
Luoto *et al.* (2014) Randomized, double-blind, controlled, single-center	94 infants (preterm; gestational age ≥32 + 0 and ≤36 + 6 weeks; birth weight >1,500; 43 M, 51 F)	***Test Formula:*** GOS and polydextrose 1:1 (Days 1-30: 1 x 600 mg/day; Days 31-60: 2 x 600 mg/day) ***Control Formula:*** Microcrystalline cellulose and dextrose anhydrate	DOL 3 - 60	Respiratory tract infections (RTI)	Significantly lower incidence of RTIs in prebiotic group. Significantly lower number of rhinovirus-induced episodes in prebiotic group.

DOL = day of life; F = female; FOS = fructo-oligosaccharides; GI = gastrointestinal; GOS = galacto-oligosaccharides; GUM = growing up milk; LCPUFA = long-chain-poly-unsaturated fatty acids; M = male; NEC = necrotizing enterocolitis; RTI = respiratory tract infection

IV.B.4 Safety of Enzyme Source Organism

IV.B.4.1 History of Safe Use

GOS manufactured by Nestlé is synthesized using a *beta*-galactosidase enzyme preparation from *Aspergillus oryzae* GL 470. *beta*-Galactosidase enzyme preparations from *A. oryzae* have a long-history of safe food use globally. For example, in the European Union, *beta*-galactosidase enzyme preparations from *A. oryzae* are currently marketed for use in food processing, and *A. oryzae* is currently listed as an authorized food enzyme in France where it is used to hydrolyze lactose in the production of milk and whey with reduced lactose, as well as cheese and fermented milk products (JORF, 2006; Amfep, 2015). In Japan, *A. oryzae* is listed on the Ministry of Health, Labour and Welfare's (MHLW) list of existing food additives (MHLW, 2014). *beta*-Galactosidase enzyme preparations derived from *A. oryzae* GL 470 have been commercially marketed in Japan since 1977. *A. oryzae* is listed as an approved source of lactase in China's National Standard on Food Safety – Standard for Use of Food Additives GB 2760-2011 (Ministry of Health of the PRC, 2011) and in the Korea Food Additives Code (KFDA, 2013). In Australia and New Zealand, *A. oryzae* is permitted for use in food processing in the manufacture of any food (FSANZ, 2015), and in Canada, lactase from *A. oryzae* var. is a permitted food enzyme in the production of the following food and beverages: lactose-reducing enzyme preparations, milk destined for use in ice cream mix, bread, flour, and whole wheat flour (Health Canada, 2015). In the U.S., GOS (Floraid GOS, International Dairy Ingredients, Inc.) produced from lactose using a *beta*-galactosidase enzyme preparation from *A. oryzae* has previously been determined to be GRAS (GRN 489; U.S. FDA, 2014b). Several carbohydrase enzyme preparations from *A. oryzae* have also previously been determined to be GRAS (GRN 90; U.S. FDA, 2002) and *A. oryzae* is listed on the FDA Partial List of Microorganisms & Microbial-Derived Ingredients Used in Food.

IV.B.4.2 Toxicogenicity and Pathogenicity

Aspergillus oryzae is generally accepted as a non-pathogenic microorganism and the species is not known to produce mycotoxins (U.S. EPA, 1997; Olempska-Beer *et al.*, 2006). Some strains of *A. oryzae*, including those used commercially for enzyme production can produce secondary metabolites that are of low to moderate toxicity in animals including 3-*beta*-nitropropionic acid, kojic acid, and cyclopiazonic acid (Olempska-Beer *et al.*, 2006; FAO, 2007). However, the production of secondary metabolites by *A. oryzae* has been greatly attenuated by most industrial enzyme producers through the use of traditional mutagenesis and other selective culture methods. The synthesis of secondary metabolites by filamentous fungi typically occurs during the stationary phase of growth, and therefore the production of toxic metabolites can be avoided by the selection of safe and suitable strains during development and through proper control of fermentation conditions. For strains of *A. oryzae* with a long-history of food use, and which have not been subjected to genetic manipulations, the use of cGLP is likely sufficient for the assurance of enzyme safety for this species. *A. oryzae* is considered a domesticated

subspecies of *Aspergillus flavus*, which is a known producer of aflatoxins (EFSA, 2007). Despite the fact that *A. oryzae* is widely recognized to have lost the capacity to synthesize aflatoxins, genetic remnants of aflatoxin synthesis genes have been detected in some strains (Blumenthal, 2004). Moreover, the species differentiation of *A. oryzae* from *A. flavus* is not readily achieved by traditional genotyping methods; therefore, new strains of *A. oryzae* used for enzyme development are typically screened for aflatoxins during product development (EFSA, 2007). The production strain *A. oryzae* GL 470 has been analytically determined not to produce any mycotoxins, including aflatoxins. Nestlé has therefore concluded that *A. oryzae* GL 470 is a non-toxicgenic and non-pathogenic production strain and is therefore safe and suitable for use in the production of *beta*-galactosidase enzyme preparations used for synthesis of Nestlé GOS.

IV.B.5 Allergy

IV.B.5.1 beta-Galactosidase

Allergic reactions to aspergilli are not uncommon, and *beta*-amylases from fungi have been implicated as causative allergens in Baker's asthma (U.S. EPA, 1997). Although case reports of allergenicity to *beta*-galactosidases have been reported in the literature (*e.g.*, Binkley, 1996), *beta*-galactosidases have a long and wide-spread history of food use. There is no evidence in the literature that *beta*-galactosidases are associated with severe cross-reactivity to a major allergen. Direct consumption of active *beta*-galactosidase enzyme preparations in dietary supplement preparations, such as Lactaid®, also has a long-history of safe use by lactose intolerant individuals.

As reported by Pariza and Foster (1983), "*Allergies and primary irritations from enzymes used in food processing should be considered a low priority item of concern except in very unusual circumstances.*" *beta*-Galactosidase enzyme preparations have a long-history of food use in the production of lactose free milk products, and are currently marketed in dietary supplement products for consumption by individuals with lactose intolerance. No reports of allergenicity associated with food uses of the enzyme preparation are known, and a search of the literature did not identify case-reports of cross reactivity to major food allergens. Although the enzyme preparation is expected to be of low allergenicity potential, for completeness, the Allergen Online database version 13 (Updated February 12, 2013) was used to conduct a preliminary screen of the *beta*-galactosidase protein for relevant matches against known putative allergens (FARRP, 2015). This database is maintained by the Food Allergy Research and Resource Program of the University of Nebraska. A FASTA3[5] overall search of Allergen Online was conducted using default settings (E cutoff = 1 and maximum alignments of 20). No sequences with E() <1.00 were identified.

An 80 amino acid sliding window (segments 1-80, 2-81, 3-82, *etc.*) was also used to scan the amino acid sequence of *beta*-galactosidase against the allergen database using FASTA3 to

[5] Algorithm used for identification of amino acid alignment matches between a pair of protein sequences.

search for matches of 35% identity or more. This 35% identity for 80 amino acid segments is a suggested guideline proposed by Codex for evaluating proteins in genetically modified crops (Codex Alimentarius Commission, 2003; Goodman *et al*., 2008). No 80 mer alignments against any putative allergen sequences in the database were identified.

IV.B.5.2 Galacto-oligosaccharides

Reports of clinically significant allergic reactions following consumption of beverage products containing GOS have been reported in Southeast Asia (Jyo *et al*., 1996; Hamahata *et al*., 1999; Chiang *et al.*, 2012; Vo *et al.*, 2012). One of the earliest case reports of GOS allergenicity reported involved a group of oyster shuckers in Hiroshima Japan who drank a GOS containing beverage (Jyo *et al*., 1996; Hamahata *et al*., 1999). All of the individuals had immediate-type hypersensitivity reactions following ingestion of the beverage, and active allergenicity to sea-squirt was established for 4 of the individuals. Skin prick and *in vitro* histamine release tests were positive to GOS isolates containing more than 4 saccharide molecules with *beta*-1-3 or 1-6 linkages. In 2012, case reports of GOS allergenicity were reported by clinicians in Vietnam and Singapore in association with the consumption of GOS containing milk formulas (Chiang *et al.*, 2012; Vo *et al.*, 2012). To date, the causative antigen(s) responsible for these cases has not been identified; however, in further testing of 5 individuals with GOS anaphylaxis, positive reactions to skin prick and basophil activation tests were reported against GOS fractions with 3 sugar units or greater (Chiang *et al*., 2012). The primary sensitizer has been speculated to be a parasitic agent specific only to the Southeast Asian region. For example, similar cases reports of sensitization leading to red meat allergy have been observed throughout the central and southern U.S. in individuals exposed to *alpha*-gal (galactose-α-1,3-galactose) glycoprotein antigens introduced to the circulation from Lone Star tick bites (Soh *et al*., 2015a).

Recent case-reports (n=12 individuals) of GOS allergenicity from consumption of a GOS containing lactic acid beverage were reported in Japan and included 4 children aged 1.5 to 14 years (Kaneko *et al*., 2014). Allergic reactions were typically mild; however, 3 patients experienced respiratory reactions requiring hospitalization. All reactions occurred in individuals with allergic predispositions to asthmatic or urticarial episodes. The authors attempted to characterize the oligosaccharides responsible for the anaphylactic responses using the histamine release test (HRT) with heparinized peripheral venous blood from 3 of the patients. Strong HRT responses were reported for tetrasaccharide isolates of GOS, and therefore the putative antigen was identified as a GOS tetramer, which is consistent with previous observations by Chiang *et al*. (2012). The authors also compared the HRT response of 2 GOS preparations, one produced by *B. circulans*, which the authors reported contained a GOS 1-4 oligomer bias, and the other produced *via* synthesis by a combination of lactases from *A. oryzae* and *Streptooccus thermophilus* (GOS 1-6 bias). Strong positive responses to GOS (µg/mL) from *B. circulans* were reported (40 to 70%), while negative (<10%) to pseudo-negative (10 to 15%) histamine release responses were reported for the GOS preparations produced by

A. oryzae and *S. thermophilus* lactases. Further isolation of select GOS molecules identified Galβ1-4Galβ1-4Galβ1-3Glc as the putative allergen based on its presence in HRT positive GOS preparations and its absence from "non-immunogenic" GOS preparations from *S. singularis* and *K. lactis*. Despite these findings, the authors were unable to identify anti-GOS IgE antibodies in the sera of the patients by the enzyme immunoassay method. Therefore, the clinical significance of the author's findings is unclear.

In response to the cases of GOS anaphylaxis reported in Singapore by Chiang *et al.* (2012), a clinical study was conducted in an atopic cohort aged 5 to 60 years (n=487) to determine the prevalence of allergy in this population (Soh *et al*., 2015b). The 30 subjects (6.2%) that were found to be sensitized to GOS (Vivinal) by skin prick test had blood drawn for basophil activation tests, and 13 of these subjects further consented to oral challenge tests with either Vivinal GOS or Oligomate 55N GOS. Six of the 13 subjects challenged orally with Vivinal GOS tested positive, whereas none of the subjects reacted to oral exposure to Oligomate 55N. Based on the findings of this one study, the authors estimated that the prevalence of allergy to Vivinal GOS in this atopic population in Singapore might be up to 3.5%; however, the authors acknowledged that "…*this estimate may not be entirely accurate as subjects were drawn from patients attending a specialized clinic in a tertiary hospital. Furthermore, due to the small number of subjects from which the ROC (Receiver Operating Characteristic) curves were derived, this limits the accuracy of the BAT (Basophil Activation Test) and, thus, the precision of this estimate.*" (Soh *et al*., 2015b). The difference in allergic response to Vivinal *versus* Oligomate 55N GOS was speculated to be related to structural differences due to the use of different β-galactosidase enzymes during their respective manufacturing processes; however, if this really is the case is unknown.

Although carbohydrates have been long known to bind to IgE, carbohydrates on their own, not conjugated to proteins, are poorly immunogenic (Altmann, 2007). Anaphylactic reactions have a basic requirement for cross-linking of IgE receptors on effector cells, a process that requires the presence of large divalent antigens (Altmann, 2007). Despite the recent case reports of GOS allergenicity and apparent induction of histamine release *in vitro* by semi-purified GOS tetramers, the etiology of GOS anaphylaxis remains a mystery as mechanistic evidence has yet to establish that a small molecular weight oligomer (*i.e*., a monovalent antigen) on its own is capable of cross-linking IgE receptors to provoke an anaphylactic response *in vivo*. Although current evidence has implicated GOS ingredients as the causative agents in case-reports of GOS allergenicity, the putative antigen(s) remains unknown. The strong geographical restriction of all cases of GOS allergenicity that have been reported to date implies that the primary sensitizer is confined to select regions of Southeast Asia, and is linked to very specific prior occupational/environmental exposure. To date GOS allergy has not been reported in children <5 years of age. GOS preparations that have been associated with GOS anaphylaxis in these specific regions of Southeast Asia have been widely consumed in other global regions for over a decade by adults, children and infants without reported incidences of anaphylaxis,

suggesting that the risk of GOS allergenicity from the introduction of GOS containing foods generally is low. Similar conclusions have been determined by other qualified experts during previous GRAS determinations (U.S. FDA 2008, 2014c).

IV.C Expert Panel Evaluation

Nestlé has determined that Nestlé GOS, as described herein, is GRAS for use in infant formula and follow-on formula as described in Section I.D, on the basis of scientific procedures. This GRAS determination is based on data generally available in the public domain pertaining to the safety of GOS, and on consensus among a panel of experts (the Expert Panel) who are qualified by scientific training and experience to evaluate the safety of GOS for use in infant and toddler formulas. The Expert Panel consisted of the following qualified scientific experts: Professor Joseph F. Borzelleca, PhD. (Virginia Commonwealth University School of Medicine), Dr. Ronald E. Kleinman, M.D. (Massachusetts General Hospital for Children & Harvard Medical School), and Professor Stephen L. Taylor, Ph.D. (University of Nebraska).

The Expert Panel, convened by Nestlé, independently and critically evaluated all data and information presented herein. Additional data and information on the safety of Nestlé GOS as deemed relevant by the Expert Panel, including information presented within previous GRAS Notified uses of GOS was also critically evaluated by the Panel (*e.g.*, U.S. FDA, 2008, 2009b, 2010, 2014b,c). The Expert Panel concluded that Nestlé GOS is GRAS for use in non-exempt term infant formula (*i.e.*, infants 0 to 12 months of age) and toddler formula, based on scientific procedures. A summary of the data and information reviewed by the Expert Panel, and evaluation of such data as it pertains to the proposed GRAS uses of Nestlé GOS is presented in Annex A.

IV.D Conclusions

Based on the above data and information presented herein, Nestlé has concluded that the intended uses of Nestlé GOS in non-exempt term infant formula and toddler formula, as described in Section I.D, is GRAS based on scientific procedures. General recognition of Nestlé's GRAS determination is supported by the unanimous consensus rendered by an independent Panel of Experts, qualified by experience and scientific training, to evaluate the use of GOS in infant formula and in food, who similarly concluded that the intended use of Nestlé GOS in non-exempt term infant formula and toddler formula as described herein is GRAS.

Nestlé GOS therefore may be marketed for its intended purpose in the U.S. without the promulgation of a food additive regulation under Title 21, Section 170.3 of the Code of Federal Regulations (U.S. FDA, 2015).

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CFR Sections Referenced (Title 21—Food and Drugs)		
Part	**Section §**	**Section Title**
106—Infant formula requirements pertaining to current good manufacturing practice, quality control	106.55	Controls to prevent adulteration from microorganisms
170—Food additives	170.3	Definitions
	170.30	Eligibility for classification as generally recognized as safe (GRAS)
184—Direct food substances affirmed as generally recognized as safe	184.1631	Potassium hydroxide

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Annex A

Expert Panel Consensus Statement

Expert Panel Consensus Statement Concerning the Generally Recognized as Safe (GRAS) Determination of Nestlé Galacto-oligosaccharides (Nestlé GOS) for Use as an Ingredient in Non-Exempt Term Infant Formula and Toddler Formula

November 19, 2015

Nestlé Ltd. (Nestlé) has determined using scientific procedures that Nestlé galacto-oligosaccharides (Nestlé GOS), as described herein, is Generally Recognized as Safe (GRAS) for addition to non-exempt term infant formula and toddler formula at use-levels providing up to 7.8 g GOS/L of the reconstituted or ready-to-drink formula.

At the request of Nestlé, a panel of independent scientists (the "Expert Panel"), qualified by their scientific training and relevant national and international experience to evaluate the safety of food ingredients, was convened on October 19th 2015 to conduct a critical and comprehensive evaluation of Nestlé GOS for use as an ingredient in non-exempt term infant formula and toddler formula. The Expert Panel consisted of Dr. Joseph F. Borzelleca, Ph.D. (Virginia Commonwealth University School of Medicine), Dr. Ronald E. Kleinman, M.D. (Massachusetts General Hospital for Children & Harvard Medical School), and Dr. Stephen L. Taylor, Ph.D. (University of Nebraska).

A comprehensive dossier (Documentation Supporting the Determination that Nestlé GOS are GRAS for Use as an Ingredient in Term Infant Formula and Toddler Formula, October 7, 2015) summarizing data and safety information related to the use of Nestlé GOS in infant formula was prepared by Nestlé and provided to the Expert Panel for their independent and collective critical evaluation. The dossier contained details regarding the method of manufacture of the ingredient, product specifications, supporting analytical data, intended use-levels, estimated exposures to Nestlé GOS under the proposed conditions of use, and a comprehensive assessment of the available scientific literature pertaining to the safety of GOS. The Expert Panel convened *via* teleconference on October 19, 2015, and following independent critical deliberation of all data available to the Panel, unanimously concluded that Nestlé GOS meeting appropriate food-grade specifications and manufactured consistent with current Good Manufacturing Practice (cGMP), is GRAS on the basis of scientific procedures for use in non-exempt infant formula and toddler formula at use-levels providing up to 7.8 g GOS/L of the reconstituted or ready-to-drink formula. A summary of the basis for the Expert Panel's conclusion is provided below.

SUMMARY AND BASIS FOR GRAS DETERMINATION

The ingredient that is the subject of this GRAS determination is Nestlé GOS. Although there is no globally-adopted definition for GOS, Food Standards Australia New Zealand (FSANZ) has defined GOS as follows: "*… the term 'galacto-oligosaccharides' (sometimes referred to as oligogalactosyl-lactose) is used consistently to describe those substances comprised of between two and eight saccharide units with one of these units being a terminal glucose and the remaining saccharide units being galactose*." (FSANZ, 2008). The European Commission Scientific Committee on Food (SCF) has described GOS in a similar manner: "*Oligogalactose is produced from lactose with the help of a bacterial β-galactosidase, it contains one molecule of glucose and typically between 1 and 7 molecules of galactose.*" (SCF, 2001a,b). GOS are commonly added to infant formula preparations as a representative source of non-digestible oligosaccharides similar to those that are present in high concentrations in human milk (Oozeer *et al*., 2013).

A number of GOS preparations have been determined to be GRAS and have been the subject of pre-market notifications to the Unites States (U.S.) Food and Drug Administration (FDA) (*i.e*., GRN 236, 285, 286, 334, 484, 489, 495, 518) (U.S. FDA, 2008, 2009a,b, 2010, 2014a-d). All GOS preparations used as ingredients in infant formula are produced in a similar manner utilizing *beta*-galactosidase enzyme preparations derived from safe and suitable sources, to convert lactose into GOS mixtures containing low-molecular weight neutral oligosaccharides of varying chain length and minimal branching. Based on the chemical similarity and physiological equivalence of all GOS preparations produced by this method, published studies supporting the safety of GOS for use in infant formula, including animal toxicity studies and published safety and suitability studies in infants, have been considered broadly applicable to the general category of GOS and are therefore relevant to Nestlé GOS.

Chemistry and Manufacturing

Nestlé GOS[1] is manufactured from partially demineralized sweet whey permeate (source of lactose) using a *beta*-galactosidase enzyme preparation derived from a non-pathogenic, non-toxicogenic strain of *Aspergillus oryzae.* The ingredient is manufactured as a powder with a total GOS content of ≥46% on a dry weight basis, combined with unreacted carbohydrates lactose (20 to 40%), glucose (max 10%), and galactose (max 5%). The enzymatic reaction produces an oligomer distribution that is characterized by a slight bias towards oligomers with *beta*-1,6, *beta*-1,3 and *beta*-1,4 linkages. Based on the raw materials, production methods, and available compositional analyses, the Expert Panel agrees that GOS synthesized by the Nestlé

[1] Nestlé GOS is used as a product specific identifier (*i.e*., GOS as manufactured by contract manufacture for Nestlé) and for differentiation between various generic GOS preparations that have been introduced to market. The trade-name Bovine Milk Oligosaccharides (BMOS) is an earlier trade name and pseudonym for Nestlé GOS and can been used interchangeably with the name Nestlé GOS.

manufacturing process produces a product that is consistent with the available global definitions of GOS discussed above and is chemically representative of other GRAS sources of GOS.

Nestlé GOS is manufactured using partially demineralized sweet whey permeate as a food grade source of lactose. Specifically, the whey permeate is concentrated by evaporation to 50% total dry matter and then incubated with a *beta*-galactosidase enzyme derived from *A. oryzae* to catalyze the hydrolysis of lactose into glucose and galactose. This is followed by the polymerization of galactose to generate a characteristic mixture of GOS with a degree of polymerization typically ranging from 2 to 5. Upon completion of hydrolysis and oligomerization, the enzyme is denatured and inactivated by heat treatment. The solution is subjected to a membrane nano-filtration step to reduce the mineral content, reduce residual lactose, and partially remove the free glucose and galactose generated during hydrolysis, thereby improving the efficiency of the spray drying step. The filtered GOS preparation is heat-treated to ensure microbial stability, further concentrated by evaporation, and finally spray-dried to produce a powdered ingredient. All raw materials, processing-aids, and additives used in the manufacturing process are food grade ingredients[2] permitted by U.S. regulation, or have been previously determined to be GRAS for their respective uses. The Expert Panel reviewed the Nestlé GOS manufacturing procedures and determined that these processes were generally consistent with those described for other GOS preparations that have been previously determined to be GRAS (*i.e.*, GRN 236, 285, 286, 334, 484, 489, 495, 518).

Nestlé has established food grade chemical and microbial specifications for its GOS ingredient, and analysis of 4 non-consecutive lots of GOS demonstrated compliance with the defined chemical specifications (see Attachment A for the specifications of Nestlé GOS). The oligosaccharide profile of Nestlé GOS is consistent with other GOS oligomers (*e.g.*, Vivinal GOS) that have been determined to be GRAS. Nestlé's GOS also contains small amounts of the sialyllactose oligosaccharides 3'-sialyllactose and 6'-sialyllactose. These oligosaccharides are not synthesized during the production process, but originate as carry-over products from the cow's milk whey permeate used as a source of lactose for GOS synthesis. In addition to their natural presence in cow's milk, 3'-sialyllactose and 6'-sialyllactose also are present within human milk. Analyses of milk samples from lactating women have reported concentrations of 170 to 600 mg/L for 3'-sialyllactose and concentrations of 120 to 590 mg/L have been reported for 6'-sialyllactose depending on the period of lactation (Wang and Brand-Miller, 2003). Infant formula preparations to which Nestlé GOS has been added at a use-level of 7.8 g/L would therefore contain quantities of 3'-sialyllactose in the range of 44 to 50 mg/L, and concentrations of 6'-sialyllactose at levels of approximately 12 to 13 mg/L. The Expert Panel noted that these concentrations are an order of magnitude below levels that are naturally occurring in human

[2] Compliant with the specifications set forth in the Food Chemicals or equivalent international food or pharmacopeia standard (*e.g.*, JECFA, CODEX, EP).

milk and therefore can be considered generally recognized as safe and suitable for addition to infant formula at these inclusion levels.

As demineralized whey permeate is used as the starting material for the manufacture of Nestlé GOS, residual quantities of minerals are present in the ingredient. Mineral content is defined within the product specifications so that inclusion rates of minerals into the finished formula can be adjusted as necessary to meet the regulatory requirements of section 350a of the U.S. Federal Food, Drug, and Cosmetic Act (U.S. FDA, 2013). The average nutrient (mineral) contribution of Nestlé GOS into the finished infant formula is presented in Table 1. Lastly, stability testing included monitoring of total moisture content, powder wettability, water content, as well as lactose, glucose, galactose, and total oligosaccharide content. The data demonstrate that Nestlé's GOS ingredient is stable for at least 1 year when stored at 25°C or 30°C (70% relative humidity). In-life storage tests up to 24 months are on-going and it is anticipated that the shelf-life of the ingredient will be 2 years.

Table 1 Nutrient (Mineral) Contribution of Nestlé GOS to Infant Formula

Nutrient	Nutrient Value Provided by a Use-Level of 7.8 g/L Nestlé GOS (per 100 kcal)[a]	Nutrient Requirements for Infant Formula under §350a of the FFDCA (per 100 kcal)
Sodium (mg)	0.4	20.0 – 60.0
Potassium (mg)	19.6	80.0 – 200.0
Chloride (mg)	≤0.07	55.0 – 150.0
Calcium (mg)	≤0.7	50.0
Phosphorous (mg)	3.5	25.0
Magnesium (mg)	0.5	6.0
Manganese (µg)	≤0.0001	5.0
Iron (mg)	≤0.001	0.15
Copper (µg)	0.001	60.0

FFDCA = Federal Food, Drug, and Cosmetic Act; GOS = galacto-oligosaccharides
[a] Values calculated based upon the average of the results listed in Attachment A and the assumption that 167 mL of infant formula provides approximately 100 kcal.

Intended Food Uses and Estimated Intake

Nestlé GOS is intended for use as a food ingredient in non-exempt term infant formula (0 to 12 months) and toddler formula at concentrations up to 7.8 grams of GOS per liter (0.78%) in the reconstituted or ready-to-drink formula. The ingredient would serve as an alternative source of GOS to existing GOS preparations that have previously been determined to be GRAS for use in infant formula in the U.S. (*e.g.*, GRN 236, 286, 334, 489, 495). The dietary exposure to GOS among infant consumers under the proposed food uses as an ingredient in term infant formula (0 to 12 months) and toddler formula has been previously estimated from dietary survey data, as described in GRN 236, 286, and 334 (U.S. FDA, 2008, 2009b, 2010). Estimates of the

dietary intake of Nestlé GOS from the intended use in non-exempt term infant formula were therefore incorporated by reference to previous GRAS determinations. As described in GRN 286, food codes representative of each proposed food use (*i.e.* infant formula and follow-on formula) were selected from the National Center for Health Statistics' 2003-2004 National Health and Nutrition Examination Survey (NHANES) (CDC, 2006; USDA, 2009) to estimate the intake of GOS. Based on a 100% market share assumption along with the assumption that GOS would be included in all infant formulas sold in the U.S., it was determined that approximately 80% of the infant population in the U.S. would be GOS consumers (202 actual users ages 0 to 6 months, 138 users aged 7 to 12 months). Toddlers were considered separately, aged 1 to 2 years, and were found to represent only 3.7% of users (19 actual users). The summary of the estimated dietary intake of GOS from infant formula and follow-on formula in the U.S. by infants and toddlers, as described in GRN 286, is provided in Table 2 below (U.S. FDA, 2009b).

Table 2 Estimated Daily Intake of GOS from Current and Proposed Uses in Infant and Follow-on Formula in the U.S. by Population Group (2003-2004 NHANES Data)[1]

Population Group	Age Group (Years)	% Users	Actual # of Total Users	All-Person Consumption		All-Users Consumption	
				Mean (g)	90th Percentile (g)	Mean (g)	90th Percentile (g)
Estimated daily consumption of GOS from current uses at 7.2 g/L (GRN 286, U.S. FDA, 2009b)							
Infants	0 to 6 months	80.8	202	4.8	8.5	5.9	8.5
Infants	7 to 12 months	81.2	138	4.5	7.6	5.2	7.9
Toddlers	1 to 2	3.7	19	*0.1	N/A	*2.8	*6.6
Estimated daily consumption of GOS from proposed uses at 7.8 g/L							
Infants	0 to 6 months	80.8	202	5.2	9.2	6.4	9.2
Infants	7 to 12 months	81.2	138	4.9	8.2	5.6	8.6
Toddlers	1 to 2	3.7	19	*0.1	N/A	*3.0	*7.1

N/A = Not available, due to small number of users in this age group.
*Due to the small sample size data may be statistically unreliable.
[1] Table adapted from GRN 286 (U.S. FDA, 2009b).

Under the conditions of intended use of Nestlé GOS in term infant and toddler formula at a concentration of 7.8 g/L, dietary intakes presented above may increase by up to 8.3% (Table 2). This intended use-level of Nestlé GOS at 7.8 g/L is representative of concentrations that have been evaluated for safety and suitability in Nestlé's product specific infant trials (*e.g.*, Cooper*,* 2014; Hascoet, 2015; Simeoni *et al.*, 2015), and are within the 8 g/L limit permitted for addition to infant formula in other countries such as Australia/New Zealand and China (FSANZ, 2008; Ministry of Health of the PRC, 2011). The introduction of Nestlé GOS to the U.S. marketplace therefore would not change overall dietary intakes in infants relative to levels that have an established history of safe in other countries. In addition, the Expert Panel also noted

that the revised use-level of 7.8 g/L is well within background exposures to resistant oligosaccharides in infants consuming human milk, where levels of human milk oligosaccharides of 25 and 12 g/L have been reported in human colostrum and mature milk samples, respectively, obtained from lactating women (Kunz *et al*., 1999, 2000).

Nestlé GOS, like all GOS preparations, also contains lactose, glucose, and galactose as carry-over products of the hydrolysis process. The Expert Panel considered the dietary exposure of these constituents from the intended uses. All these constituents are common nutrients present at significant concentrations in the normal diets of term infants and toddlers and the concentrations of these sugars are comparable to levels that have been reported for other GRAS sources of GOS currently used within infant formula. The Expert Panel noted that GOS ingredients in general are not appropriate for use by infants with galactosemia, and identification of galactose within the product specification should be a requirement for all GOS preparations.

Information to Establish Safety

The first GRAS determination notified to the FDA for a GOS preparation was submitted by Friesland Foods Domo in 2007 for their ingredient named Vivinal GOS (GRN 236, U.S. FDA, 2008). A critical and comprehensive review of the publically available data and information pertaining to the safety of GOS for use as an ingredient in infant formula was presented in this dossier. The information presented by Friesland has served as the basis for a number of GRAS determinations for the use of various GOS preparations in infant formula (U.S. FDA, 2009b, 2010, 2014b,c) as well as within food and beverage products across multiple categories (U.S. FDA, 2009a,b, 2010, 2014a-d). All of these GOS preparations are produced from lactose using fungal or bacterial derived *beta*-galactosidase, and based on the chemical similarity and physiological equivalence of these GOS preparations; General Recognition of Safety of GOS has been supported using an equivalence approach. In this regard, publically available data and information supporting the GRAS use of GOS as an ingredient in term infant formula have been critically reviewed by a number of qualified scientific experts including the FDA. International regulatory bodies, including the European Commission SCF and FSANZ, have issued opinions supporting the safe use of GOS as an ingredient in infant formula (SCF, 2003; FSANZ, 2008).

Based on the chemical and compositional similarity of Nestlé GOS to other GRAS GOS preparations (*e.g*., Vivinal GOS), publically available data and information establishing General Recognition of Safety of GOS preparations, including toxicological studies in animals and safety and tolerance reports in healthy adults and infants, are therefore incorporated by reference to previous GRAS determinations (U.S. FDA, 2008, 2009a,b, 2010, 2014a-d). An updated search of the publically available scientific literature was conducted to identify new information relevant to the safety of GOS, and the following databases were queried for articles

published in 2014 and 2015: Medline, ToxFile, AGRICOLA, AGRIS, BIOSIS Toxline, FOODLINE: Science, CAB Abstracts, BIOSIS Previews, FSTA (Food Science and Technology Abstracts), NTIS (National Technical Information Service), EMBASE, and Adis Clinical Trial Insight. A summary of the historical basis for General Recognition of Safety and newly identified studies relevant to GOS safety is provided below.

Metabolic Fate and Toxicity

The metabolism of GOS has been previously described in detail and the related physiological effects of GOS consumption on gastrointestinal physiology have been well characterized (U.S. FDA, 2008, 2009a, 2010). Briefly, with the exception of lactose that is hydrolyzed by small intestinal brush border lactases, *beta*-linked sugars are not digested by human pancreatic or intestinal enzymes. GOS are therefore not absorbed and are transported intact to the large intestine where they are subjected to fermentation by the indigenous microbiota. Although *in vitro* studies have reported slight differences in the efficiency by which particular bacterial species metabolize GOS (Ishikawa *et al.*, 1995; German *et al.*, 2008), they are ultimately hydrolyzed to glucose and galactose. These monosaccharides are subsequently metabolized by the anaerobic microbiota by the Embden-Meyerhof-Parnas pathway resulting in the production of short chain fatty acids, CO_2 and H_2 gas (common and innocuous metabolites) (Miller and Wolin, 1996; Suarez *et al.*, 1999; Smiricky-Tjardes *et al.*, 2003). These products of microbial fermentation, short-chain fatty acids in particular, result in the reduced pH and osmotic effects within the colon that are characteristic of GOS consumption. Since all GOS oligomers are comprised exclusively of the same basic monomeric units of galactose and glucose, they are ultimately expected to be physiologically equivalent in their metabolic profiles.

Toxicological Studies

GOS preparations have consistently demonstrated to be without evidence of toxicity in rodent toxicity studies. No-observed-adverse-effect levels (NOAELs) of 2,000 (Oligomate GOS) and 2,250 (Vivinal GOS) mg/kg body weight were reported for mature Sprague-Dawley rats administered GOS preparations by gavage (Anthony *et al*., 2006; Kobayashi *et al.*, 2009, 2014a). A NOAEL of 6,900 mg/kg body weight was reported for dietary administration of Vivinal GOS in mature Wistar rats (GRN 236, U.S. FDA, 2008). In all studies, the NOAELs were the highest doses tested. GOS related effects reported in these studies (transient diarrhea, increased cecal weights) are well established physiological effects that are consistent with the transport of resistant sugars/carbohydrates to the colon and are widely recognized as not being toxicologically relevant to humans (WHO, 1987).

The subacute toxicity of Nestlé GOS in Wistar rats was reported by Penard (2015). The final study report, including all raw data, was made available for review by the Expert Panel. In brief, male and female rats (10/sex/group) were administered Nestlé GOS by gavage for 30 consecutive days at doses of 0, 500, 1,000, or 2,000 mg/kg/day. Half of the control and

high-dose groups were followed for a 2-week recovery period to evaluate the regression of any toxic signs. There were no deaths, no relevant clinical signs, and no test item-related ophthalmological findings reported during the study. There was no variation in body weight or food consumption between groups. At study termination, there were no relevant changes reported in hematology, coagulation, serum clinical chemistry or urine parameters between groups. GOS consumption did not cause any significant organ weight, macroscopic, or histopathological changes. Under the defined experimental conditions, the oral administration of Nestlé GOS for 30 days in the Wistar rat at doses of 500, 1,000, and 2,000 mg/kg/day was well-tolerated clinically and histologically and did not induce any treatment-related effects. The NOAEL was 2,000 mg/kg/day, the highest dose tested. The Expert Panel agreed that this NOAEL determination was appropriate and that findings from this study are consistent with publically available information characterizing the toxicity of GOS (*i.e.*, Anthony *et al.*, 2006; U.S. FDA, 2008; Kobayashi *et al.*, 2009; Desbuards *et al.*, 2012; Kobayashi *et al.*, 2014a,b). The Expert Panel therefore considered findings from this study to corroborate available published studies characterizing the toxicity of GOS.

The developmental and reproductive effects of GOS were recently evaluated by Kobayashi *et al.* (2014b) in male and female parental rats, pregnant females, and their offspring. Male and female Sprague-Dawley rats (24 per sex per group) were administered Oligomate GOS by gavage at doses of 0, 500, 1,000, or 2,000 mg/kg/day as follows: males were dosed 10 weeks prior to mating and 3 weeks thereafter; females were dosed 2 weeks before mating and GOS administration continued through pregnancy to Day 20 of lactation. GOS consumption did not produce any toxicological effects on male or female parental animals and did not adversely affect reproduction/development from premating, copulation, implantation, or maintenance of pregnancy. The offspring were unaffected by the maternal consumption of GOS. No effects were reported on the number of live births, sex ratio, external observation at the time of birth, body weight, pup survival, or external differentiation during lactation. The Expert Panel considered this study to be supportive of GOS safety for use as an infant formula ingredient, but did not appreciably add to the overall body of data considered within the safety assessment.

It is generally recognized that GOS are inert materials with low potential for genotoxicity. The molecular constituents of GOS, glucose and galactose, are commonly consumed as part of the human diet and are therefore without concern for genotoxicity. The genotoxic potential of GOS (*e.g.,* Oligomate GOS) has been evaluated in several studies including the bacterial reverse mutation assay, mammalian chromosomal aberration test, and *in vivo* micronucleus assay in mice. The outcome of all investigations has been uniformly negative (Kobayashi *et al.*, 2009; Narumi *et al.,* 2014). The mutagenic activity of Nestlé GOS was evaluated in the *S.* Typhimurium (TA1535, TA1537, TA98, and TA100) and *Escherichia coli* (WP_2uvrA) reverse mutation assays in the presence and absence of a metabolizing system (rat liver s9-mix induced by Aroclor 1254). Nestlé GOS was tested at concentrations up to the limit dose of

5,000 µg/ plate in triplicate and no significant increase in the number of revertant colonies were reported (Verspeek-Rip, 2015).

Nestlé GOS was also tested for clastogenic and aneugenic potential in an *in vitro* micronucleus assay using cultured peripheral human lymphocytes (Verbaan, 2015). Lymphocytes were prepared by collecting whole blood samples from healthy male subjects into heparin-coated tubes and the blood cells were then cultured in the presence of mitogen phytohaemagglutinin to generate stimulated lymphocytes. The stimulated lymphocytes were incubated with the test substance, Nestlé GOS, in 2 independent experiments. In the first test, stimulated lymphocytes were exposed to 512, 1,600, or 5,000 µg/mL GOS in the culture medium for 3 hours, both in the presence and absence of a metabolizing system (phenobarbital and β-naphthoflavone-induced rat liver S9-mix). Following exposure to GOS, the lymphocytes were cultured for 27 hours to allow chromosome or spindle damage to induce micronuclei formation in interphase cells. No significant increase was reported in the GOS-treated cells with respect to the number of mono- and bi-nucleated cells with micronuclei. In the second assay, stimulated lymphocytes were exposed to GOS at the same concentrations but for a longer time period, 24 hours, only in the absence of metabolic activation. Following exposure to GOS, the lymphocytes were cultured for 24 hours and as was reported in the first test, Nestlé GOS was not found to significantly increase the number of mono- and bi-nucleated cells with micronuclei. The results from these micronucleus assays indicate that Nestlé GOS is not clastogenic or aneugenic in human lymphocytes.

The Expert Panel noted that findings from the above *in vitro* studies evaluating the genotoxicity of Nestlé GOS are corroborative of published data demonstrating that GOS preparations, synthesized from lactose by enzymatic synthesis, do not represent a genotoxic hazard.

Studies in Humans/Infants

Studies evaluating the safety of GOS consumption in adults and infants have assessed a number of biological endpoints, including the effects of GOS on gastrointestinal physiology, fecal microbiota, the immune system, and tolerance (U.S. FDA, 2008, 2009a,b, 2010, 2014a-d). Findings from these studies have consistently demonstrated that GOS is safe to consume and well-tolerated in adults and infants. Due to the metabolic profile of GOS and the fact that GOS are transported intact to the large intestine, excess consumption of GOS can produce transient gastrointestinal effects including osmotic diarrhea (Torres *et al.*, 2010). The laxative threshold for GOS has been reported to be approximately 0.3 g/kg body weight/day in adult subjects (Sako *et al.,* 1999; Kimura *et al.,* 2004). The Expert Panel noted that studies establishing tolerance levels of GOS in adult subjects on the basis of laxative thresholds were not applicable to infants where higher dietary intakes of GOS on a g/kg body weight basis (*i.e.*, ≥ 1.5 g/kg body weight) are not only well-tolerated but represent a desirable levels that have the effect of

simulating the osmotic effects of human milk oligosaccharides that are present within human milk. An upper tolerance limit for GOS in infants has not been established.

The Expert Panel noted that an extensive body of peer-reviewed scientific literature characterizing the safety and suitability of various GOS preparations for consumption by infants at use-levels of up to 10 g/L has been published. These studies have been the subject of previous systematic and comprehensive reviews by various qualified Experts and authoritative bodies and are therefore incorporated by reference to previous GRAS determinations and regulatory opinions (FSANZ, 2008; U.S. FDA, 2008, 2009a,b, 2010, 2014a-d). Studies evaluating the addition of GOS to infant formula, alone, or in combination with other resistant carbohydrates [*e.g*., fructo-oligosaccharides (FOS)] or synergistic 'probiotic' ingredients have repeatedly demonstrated that the addition of GOS to infant formula across a range of use-levels from 2 to 10 g/L is safe (*e.g*., FSANZ, 2008; U.S. FDA, 2008, 2009a,b, 2010, 2014a-d). These studies also have consistently demonstrated that the addition of GOS to infant formula is a suitable alternative to human milk oligosaccharides consumed by breast fed infants and results in number of desirable physiological effects in the infant including elevated levels of *Bifidobacterium* in the gastrointestinal tract, higher short-chain fatty acid concentrations in the stool leading to decreased pH, and improved stool consistency approaching that of stools from breast-fed infants (*e.g*., Veereman-Wauters, 2005; Roberfroid *et al.*, 2010; Oozeer *et al.,* 2013).

Based on the similar metabolic fate of GOS and FOS, and the totality of evidence characterizing the physiological effects of GOS and FOS consumption in a large body of infant trials, the Expert Panel concluded that studies evaluating the co-administration of GOS and FOS in a 9:1 ratio at use-levels of up to 8.0 g/L would be applicable to the use of GOS alone at a level providing 7.8 g/L, provided that the total oligosaccharides content (GOS + FOS) did not exceed 8 g/L. This conclusion is consistent with those determined by other qualified Experts (FSANZ, 2008[3]). The Expert Panel also noted that this conclusion is further corroborated by available published and unpublished product specific studies evaluating the administration of Nestlé GOS in infants at a use-level of between 8 and 10 g/L. These studies are discussed further below.

Meli *et al*. (2014) evaluated the effects of consuming infant formula containing Nestlé GOS in a double-blind parallel group placebo controlled study in 281 infants conducted in Italy. Infants (≤14 days old; 2,500 to 4,500 g birth weight; gestational age ≥37 weeks) were randomized to 1 of 3 groups and consumed one of three infant formula preparations for 4 months: 1) control infant formula (n=84); 2) infant formula supplemented with Nestlé GOS (10 g/L) (n=99); or 3) infant formula supplemented with Nestlé GOS (10 g/L) + probiotics (n=98). The primary

[3] "*These soluble oligosaccharides [GOS and FOS], like naturally occurring HMOs [human milk oligosaccharides], are not digested to any great extent in the small intestine, and reach the large intestine intact where they are also fermented by colonic bacteria to SCFAs and carbon dioxide*." And "…*based on the available evidence, FSANZ concludes that infant and follow-on formula containing up to 8 g/L of inulin derived substances and/or GOS, singularly or combined, in any ratio, are unlikely to pose a risk to infants.*" (FSANZ, 2008).

outcome was mean weight gain per day between 14 days and 4 months (112 days) of age. The study also included general measures of tolerance, clinical chemistry and hematology safety analyses, as well as observation of fecal characteristics. No differences in growth, adverse events, or clinical safety indices were reported between groups. Infants consuming infant formula supplemented with Nestlé GOS (prebiotic), with and without probiotics, had more frequent and less hard stools compared to the control group. The authors reported a statistically significant increase in investigator diagnosed colic in the prebiotic group relative to control group (OR = 0.38; 95% CI 0.18, 0.81; p=0.01). These findings were recorded despite the fact that no statistically significant differences in caregivers' records of flatulence, spitting up, vomiting, duration of crying, fussing, episodes of colic, or illness were reported. An explanation for this discrepancy was not discussed, and the authors suggested that the observed increase in colic in the prebiotic group may be related to the higher GOS use-level of 10 g/L, which is significantly higher than the usual concentrations of GOS used in infant studies that typically range from 4 to 8 g/L. The Expert Panel considered the authors conclusions on tolerance (*i.e.*, colic) to be complicated by limitations in the study design[4] and placed greater emphasis on the care-giver records over investigator reported outcomes. The Expert Panel noted that the study was adequately powered to evaluate safety measures of growth in accordance with the American Academy of Pediatrics requirements where a non-inferiority margin of -3 g/day is recommended (AAP, 1988).

The Expert Panel reviewed full study reports for three additional product specific studies evaluating the safety and suitability of Nestlé GOS in infant formula a use-levels (~ 8 g/L[5]) that were consistent with the proposed use-level of Nestlé GOS in term infant formula (*i.e.*, 7.8 g/L). The first study was conducted in 421 infants (≤3 days old; 2,500 to ≤4,500 g birth weight; gestational age ≥37 to ≤ 2 weeks) delivered by normal birth or caesarean section from HIV+ mothers in South Africa (Cooper*,* 2014). Infants received either the control formula (n=214) or the test formula supplemented with Nestlé GOS (8 g/L) and probiotic (n=207) for a duration of 6 months and then were transferred to a standard follow-on formula for a 6-month observation period. In the second study conducted in the Netherlands, Germany and France, 413 infants (≤13 days old; 2,500 to ≤4,500 g birth weight; gestational age ≥37 to ≤42 weeks) born by normal birth or caesarean section were randomized to 1 of 2 groups: 1) control infant formula (n=206); or 2) Nestlé GOS (8 g/L) + probiotic (n=207) (Hascoet, 2015). Formula was provided for 6 months and all subjects were followed for an additional 6 months. The last study, conducted in France and Poland, enrolled 115 healthy term infants (≤14 days old; 2,500 to

[4] Caregiver-reported colic was not significantly different between infants administered infant formula with GOS *versus* without GOS; however, caregivers were asked to record episodes of colic (defined as bouts of intense, inconsolable crying with painful facial expressions and pulling up of the legs) for only 3 days prior to each visit at 14 days and at ages 1, 2, 3, 4, 6 and 12 months. The 3-day collection period may not have been sufficient to identify episodes of colic. The investigator-reported definition of colic required the occurrence of colic for 3 or more hours per day and for at least 3 days per week for at least 1 week. It is not clear how this information could have been collected by the investigator, given that it was not collected as such in the caregiver diaries.

[5] Use levels of the test articles were intended to deliver 8 g GOS/L infant formula; however, analyses of the test formulas resulted in concentrations of 7.8 to 7.9 g/L.

≤4,500 g birth weight; gestational age ≥37 to ≤42 weeks) in a 12-week study comparing the consumption of starter formula supplemented with Nestlé GOS (8 g/L) and probiotic (n=39) to a control group administered a starter formula (n=37) (Simeoni *et al.,* 2015). The test formulas containing Nestlé GOS with probiotics were well-tolerated in all 3 studies. No adverse effects on growth or other safety related endpoints were reported in the Nestlé GOS groups relative to controls. Measures of fecal characteristics (*i.e*., fecal pH, consistency, frequency, and fecal microbiota, and stool immune markers) were consistently found to approach those of breast-fed infants rather than the controls and therefore provide corroborating evidence on the suitability of Nestlé GOS, at a use-level of 7.8 g/L, as source of resistant oligosaccharides in infant formula in a manner that is consistent with current efforts to produce infant formula preparations that are representative of the composition of human milk (Hascoet, 2015; Simeoni *et al.,* 2015).

Overall, the Expert Panel concluded that the available published (Meli *et al*., 2014) and unpublished studies (Cooper*,* 2014; Hascoet, 2015; Simeoni *et al.,* 2015) evaluating the safety and tolerance of Nestlé GOS in term infants demonstrate that Nestlé GOS is representative of other GOS preparations that have been previously determined to be GRAS (*e.g*., GRN 236, 286, 334, 489, 495), and therefore provide corroborating evidence of safety under the conditions of intended use in non-exempt infant formula at a use-level of 7.8 g/L.

An updated survey of the published scientific literature (2014 and 2015) conducted since GRN 518 in 2014 identified 2 new studies evaluating GOS consumption in adults (Ladirat *et al*., 2014; Schmidt *et al.,* 2015) and 10 studies in infants and children (Armanian *et al.*, 2014; Chatchatee *et al.,* 2014; Giovannini *et al*., 2014; Luoto *et al.,* 2014; Meli *et al*., 2014; Underwood *et al*., 2014; Williams *et al.*, 2014; da Costa Ribeiro *et al*., 2015; Dasopoulou *et al*., 2015; Lee *et al*., 2015). GOS consumption was reported to be safe and well-tolerated in all of these studies, and continue to support the long-history of safe use of GOS in infant formula.

Safety of the Enzyme Source Organism

GOS manufactured by Nestlé is synthesized using a *beta*-galactosidase enzyme preparation from *Aspergillus oryzae* GL 470. *beta*-Galactosidase enzyme preparations from *A. oryzae* have a long-history of safe food use for the processing of milk products in the U.S. and globally. Several *beta*-galactosidase enzyme preparations from non-pathogenic and non-toxicogenic bacteria and fungi, including preparations sourced from *A. oryzae*, have been previously determined to be GRAS for use in the manufacture of GOS (*e.g*., GRN 489; U.S. FDA, 2014b). Carbohydrase enzyme preparations from *A. oryzae* have also been previously determined to be GRAS (GRN 90; U.S. FDA, 2002) and enzyme preparations from *A. oryzae* are listed on the FDA Partial List of Microorganisms & Microbial-Derived Ingredients Used in Food. *A. oryzae* is generally accepted as a non-pathogenic microorganism and the species is not known to produce mycotoxins (U.S. EPA, 1997; Olempska-Beer *et al.*, 2006). The Expert Panel

reviewed analytical data demonstrating that the production strain, *A. oryzae* GL 470, does not produce mycotoxins. The Expert Panel considered enzyme preparations used for food applications to be of low toxic potential (Pariza and Foster 1983; Pariza and Johnson, 2001), and following evaluation of the enzyme preparation using the Pariza-Johnson decision tree, concluded that it was safe and acceptable for use in food (see Attachment B).

Allergy

beta-Galactosidase enzyme preparations have a long-history of use in the production of lactose free milk products, and are currently marketed in dietary supplement products for consumption by individuals with lactose intolerance. Although case reports of allergenicity to *beta*-galactosidases found in dietary supplements such as Lactaid® have been reported in the literature (Binkley, 1996), no reports of allergenicity associated with food uses of *beta*-galactosidase are known, and a search of the literature did not identify case-reports of cross reactivity to major food allergens. The Allergen Online database version 13 (Updated February 12, 2013) was used to conduct a preliminary screening of the *beta*-galactosidase protein for relevant matches against known putative allergens (FARRP, 2013). No matches were identified.

Some case reports of clinically significant allergic reactions following consumption of beverage products containing GOS have been reported in Southeast Asia (Jyo *et al*., 1996; Hamahata *et al*., 1999; Chiang *et al.*, 2012; Vo *et al.*, 2012; Kaneko *et al*., 2014). The causative antigen(s) responsible for these cases has not been identified; however, in further testing of 5 individuals with GOS anaphylaxis, positive reactions to skin prick and basophil activation tests were observed against GOS fractions with 3 sugar units or greater (Chiang *et al*., 2012). The primary sensitizer has been speculated to be a parasitic agent specific to the Southeast Asian region. Similar case reports of sensitization leading to red meat allergy, for example, have been reported in individuals located in the central and southern U.S. exposed to *alpha*-gal (galactose-α-1,3-galactose) glycoprotein antigens introduced to the circulation from Lone Star tick bites (Soh *et al*., 2015a).

Case-reports of GOS allergenicity from consumption of a GOS containing lactic acid beverage were recently reported in 12 individuals from Japan and included 4 children aged 1.5 to 14 years (Kaneko *et al*., 2014). Allergic reactions were typically mild; however, 3 patients experienced respiratory reactions requiring hospitalization. All reactions occurred in individuals with allergic predispositions to asthmatic or urticarial episodes. The authors attempted to characterize the oligosaccharides responsible for the anaphylactic responses *in vitro* using the histamine release test (HRT) with heparinized peripheral venous blood from 3 of the patients. Strong HRT responses were observed for tetrasaccharide isolates of GOS, and therefore the putative antigen was identified as a GOS tetramer, which is consistent with previous observations by Chiang *et al*. (2012). Strong HRT responses to GOS containing a 1-4 oligomer

bias (produced by *Bacillus circulans*) were reported, whereas negative to pseudo-negative responses were found to GOS containing a 1-6 oligomer bias (produced by a combination of *A. oryzae* and *Streptococcus thermophilus*). Despite these findings, the authors were unable to identify anti-GOS IgE antibodies in the sera of the patients and therefore, the clinical significance of the findings remains unclear.

In response to the cases of GOS anaphylaxis reported by Chiang *et al.* (2012) in Singapore, a clinical study was conducted in Singapore in an atopic cohort aged 5 to 60 years (n=487) to determine the prevalence of allergy in this specific population (Soh *et al*., 2015b). The 30 subjects (6.2%) that were found to be sensitized to GOS (Vivinal) by skin prick test had blood drawn for basophil activation tests, and 13 of these subjects further consented to oral challenge tests with either Vivinal GOS or Oligomate 55N GOS. Six of the 13 subjects challenged orally with Vivinal GOS tested positive, whereas none of the subjects reacted to oral exposure to Oligomate 55N. The difference in allergic response to Vivinal *versus* Oligomate 55N GOS was speculated to be related to structural differences in the oligomer composition attributable to the use of different β-galactosidase enzymes and processing conditions specific to the manufacturing processes of each ingredient. Based on these findings, the authors estimate that the prevalence of allergy to Vivinal GOS in this atopic population in Singapore may be approximately 3.5%; however, the authors acknowledged that "…*this estimate may not be entirely accurate as subjects were drawn from patients attending a specialized clinic in a tertiary hospital. Furthermore, due to the small number of subjects from which the ROC* [Receiver Operating Characteristic] *curves were derived, this limits the accuracy of the BAT* [Basophil Activation Test] *and, thus, the precision of this estimate*" (Soh *et al*., 2015b). The Expert Panel noted that the atopic population of Singapore constitutes approximately 1/5th of the total population of the country; therefore a prevalence of GOS allergy of up to 3.5% within the Singapore population would tend to argue against the sensitizing agent being of parasitic origin as this prevalence of parasitic infection is unlikely in developed populations.

Although carbohydrates have been long known to bind to IgE, carbohydrates on their own (*i.e.,* not bound to protein) are poorly immunogenic. Anaphylactic reactions have a basic requirement for cross-linking of IgE receptors on effector cells, a process that requires the presence of large divalent antigens (Altmann, 2007). Despite the recent case reports of GOS allergenicity and apparent induction of histamine release *in vitro* by semi-purified GOS tetramers, the etiology of GOS anaphylaxis remains a mystery as mechanistic evidence has yet to establish that a small molecular weight oligomer (*i.e*., a monovalent antigen) on its own is capable of cross-linking IgE receptors to provoke an anaphylactic response *in vivo*. Although current evidence has implicated GOS ingredients as the causative agents in case-reports of GOS allergenicity, the putative antigen(s) remains unknown. The Expert Panel noted that the strong geographical restriction of all cases of GOS allergenicity that have been reported to date implies that the primary sensitizer is confined to very local regions of Southeast Asia, and is linked to specific prior occupational/environmental exposure. GOS preparations that have been

associated with GOS anaphylaxis in these localized regions have been widely consumed by populations elsewhere for over a decade by adults, children and infants without reported incidences of anaphylaxis, suggesting that the risk of GOS allergenicity from the introduction of GOS containing foods generally is low. Similar conclusions have been determined by other qualified experts during previous GRAS determinations (U.S. FDA 2008, 2014c).

CONCLUSIONS

We, the members of the Expert Panel, have independently and collectively, critically evaluated the data and information summarized above, as well as other information that we deemed pertinent to the safety of the proposed uses of Nestlé GOS. We unanimously conclude that Nestlé GOS, meeting appropriate food-grade specifications and manufactured in accordance with current Good Manufacturing Practice, is safe and suitable and Generally Recognized as Safe (GRAS) for use in term infant formula (0 to12 months) and toddler formula at a use-level providing up to 7.8 grams of GOS/L in the reconstituted or ready-to-drink formula.

It is our opinion that other qualified experts, critically evaluating the same information, would concur with these conclusions[6].

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20 November 2015
Date

[6] *E.g. "based on the available evidence, FSANZ concludes that infant and follow-on formula containing up to 8 g/L of inulin derived substances and/or GOS, singularly or combined, in any ratio, are unlikely to pose a risk to infants"* (FSANZ, 2008).

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(b) (6)

21 November 2015

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Attachment A

Nestlé GOS Specifications

Table A-1 Specifications for Nestlé GOS		
Specification Parameter	**Specification**	**Method**
Dry matter (DM)	≥ 96 %	AS-INC-012
Total moisture (Karl-Fisher)	Max 5.5%	Nestlé LI-08.055
Protein (N x 6.38) on DM	Max 4.47	Nestlé LI-00.556; Nestlé LI-00.561
Total oligosaccharides/GOS (on DM) Sialyllactose (on DM)	Min 46% Min 0.2%	Nestlé LI-00.590 (Austin *et al.,* 2014); AOAC 2001.02; Nestlé LI-08.007
Total Nitrogen on DM	Max 0.7%	Nestlé LI-00.556
Ash content on DM	Max 4%	Nestlé LI-00.565
Lactose on DM	20 – 40%	Nestlé LI-00.593
Glucose on DM	Max 10%	Nestlé LI-00.593
Galactose on DM	Max 5%	Nestlé LI-00.593
Nitrite	Max 2 mg/kg	ISO 14673-2: 2004
Nitrate	Max 50 mg/kg	ISO 14673-2: 2004
pH (10% solution)	5 - 6	Nestlé LI-00.908
Sodium (mg/100g on DM)	≤ 50	AOAC 2011.14
Potassium (mg/100g on DM)	1,200 – 2,100	AOAC 2011.14
Chloride (mg/100g on DM)	≤ 100	Nestlé LI-00.580
Calcium (mg/100g on DM)	≤ 100	AOAC 2011.14
Phosphorus (mg/100g on DM)	150 – 350	AOAC 2011.14
Magnesium (mg/100g on DM)	≤ 100	AOAC 2011.14
Manganese (mg/kg on DM)	≤ 0.2	AOAC 2011.14
Iron (mg/kg on DM)	≤ 5	AOAC 2011.14
Copper (mg/kg on DM)	≤ 2.5	AOAC 2011.14
Microbial Specifications		
Aerobic mesophilic microorganisms (per g)	10,000	ISO 4833
Aerobic mesophilic spores (per g)	500	80°C, 10 min – Nestlé LI-00.718
Enterobacteriaceae (per g)	10	ISO 21528 Incubation temperature 37°C
Salmonella sp. (per 25 g)	Negative	ISO 6579

DM = dry matter; ISO = International Organization for Standardization (Betts RP, Oscroft CA, Baylis CL (2004). *A Code of Practice for Microbiology Laboratories Handling Food, Drink and Associated Samples, 3rd revised edition*. Gloucestershire, UK: Campden & Chorleywood Food Research Association.)

Attachment B

Pariza-Johnson Decision Tree

This analysis is based on the Decision Tree of MW Pariza and EA Johnson (2001): *Evaluating the Safety of Microbial Enzyme Preparations Used in Food Processing: Update for a New Century*, Regulatory Toxicology and Pharmacology, 33:173-186. Decision points that do not pertain are included for completeness but crossed out.

1. Is the production strain genetically modified?
If yes, go to 2. If no, go to 6. **No**

~~2. Is the production strain modified using rDNA techniques?~~
~~If yes, go to 3. If no, go to 5.~~

~~3. Issues relating to the introduced DNA are addressed in 3a–3e.~~

~~3a. Do the expressed enzyme product(s) which are encoded by the introduced DNA have a history of safe use in food?~~
~~If yes, go to 3c. If no, go to 3b~~

~~3b. Is the NOAEL for the test article in appropriate short-term oral studies sufficiently high to ensure safety?~~
~~If yes, go to 3c. If no, go to 12.~~

~~3c. Is the test article free of transferable antibiotic resistance gene DNA?~~
~~If yes, go to 3e. If no, go to 3d.~~

~~3d. Does the resistance gene(s) code for resistance to a drug substance used in treatment of disease agents in man or animal? If yes, go to 12. If no, go to 3e.~~

~~3e. Is all other introduced DNA well characterized and free of attributes that would render it unsafe for constructing microorganisms to be used to produce food-grade products?~~
~~If yes, go to 4. If no, go to 12.~~

~~4. Is the introduced DNA randomly integrated into the chromosome?~~
~~If yes, go to 5. If no, go to 6.~~

~~5. Is the production strain sufficiently well characterized so that one may reasonably conclude that unintended pleiotropic effects which may result in the synthesis of toxins or other unsafe metabolites will not arise due to the genetic modification method that was employed?~~
~~If yes, go to 6. If no, go to 7.~~

6. Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment *via* this evaluation procedure? **YES, the *Aspergillus oryzae* production strain lineage has a long-history of safe use in the production of lactases and other food enzymes.**
If yes, the test article is ACCEPTED. If no, go to 7. **TEST ARTICLE IS ACCEPTED**

~~7. Is the organism nonpathogenic?~~
~~If yes, go to 8. If no, go to 12.~~

~~8. Is the test article free of antibiotics?~~
~~If yes, go to 9. If no, go to 12.~~

9. Is the test article free of oral toxins known to be produced by other members of the same species?
If yes, go to 11. If no, go to 10.

10. Are the amounts of such toxins in the test article below levels of concern?
If yes, go to 11. If no, go to 12.

11. Is the NOAEL for the test article in appropriate oral studies sufficiently high to ensure safety?
If yes, the test article is ACCEPTED.

12. An undesirable trait or substance may be present and the test article is not acceptable for food use. If the genetic potential for producing the undesirable trait or substance can be permanently inactivated or deleted, the test article may be passed through the decision tree again.

SUBMISSION END