Generally Recognized As Safe Determination for the Use of VITAGOS™ in Non-Exempt Term Infant Formula and Selected Conventional Foods

Prepared for:

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June 8, 2017

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LIST OF ABBREVIATIONS

CCP: Critical Control Points

CFR: Code of Federal Regulations

DP: Degree of Polymerization

EPA: United States Environmental Protection Agency

FDA: United States Food and Drug Administration

FFDCA: Federal Food, Drug, and Cosmetic Act

FOS: Fructo-oligosaccharides

FOSHU: Food for Specified Health Uses

FSANZ: Food Standards of Australia and New Zealand

FSSC: Food Safety System Certification

GLP: Good Laboratory Practices

GMO: Genetically Modified Organisms

GOS: Galacto-oligosaccharides

GRAS: Generally Recognized As Safe

GRN: GRAS Notification

HACCP: Hazard analysis critical control point

HDPE: High Density Polyethylene

OVA: Ovalbumin

SCF: Scientific Committee on Food

USP: United Stated Pharmacopeia

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I. SIGNED STATEMENT OF THE CONCLUSION OF GENERALLY RECOGNIZED AS SAFE (GRAS) AND CERTIFICATION OF CONFIRMITY TO 21 CFR §170.205-170.260

A. SUBMISSION OF GRAS NOTICE

Vitalus Nutrition Inc. is hereby submitting a GRAS notice in accordance with subpart E of part 170.

B. NAME AND ADDRESS OF THE SPONSOR

Vitalus Nutrition Inc.
3911 Mt. Lehman Road
Abbotsford, BC V2T 5W5
Canada

C. COMMON OR USUAL NAME

Galacto-oligosaccharides (GOS), also known as oligogalactosyllactose, oligogalactose, oligolactose, transgalactosylated oligosaccharide, and transgalacto-oligosaccharide.

D. TRADE SECRET OR CONFIDENTIAL INFORMATION

This notification does not contain any trade secret or confidential information.

E. INTENDED USE

VITAGOS™ will be added to powdered, ready-to-feed and concentrated liquid versions of milk-based non-exempt term infant formulas, and selected conventional foods.

F. BASIS FOR GRAS DETERMINATION

This GRAS determination for the use of GOS for the intended uses specified above has been shown to be safe and GRAS, using scientific procedures, under the Federal Food, Drug, and Cosmetic Act (FFDCA), as described under 21 CFR §170.30(b). The safety of the intake of VITAGOS™ has been determined to be GRAS by demonstrating that the safety of this level of intake is generally recognized by experts qualified by both scientific training and experience to evaluate the safety of substances directly added to food, and is based on generally available and accepted information.

The proposed use of VITAGOS™ as an ingredient for the intended uses in foods and infant formulas has been determined to be safe through scientific procedures set forth under 21 CFR §170.30(b) based on the following:

1. GOS are non-digestible oligosaccharides consisting of 1 to 7 galactose units linked via β(1-2), β(1-3), β(1-4), or β(1-6) glycosidic bonds to either a terminal glucose or galactose. Although tri- to hexa-saccharides with 2 to 5 galactose units (degree of polymerization (DP) of 3 to 6) tend to be the main components of GOS-containing products, disaccharides (DP2) consisting of galactose and glucose with different β-glycoside bonds from lactose are also present and defined as GOS because they have physiological characteristics that are similar to longer GOS.
    a. VITAGOS™ is a GOS-containing product manufactured using lactose and β-galactosidases derived from *Aspergillus oryzae* and *Kluyveromyces lactis* in a manner similar to other GOS-containing products that have received "no questions" letters from the United States Food and Drug Administration.
    b. All processing aids used to produce VITAGOS™ comply with appropriate federal regulations.
    c. A comparison of the manufacturing processes and product specifications for VITAGOS™ and other GOS-containing products shows that VITAGOS™ is essentially equivalent to the other GOS-containing products currently marketed in the United States for use in infant formulas and conventional foods.
2. GOS are transported through the upper gastrointestinal tract to the colon where they are fermented by the resident microbiota into short-chain fatty acids, carbon dioxide, methane, and hydrogen.
3. GOS present in food are either naturally occurring in human milk and colostrum, bovine colostrum, and fermented milk products or synthetic, which are then added to the food during processing and formulation.
4. Synthetic GOS have a long history of use worldwide.
    a. In Japan, GOS have been commercially available since 1995 and are considered as Food for Specified Health Uses (FOSHU).
    b. In the United States, the first GOS product was determined GRAS for use in term infant formula and selected conventional foods, and received a "no questions" letter from the FDA in 2008 (GRN 236). Since then, six additional GOS-containing products have been determined GRAS for use in infant formulas and selected conventional foods at levels up to 7.8 g/L and 11 g/serving, respectively, resulting in ten GRAS Notifications (GRN) to the FDA (GRN 236, 285, 286, 334, 484, 489, 495, 518, 569, and 620).

c. In the European Union, the safety of GOS was reviewed by the Scientific Committee on Food (SCF) in 2003 and is approved for use in infant and follow-on formulas in combination with fructo-oligosaccharides (FOS) at levels up to 8 g (90% GOS and 10% FOS)/L (7.2 g GOS and 0.8 g FOS/L) (Select Committee on Food EU 2016/127).

d. In Australia and New Zealand, the safety of GOS was reviewed by the Food Standards of Australia and New Zealand (FSANZ) in 2008 and is permitted in infant and follow-on formulas at levels up to 290 mg/100 kJ, or approximately 8 g/L (Australia New Zealand Food Standards Code – Standard 2.9.1 – 7).

5. The safety of VITAGOS™ was determined in a pivotal published 90-day toxicology study that identified a no observed adverse effect level (NOAEL) of 4082 mg/kg/day (2000 mg GOS/kg/day), which was the highest dose tested.

6. The safety of VITAGOS™ is corroborated by multiple subchronic, developmental, reproductive, and genotoxicology studies conducted on other GOS-containing products.

7. GOS-containing products are well tolerated in humans and have been reported to increase the abundance of bifidobacteria and lactobacilli in the gastrointestinal tract in infants and adults, increase fecal short-chain fatty acid concentrations in infants and adults, improve stool consistency in infants, reduce the incidence of atopic dermatitis in infants, and alleviate the symptoms of irritable bowel syndrome in adults.

8. GOS is available worldwide and, although GOS-containing products have been reported to provoke allergic reactions in sensitized individuals living in Vietnam and Singapore, there have been no reported reactions to GOS-containing products outside of Vietnam and Singapore. Thus, the reported cases likely represent unique, rare, geographically localized allergic reactions to GOS-containing products.

9. The addition of VITAGOS™ to infant formula at 7.2 g GOS/L is the same use level of other GOS products in infant formula that have been determined GRAS and received "no questions" letters from the FDA (GRN 286, 334, 569) and will result in intakes of approximately 5.1 and 6.9 g GOS/day for one-month-old and six-month-old infants, respectively.

10. VITAGOS™ will be used as an alternative source of GOS in selected conventional foods at the intended use levels specified for Oligomate in GRN 334. Thus, the dietary exposure to VITAGOS™ from the intended uses will not increase GOS-intake in the United States. The estimated mean and 90th percentile exposure to VITAGOS™ from the intended uses in selected conventional foods are 12.2 and 25.3 g per person per day (g/p/d), respectively.

Determination of the GRAS status of VITAGOS™ under the intended conditions of use has been made through the deliberations of Roger Clemens, DrPH, CNS, CFS, FACN, FIFT, A. Wallace Hayes, PhD, DABT, FATS, ERT, CNS, FACN, and Thomas Sox PhD, JD. These individuals are qualified by scientific training and experience to evaluate the safety of food and food ingredients. These experts have carefully reviewed and evaluated the publicly available information summarized in this document, including the safety of VITAGOS™ and the human exposure to VITAGOS™ resulting from its intended use as an ingredient in powdered non-exempt term infant formula and selected conventional foods:

> *There is no evidence in the available information on VITAGOS™ that demonstrates, or suggests reasonable grounds to suspect, a hazard to the public when VITAGOS™ is used at levels that might reasonably be expected from the proposed applications of VITAGOS™ for use in powdered non-exempt term infant formulas and selected conventional food as proposed by Vitalus Nutrition Inc.*

Therefore, VITAGOS™ is safe and GRAS at the proposed levels of addition to the intended foods. VITAGOS™ is, therefore, excluded from the definition of a food additive, and may be used in the U.S. without the promulgation of a food additive regulation by the FDA under 21 CFR.

G. PREMARKET APPROVAL

The notified substance is not subject to the premarket approval requirements of the FD&C Act based on our conclusion that the substance is GRAS under the conditions of intended use.

H. AVAILABILITY OF INFORMATION

The data and information that serve as the basis for this GRAS determination will be available for review and copying at reasonable times at the office of Claire L. Kruger, PhD, DABT, President, ChromaDex Spherix Consulting, A Business Unit of ChromaDex, Inc., at 11821 Parklawn Drive, Suite 310, Rockville, MD 20852. Telephone: 301-230-2180; Email: clairek@chromadex.com, or be sent to FDA upon request.

I. FREEDOM OF INFORMATION ACT (FOIA)

Parts 2 through 7 of this notification do not contain data or information that is exempt from disclosure under the FOIA.

J. INFORMATION INCLUDED IN THE GRAS NOTIFICATION

To the best of our knowledge, the information contained in this GRAS notification is complete, representative and balanced. It contains both favorable and unfavorable information, known to Vitalus Nutrition Inc. and pertinent to the evaluation of the safety and GRAS status of the use of this substance.

(b) (6)

Signature
Authorized Representative of Vitalus Nutrition Inc.

June 14, 2017
Date

II. IDENTITY, METHOD OF MANUFACTURE, SPECIFICATIONS, AND PHYSICAL OR TECHNICAL EFFECT OF THE NOTIFIED SUBSTANCE

A. COMMON OR USUAL NAME

Galacto-oligosaccharides (GOS), also known as oligogalactosyllactose, oligogalactose, oligolactose, transgalactosylated oligosaccharide, and transgalacto-oligosaccharide.

B. TRADE NAME

VITAGOS™

C. DESCRIPTION OF GALACTO-OLIGOSACCHARIDES

As stated in GRN 495 and 620, "there is no globally-adopted definition of galacto-oligosaccharides". Galacto-oligosaccharides have been defined as a mixture of those substances produced from lactose, comprising between 2 and 8 saccharide units, with one of these units being a terminal glucose and the remaining saccharide units being galactose, and disaccharides comprising two units of galactose (Tzortzis and Vulevic, 2009; FSANZ, 2008; Figure 1), and substances that are produced from lactose with the help of a bacterial β-galactosidase and contain one molecule of glucose and typically between 1 and 7 molecules of galactose (Scientific Committee on Food, 2001). Typically, the oligosaccharides in GOS preparations are linked via β-glycosidic bonds with 1-3, 1-4, or 1-6 anomeric configurations, which are determined by the type of β-galactosidase used during manufacturing and manufacturing conditions (reviewed in Torres et al., 2010). Although tri- to hexa-saccharides with 2 to 5 galactose units (degree of polymerization (DP) of 3 to 6) tend to be the main components of GOS-containing products, disaccharides (DP2) consisting of galactose and glucose with β-glycoside bonds different from lactose are also present and, because these disaccharides have the same physiological characteristics as longer GOS, they are considered GOS (Sangwan et al., 2011; Sako et al., 1999).

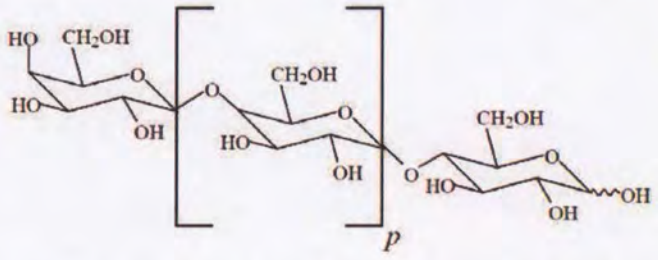

Figure 1. Structure of Galacto-oligosaccharides
Brackets denote the repeating units, $p = 0$ to 6 to generate galacto-oligosaccharides consisting of 1 to 7 galactose units linked to a terminal glucose or galactose via β(1→3), β(1→4), or β(1→6) glycosidic bonds.

VITAGOS™ is a GOS-containing syrup sythesized from lactose using β-galactosidases derived from *Aspergillus oryzae* and *Kluyveromyces lactis*. VITAGOS™ contains minimum of 62% GOS, among which DP3 GOS predominate (Table 1).

Table 1. Total Sugar Composition of VITAGOS™

Saccharide (DP)	Carbohydrate	Relative Amount (% DM)[1]
Monosaccharides (DP1)	Galactose	2 ± 0.34
	Glucose	17.5 ± 0.37
Disaccharides (DP2)	Lactose	14.9 ± 0.32
	GOS	17.0 ± 0.42
Trisaccharides (DP3)	GOS	33 ± 0.48
Tetrasaccharides (DP4)	GOS	11.5 ± 0.20
Pentasaccharides and higher oligomers (DP≥5)	GOS	3.8 ± 0.11

DM = dry matter; DP = Degree of Polymerization; GOS = Galacto-oligosaccharides
[1]Relative amount was determined using data from five batches.

D. PRODUCTION PROCESS

VITAGOS™ is manufactured using a standardized procedure that has been widely reviewed in the scientific literature (Sangwan et al., 2011; Torres et al., 2010). It consists of three basic steps: preparation of highly concentrated solution of lactose; treatment of the lactose solution with β-galactosidases to produce galacto-oligosaccharides (GOS); and termination of the enzymatic reaction with heat. The β-galactosidases perform two functions; the hydrolysis of lactose to the monosaccharides glucose and galactose, and transgalactosylation of lactose, producing GOS. Importantly, the concentration, chain length, and type of β-glycosidic bonds of GOS are determined by the rate of hydrolysis, degree of transgalactosylation, and source of the β-galactosidase, and manipulation of these factors during production results in products containing GOS with different concentrations, varying chain lengths, and different β-glycosidic bonds (GRN 489).

1. Compliance

VITAGOS™ is manufactured by Vitalus Nutrition Inc., located at 3911 Mt. Lehman Rd. Abbotsford, British Columbia, V2T 5W5, Canada under food grade conditions. Products manufactured by Vitalus Nutrition Inc. are certified as meeting kosher and halal specifications, and do not contain genetically modified organisms (GMOs) or ingredients derived from GMO-derived products. Vitalus Nutrition Inc. has a hazard analysis critical control point (HACCP) management system in place and their manufacturing facility has been audited and determined to be compliant with the Food Safety System Certification (FSSC) 22000 standards by a third party. All food contact surfaces used in manufacturing VITAGOS™ are either stainless steel,

aluminum or suitable for use in the production of food ingredients. The whey used to produce the lactose is free of antibiotics and all raw materials, enzymes, and processing aids are either Food Chemical Codex grade, comply with conditions of use stipulated in Parts 168, 173, 177, 182 and 184 of Title 21 of the United States Code of Federal Regulations or have been determined GRAS (GRN 90; GRN 489; GRN 620, FDA, 2013) (Table 2). All ingredients and processing aids also comply with European Union and Codex requirements, and, because current Canadian legislation prohibits the use of bovine growth hormones in dairy cattle, are free of recombinant bovine somatotropic and growth hormones. In addition, the β-galactosidases are derived from edible, nontoxic sources without the use of ionizing radiation.

Table 2. Regulatory Compliance of Ingredients and Processing Aids

Material	Regulatory Status
Lactose	21 CFR §168.122
β-Galactosidase (derived from *Kluyveromyces lactis*)	21 CFR §184.1388
β-Galactosidase (derived from *Aspergillus oryzae*)	GRN 90, GRN 489, GRN 620, FDA Partial List of Enzyme Preparations Used in Food (FDA, 2013)
Ion Exchange Resins	21 CFR §173.25
Potassium Hydroxide	21 CFR §184.1631
Magnesium Chloride	21 CFR §184.1426
Citric Acid	21 CFR §182.1033
Hydrochloric Acid	21 CFR §182.1057
Sodium Hydroxide	21 CFR §184.1763
Tubing Materials	21 CFR 177.2600, 21 CFR 177.2490, 21 CFR 177.1550, 21 CFR 177.1520
CFR = Code of Federal Regulations; GRN = Generally Recognized As Safe (GRAS) Notification	

2. Manufacturing Process

To produce VITAGOS™, food-grade lactose is dissolved in municipal drinking water and heated to a temperature greater than 80°C under agitation (Figure 2). The temperature and pH of the solution are then adjusted to optimum conditions for transgalactosylation. β-Galactosidase derived from *A. oryzae* is added and the solution is agitated for a set period of time to convert the lactose to GOS. The temperature and pH are then adjusted to minimize subsequent transgalactosylation, maximize the hydrolysis of the remaining lactose, and achieve the desired final GOS purity using a β-galactosidase derived from *K. lactis.* Both enzymes are deactivated at 80°C. Filtration and adsorption processes using several resins then remove the enzyme residues

and other impurities. The resulting material is concentrated by evaporation and further heated to 80°C for 1 min. to destroy pathogens in the finished product prior to packaging. The material is passed through a screen to ensure a homogenous syrup and packaged in containers lined with ultra-low density polyethylene food-grade bags under hygienic conditions. Packaged VITAGOS™ is stored under ambient conditions and when compliance with the product specifications is determined, cleared for distribution.

The quality of the finished product is controlled by two critical control points (CCPs). The first CCP (CCP1) occurs at the concentration step where Vitalus Nutrition Inc. heats the material to 80°C for 1 min to destroy pathogens in the finished product. The second, CCP2, occurs at the packaging step where Vitalus Nutrition Inc. passes the material through a screen to ensure a homogenous syrup. In addition, the quality of the product is also monitored during processing with in-line testing for solids, conductivity, pH, color, and sugar profile.

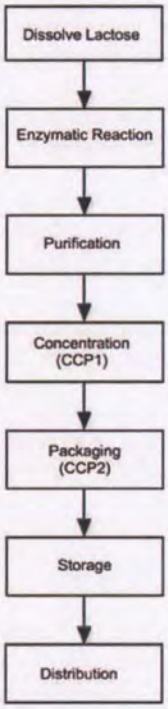

Figure 2. Production Process for VITAGOS™

Lactose is dissolved in water and mixed with β-galactosidases from *Aspergillus oryzae* and *Kluyveromyces lactis*. The enzymes are then deactivated and GOS are purified by filtration and adsorption with resins. VITAGOS™ is concentrated by evaporation, heat-treated, packaged in containers lined with food grade bags, and stored under ambient conditions. When compliance with the product specifications is met, VITAGOS™ is distributed to customers.

E. FINISHED PRODUCT SPECIFICATIONS AND OTHER QUALITY ATTRIBUTES

1. Product Specifications

To ensure a consistent food-grade product, each batch of VITAGOS™ is evaluated against an established set of product specifications (Table 3) using validated methods. Data from five pilot batches demonstrate control of the production process and compliance with the product specifications.

Table 3. VITAGOS™ Product Specifications and Batch Data

Parameter	Specification	Method	Batch Number (b) (6)				
Physical Characteristics							
Appearance	Clear to slight yellow	Visual	Clear to slight yellow	Clear to slight yellow	Clear to slight yellow	Clear to slight yellow	Clear to slight yellow
pH	2.7 - 3.7	pH Meter*	2.9	3.1	2.7	2.9	3.0
Viscosity (cPs @ 26°C)	1000 - 5000 cPs	Rotational Shear*	2000	2000	1800	1950	2000
Dry Matter (Total %)	74-76	Vacuum Oven Solids*	74.7	74.3	74.8	74.3	74.3
Chemical Composition							
Galacto-oligosaccharides (% DM)	≥ 62	HPLC-RID*	65.9	65.8	65.7	65.3	65.4
Lactose (% DM)	≤ 16	HPLC-RID*	15.0	14.4	15.0	15.3	14.7
Glucose (% DM)	≤ 22	HPLC-RID*	17.1	17.2	17.7	17.5	18.0
Galactose (% DM)	≥ 1	HPLC-RID*	2.0	2.5	1.6	1.8	1.9
Sulfated Ash (% DM)	≤ 0.3	USP / NF Current Version	0.01	0.01	0.01	0.03	0.03
Protein (% DM)**	≤ 0.2	AOAC 991.20.I	0.16	0.16	0.16	0.16	0.16
Microbiological Parameters							
Standard Plate Count (cfu/g)	< 3000	MFHPB-33†	ND	ND	ND	ND	ND
Enterobacteriaceae (cfu/ g)	< 10	MFLP-43†	ND	ND	ND	ND	ND
Escherichia coli (cfu/g)	< 10	MFHPB-34†	ND	ND	ND	ND	ND
Yeast and Mold (cfu/g)	< 100	MFHPB-22†	ND	ND	ND	ND	ND
Staphylococcus aureus (cfu/g)	< 10	MFHPB-21†	ND	ND	ND	ND	ND
Salmonella (per 25g)	Negative	MFLP-29†	ND	ND	ND	ND	ND
Heavy Metals							
Arsenic (ppm; w/w)[1]	< 0.4	EPA 3050/6020, USP 730	ND	ND	ND	ND	ND
Lead (ppm; w/w)[1]	< 0.2	EPA 3050/6020 USP 730	0.02	0.04	0.03	0.03	0.06
Cadmium (ppm; w/w)[1]	< 0.06	EPA 3050/6020 USP 730	0.001	0.001	ND	ND	ND
Mercury (ppm; w/w)[1]	< 0.005	EPA 3050/6020 USP 730	ND	ND	ND	ND	ND

cPs = centipoises; cfu = colony forming units; USP = United Stated Pharmacopeia; NF = National Formulary; EPA = United States Environmental Protection Agency; AOAC = Association of Analytical Communities; MFHPB = Methods for the Microbiological Analysis of Foods; MFLP = Laboratory Procedures for the Microbiological Analysis of Foods; ppm = parts per million; w/w = weight/weight; g = gram; DM = dry matter; ND = not detected; HPLC-RID = High Performance Liquid Chromatography-Refractive Index Detector.
[1]Limit of detection: Arsenic = 0.01 ppm; lead = 0.01 ppm; cadmium = 0.001 ppm; mercury = 0.005 ppm.
†Obtained from the Compendium of Analytical Methods prepared by the Evaluation Division Bureau of Microbiological Hazards, Food Directorate, Health Products and Food Branch, Health Canada (http://www.hc-sc.gc.ca/fn-an/res-rech/analy-meth/microbio/index-eng.php).
*Validated by Vitalus Nutrition, Inc.
**N=6.38

2. Other Quality Attributes

a. Degree of Polymerization

To demonstrate control of the production process, Vitalus Nutrition Inc. analyzed the GOS DP content of five batches of VITAGOS™ by HPLC (Table 4). Each batch had similar amounts of the different GOS DP fractions, indicating that the manufacturing process produces a consistent product. Importantly, Vitalus Nutrition Inc. monitors the GOS DP content of the finished product on a quarterly basis.

Table 4. GOS Content of VITAGOS™

Saccharide (DP)[1]	Batch Number (b) (6)					Average +/- St. Dev. (%)
DP2	16.7	16.5	17.1	17.1	17.6	17.0 +/- 0.42
DP3	33.8	33.8	33.2	32.8	32.9	33.3 +/- 0.48
DP4	11.6	11.7	11.5	11.4	11.2	11.5 +/- 0.20
DP≥5	3.8	3.8	3.9	4.0	3.7	3.8 +/- 0.11
Total GOS (% DM)[2]	65.9	65.8	65.7	65.3	65.4	65.6 +/- 0.25

DP = Degree of Polymerization; GOS = Galacto-oligosaccharides; DM = dry matter.
[1]Does not include lactose. Determined by HPLC-RID (High Performance Liquid Chromatography-Refractive Index Detector), which was validated by Vitalus Nutrition, Inc.
[2]Corresponds to the data presented for the GOS specification in Table 3.

b. Pathogenic Bacteria

To confirm the absence of *Cronobacter sakazakii* and *Bacillus cereus*, Vitalus Nutrition Inc. analyzed five batches of VITAGOS™ using the appropriate validated microbiological techniques (Table 5). *C. sakazakii* and *B. cereus* were undetectable in each batch. Importantly, Vitalus Nutrition Inc. monitors VITAGOS™ for the presence of these pathogenic bacteria on a quarterly basis.

Table 5. Pathogenic Bacteria in VITAGOS™

Bacteria	Method	LOD	Batch Number (b) (6)				
Cronobacter sakazakii	MFLP-42†	<10 cfu/g	ND	ND	ND	ND	ND
Bacillus cereus	MFLP-27†	Neg./25 g	ND	ND	ND	ND	ND

ND = not detected;; LOD = limit of detection; Neg = negative; MFLP = Laboratory Procedures for the Microbiological Analysis of Foods.
†Obtained from the Compendium of Analytical Methods prepared by the Evaluation Division Bureau of Microbiological Hazards, Food Directorate, Health Products and Food Branch, Health Canada (http://www.hc-sc.gc.ca/fn-an/res-rech/analy-meth/microbio/index-eng.php).

c. Protein Allergens

VITAGOS™ is manufactured on a production line that processes only milk products. No other potentially allergenic substances are used. For due diligence purposes, Vitalus Nutrition Inc. determined the amount of casein, a major milk allergen, in VITAGOS™ by SDS-PAGE. The limit of detection for the assay was 2.6 ppm. Casein was not detected.

d. Minerals

The mineral content of 5 batches of VITAGOS™ was determined using the Environmental Protection Agency (EPA) 3050/6020, United States Pharmacopeia (USP) 730 validated method and confirms the food grade status of the finished product (Table 6).

Table 6. Minerals in VITAGOS™

Minerals[1]	Batch Number (b)(6)				
Aluminum (ppm)	0.7	1.5	2.8	0.4	0.6
Antimony (ppm)	<0.01	<0.01	<0.01	<0.01	<0.01
Barium (ppm)	<0.02	0.03	0.03	<0.02	0.02
Beryllium (ppm)	<0.01	<0.01	<0.01	<0.01	<0.01
Bismuth (ppm)	<0.02	<0.02	<0.02	<0.02	<0.02
Boron (ppm)	0.5	0.4	0.4	<0.2	<0.2
Calcium (ppm)	45.3	54.5	5.5	11.7	7.2
Chromium (ppm)	0.45	0.5	0.03	0.03	0.03
Cobalt (ppm)	<0.01	<0.01	<0.01	<0.01	<0.01
Copper (ppm)	0.29	0.5	0.23	0.29	0.43
Iron (ppm)	6.0	5.7	<0.5	<0.5	<0.5
Lithium (ppm)	<0.1	<0.1	<0.1	<0.1	<0.1
Magnesium (ppm)	<0.05	0.05	1.97	1.91	2.55
Manganese (ppm)	0.10	0.11	0.05	0.06	0.03
Molybdenum (ppm)	0.02	0.02	0.01	<0.01	<0.01
Nickel (ppm)	0.06	0.08	0.06	0.14	0.1
Phosphorus (ppm)	<1.0	1	3.3	2.1	3.0
Potassium (ppm)	56.2	64.8	64.9	214	60.2
Selenium (ppm)	<0.1	<0.1	0.1	0.1	<0.1
Silver (ppm)	<0.02	<0.02	<0.02	<0.02	<0.02
Strontium (ppm)	0.04	0.1	0.01	0.02	0.02
Sodium (ppm)	64.4	67.7	<0.1	4.0	3.7
Thallium (ppm)	<0.01	<0.01	<0.01	<0.01	<0.01
Thorium (ppm)	<0.1	<0.1	<0.1	<0.1	<0.1
Tin (ppm)	<0.01	<0.01	<0.01	<0.01	<0.01
Titanium (ppm)	<0.02	0.04	0.31	0.03	0.08

Table 6. Minerals in VITAGOS™					
	Batch Number				
Minerals[1]	(b) (6)				
Uranium (ppm)	<0.01	<0.01	0.30	0.28	0.30
Vanadium (ppm)	0.01	0.02	0.02	0.02	0.01
Zinc (ppm)	3.4	0.79	0.48	0.78	0.52
Zirconium (ppm)	<0.1	<0.1	<0.1	<0.1	<0.1
ppm = parts per million; "<" denotes that the amount of the mineral was below the limit of detection. [1]Determined using Environmental Protection Agency (EPA) 3050/6020 United States Pharmacopeia (USP) 730.					

F. STABILITY OF VITAGOS™

The intended shelf-life of VITAGOS™ is at least 12 months. To support this, one batch of VITAGOS™ was stored in high-density polyethylene (HDPE) bottles under ambient conditions (18-25°C). Oligosaccharide content, microbiological content, and pH were determined at various time points and compared to the acceptance limits stipulated in the product specifications. Over the course of 18 months, GOS, galactose, glucose, and lactose content were similar to freshly made VITAGOS™ and at all time points complied with the product specifications (Table 7). The distribution of GOS in DP2, DP3, DP4, and DP5 or greater was similar to VITAGOS™ at the beginning of the testing period. Microbiological content and pH were determined over the course of 12 months and, although not all parameters were determined at each time point, all complied with the product specifications over the course of the testing period (Table 8). Importantly, determining the stability of VITAGOS™ is an ongoing process and will continue to be monitored to support the intended shelf-life of the finished product.

Table 7. Oligosaccharide Stability of VITAGOS™					
		Time (Months)			
Parameter	Specification[1]	0	3	12	18
Galacto-oligosaccharides (% DM)	≥ 62	65.1	65.1	64.7	65.2
Galactose (% DM)	≥ 1	2.9	3.3	3.3	3.3
Glucose (% DM)	≤ 22	16.2	16.3	16.6	16.3
Lactose (% DM)	≤ 16	15.7	15.3	15.4	15.2
DP2 (% GOS)	ns	18.4	18	18.1	18.4
DP3 (% GOS)	ns	32.5	32.7	32.5	32.5
DP4 (% GOS)	ns	11.1	10.9	10.7	10.9
≥DP5 (% GOS)	ns	3.1	3.5	3.4	3.35
DM = dry matter; GOS = galacto-oligosaccharides; DP = degree of polymerization; ns = no specification. [1]Determined by HPLC-RID (High Performance Liquid Chromatography-Refractive Index Detector), which has been validated by Vitalus Nutrition, Inc.					

Table 8. Microbiological Stability of VITAGOS™

Parameter	Specification[1]	Time (Months)											
		1	2	3	4	5	6	7	8	9	10	11	12
Standard Plate Count	< 3000 (cfu/g)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Enterobacteriaceae	< 10 (cfu/g)	ND	ND	ND	ND	-	-	-	-	-	ND	ND	ND
Escherichia coli	< 10 (cfu/g)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Yeast	< 100 (cfu/g)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Mold	< 100 (cfu/g)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Staphylococcus aureus	< 10 (cfu/g)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Salmonella	Negative/25 g	ND	ND	ND	ND	-	ND	ND	ND	ND	ND	ND	ND
pH	2.7 - 3.7	3.2	3	2.8	-	2.8	-	2.7	3.1	2.7	3.4	-	3.2

cfu = colony forming units; "-" = not tested; ND = not detected
[1]Methods used are the same as those cited in Table 3.

III. DIETARY EXPOSURE

A. INTENDED EFFECT

The intended effect of adding GOS to powdered, ready-to-feed, and concentrated liquid versions of milk-based non-exempt term infant formulas and selected conventional foods is to increase oligosaccharide intake in formula-fed infants and the general population and promote the growth of beneficial bacteria, including, but not limited to bifidobacteria and lactobacillus.

B. HISTORY OF USE

GOS present in food are either naturally occurring or synthetic forms added to food during processing and formulation. Naturally occurring GOS are present in human milk and colostrum, bovine colostrum, and fermented milk products (Kunz et al., 2000; Coppa et al., 1991; Coppa et al., 1997; Toba et al., 1982; Saito et al., 1987). Synthetic GOS are found in a wide variety of products (Table 6). The levels of naturally occurring GOS range from 5 – 15 g/L, 8.5 g/L, and 0.03 – 0.09% in human milk, bovine colostrum, and fermented milk products, respectively (Kunz et al., 2000; Coppa et al., 1991; Coppa et al., 1997; Saito et al., 1987; Toba et al., 1982). It is important to note that, although synthetic GOS are structurally and compositionally less diverse than naturally occurring GOS, both types contain glycosidic bonds, which render them resistant to the digestive enzymes in the stomach and small intestine, and fermentable by the gastrointestinal microbiota present in the small intestine and colon (Wisker et al., 1985; Ohtsuka et al., 1990; Chonan et al., 2004).

GOS have a long history of safe use worldwide.

In Japan, GOS have been commercially available since 1995 and are considered as Food for Specified Health Uses (FOSHU).

In the United States, the first GOS product was determined GRAS for use in term infant formula and selected conventional foods, and received a "no questions" letter from the FDA in 2008 (GRN 236). Since then, six additional GOS-containing products have been determined GRAS for use in infant formulas and selected conventional foods at levels up to 7.8 g/L and 11 g/serving, respectively, resulting in ten GRAS Notifications (GRN) to the FDA (GRN 236, 285, 286, 334, 484, 489, 495, 518, 569, and 620). All GRNs received "no questions" letters from FDA.

In the European Union, the safety of GOS was reviewed by the Scientific Committee on Food (SCF) in 2003. GOS is currently approved for use in infant and follow-on formulas GOS in

combination with fructo-oligosaccharides (FOS) at levels up to 8 g (90% GOS and 10% FOS)/L (7.2 g GOS and 0.8 g FOS/L) (Scientific Committee on Food, EU 2016/127).

In Australia and New Zealand, the safety of GOS was reviewed by the Food Standards of Australia and New Zealand (FSANZ) in 2008 and, similar to the EU, GOS is currently permitted in infant and follow-on formulas at levels up to 290 mg/100 kJ, or approximately 8 g/L (Australia New Zealand Food Standards Code – Standard 2.9.1 – 7).

C. INTENDED USE

Vitalus Nutrition Inc. intends to use VITAGOS™ as a substitute for the GOS product that was the subject of GRN 334. Thus, the intended uses for VITAGOS™ will be the same as those specified in GRN 334, which includes powdered, ready-to-feed, and concentrated liquid versions of milk-based non-exempt term infant formulas and selected conventional foods. The infant formulas will not exceed 7.2 g GOS/L reconstituted infant formula and the selected conventional foods will not exceed the maximum use levels listed in Table 9.

Table 9. Intended Uses of VITAGOS™

Food Group	Proposed Food Uses[2]	Maximum Use Level (g GOS/100 g Product)[1]
Milk and milk products	Milk, milk substitute such as soy milk	2
	Milk drink	4
	Yogurt	3.4
	Milk based meal replacement	2
	Infant formula	NA[3]
	White sauces, milk gravies and cheese sauces	1.25
	Milk desserts, frozen like ice creams	2
	Pudding and custards including baby foods	1.4
	Cheese soups	0.62
Soups	Egg soups; soups with legumes as major ingredient; soups with grain products as major ingredient; potato soups; deep-yellow vegetable soups; tomato soups; other vegetable soups	0.62
Nut beverages	Coconut beverages	1.6
Bakery products	Bread	1
	Brownies	1
	Cakes, heavy weight	1
	Cakes, medium weight	1
	Cakes, light weight	1

Table 9. Intended Uses of VITAGOS™		
Food Group	**Proposed Food Uses[2]**	**Maximum Use Level (g GOS/100 g Product)[1]**
	Coffee cakes, crumb cakes, doughnuts, Danish, sweet rolls, sweet quick type breads, muffins, toaster pastries	1
	Cookies	1
	Crackers that are usually used as snacks	1
	French toast, pancakes	1
	Pies, cobblers, fruit crisps, turnovers, other pastries	1
	Waffles	1
	Grain-based bars with or without filling or coating, e.g., breakfast bars, granola bars, rice cereal bars	1
Cereals	Ready-to-eat cereals	2
	Ready-to-eat cereals (dry) for baby food	4
	Ready-to-serve cereals (wet) for baby food	0.55
Fruit and vegetable juices	Fruit juices (including citrus fruit juices) and nectars	1.6
	Vegetable juices	1.6
	Fruit juices, vegetable juices and juice mixtures baby food	1.6
Sugars and sweets	Jellies, jams, preserves	25
Nonalcoholic beverages	Fruit drinks such as fruit juice drinks, fruit flavored drinks, sports drinks, etc.	2
	Non-fruit beverages including energy drinks	4.4
	Beverage concentrate (powder)	33.4

NA = not applicable
[1]Use levels are consistent with those specified in GRN 334.
[2]Food groups were obtained from the Food and Nutrition Database for Dietary Studies (FNDDS).
[3]Maximum amount of GOS ingested is based on the caloric need of the infant (see Chapter IV, Section C.1.).

D. ESTIMATED DAILY INTAKE

1. Infant Formula

The powdered, ready-to-feed, and concentrated liquid versions of milk-based non-exempt term infant formulas will contain 7.2 g GOS/L as consumed. Infant formulas in the US market provide approximately 670 kcal/L (20 kcal/fl oz) (Martinez and Ballew, 2011). Assuming infant formulas are the sole source of nutrition, reconstituted at 141 g/L, or a caloric density of 670 kcal/L, and the caloric requirements of a one month-old and six month-old infants are 472 kcal/day and 645 kcal/day, respectively (Institute of Medicine (US) Panel on Macronutrients and Institute of Medicine (US) Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, 2005), 1 and 6 month-old infants consume approximately 0.704 and 0.963 L formula/day. The addition of 7.2 g GOS/L in infant formula will therefore result in a GOS intake of approximately 5.1 and 6.9 g/day for one-month-old and six-month-old infants, respectively. Importantly, the use of other GOS products in infant formula at this intended use level has been determined GRAS and received "no questions" letters from the FDA (GRN 286, 334, 569).

2. Selected Conventional Food Uses

Because Vitalus Nutrition Inc. intends to use VITAGOS™ as a substitute for the GOS preparation specified in GRN 334, the exposure to GOS from the ingestion of VITAGOS™ will not increase compared to those resulting from the ingestion of Oligomate 55N/55NP. Per GRN 334, the GOS EDIs were calculated using food consumption data reported in the United States Department of Health and Human Service's 2003-2004 National Health and Nutrition Examination Survey (NHANES) and the mean and 90th percentile GOS EDIs for the total U.S. population from the ingestion of all GOS-containing foods are 12.2 g/person/day (0.28 g/kg body weight/day) and 25.3 g/person/day (0.7 g/kg body weight/day), respectively. On an individual basis, the greatest mean and 90th percentile GOS EDIs occur in children and male teenagers at 18.1 and 33.0 g/person/day. On a body weight basis, the greatest mean and 90th percentile GOS EDIs occur in infants at 1.44 and 2.42 g/kg body weight/day. Importantly, intake in infants included exposures from both infant formula and conventional food uses.

IV. SELF-LIMITING LEVELS OF USE

This part does not apply.

V. COMMON USE IN FOOD BEFORE 1958

This part does not apply.

VI. NARRATIVE ON THE CONCLUSION OF GRAS STATUS

The safety and GRAS status of VITAGOS™ is supported by a publicly available 90-day toxicology study conducted with VITAGOS™ (Zhou et al., 2017). Numerous corroborative *in vitro*, toxicology, animal, and clinical studies, and the opinions of regulatory bodies throughout the world on the use of GOS in infant formulas and selected conventional foods (GRN 236; GRN 285; GRN 286; GRN 334; GRN 484; GRN 489; GRN 495; GRN 518; GRN 569; GRN 620; Scientific Committee on Food, 2001; FSANZ, 2008) support the safety of GOS. Although a limited number of allergic reactions to a GOS-containing product manufactured using β-galactosidase derived from *B. circulans* have been reported in individuals residing in Southeast Asia, the GOS present in VITAGOS™ are produced with β-galactosidase that does not produce the oligosaccharides that are believed to provoke allergic reactions, particularly because *B. circulans* is not used by the sponsor. Therefore, the use of VITAGOS™ in conventional foods is GRAS.

A. ABSORPTION, DISTRIBUTION, METABOLISM, AND EXCRETION

GOS including VITAGOS™ are non-digestible oligosaccharides consisting of 1 to 7 galactose units linked via 1-3, 1-4, or 1-6 β-glycosidic bonds to either a terminal glucose or galactose. The absorption, distribution, metabolism and excretion of GOS and their metabolites have been extensively reviewed in GRNs 236, 286, and 334, and by the Scientific Committee on Food (2001) and FSANZ (2008). It is generally recognized that with the exception of lactose, which is hydrolyzed by small intestinal brush border lactase, beta-linked sugars are not digested by human pancreatic or intestinal enzymes (Ohtsuka et al., 1990; Wisker et al., 1985; Chonan et al., 2004). 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, 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) (cited in GRN 620;. Ohtsuka et al., 1991; Suarez et al., 1999; Smiricky-Tjardes et al., 2003). Importantly, short-chain fatty acids, carbon dioxide, methane and hydrogen are the same metabolites as those produced by the microbiota following the ingestion of other foods and are either absorbed, exhaled, or excreted (reviewed in Slavin, 2013). Therefore, VITAGOS™ is expected to be unabsorbed followed by fermentation by the microflora in the colon, producing short chain fatty acids, CO_2 and H_2 gas.

B. GENOTOXICITY STUDIES

The genotoxicity of GOS-containing products has been extensively reviewed in GRNs 334 and 620. As summarized in GRN 334, Kobayashi et al. (2009) showed that GOS are not mutagenic, genotoxic, or clastogenic using a bacterial reverse mutation, a chromosomal aberration assay, and an *in vivo* micronucleus study. As summarized in GRN 620, Narumi et al. (2014) showed that GOS are not genotoxic using an *in vivo* comet assay. In addition, two non-publicly available bacterial reverse mutation assay and an *in vitro* micronucleus assay were reviewed in GRN 620 and, importantly, corroborate the lack of genotoxicity reported by Kobayashi et al. (2009) and Narumi et al (2014). Therefore, VITAGOS™ is not genotoxic.

C. TOXICOLOGY STUDIES

1. Subchronic Study Conducted with VITAGOS™ (Zhou et al., 2017)

A subchronic toxicity of VITAGOS™ GOS was assessed in Sprague-Dawley rats (10 per sex per group) for 90 consecutive days by oral gavage at 0, 1020, 2041, and 4082 mg GOS syrup/kg/day, which resulted in daily intakes of 0, 500, 1000, and 2000 mg GOS/kg/day. The study was performed in accordance with cGLP [OECD c(97)/186Final and US FDA (21 CFR Part 58)] and as per OECD guideline No. 408.

General clinical observations were performed once daily and morbidity/mortality assessments were performed twice daily. Detailed clinical examination was done on Day 1 prior to treatment of test article and weekly thereafter. Ophthalmological examination was carried out prior to treatment and prior to the animals being euthanized. Individual body weight was recorded on Day 1 prior to test article administration and at weekly intervals. Fasting (12 hrs) body weight was recorded prior to termination. Feed consumption was measured at weekly intervals. On Day 91, blood was collected for hematology, coagulation, and clinical chemistry evaluations and urine was collected for urinalysis. All animals were then euthanized by exsanguination and were subjected to detailed necropsy and gross pathological examination. Selected tissues and organs were collected, weighed, and preserved from all animals. In accordance with the OECD No. 408 guidelines, histopathological examination was carried out on all the preserved organs and tissue of control and high-dose (2000 mg GOS /kg/day) group animals. Additionally, the cecum was examined in the respective lower-groups (500 and 1000 mg GOS /kg/day) as test article-related histopathological change was observed in the high-dose group.

There were no deaths, relevant clinical signs, or abnormal ophthalmological findings reported at any dose levels in this study. Body weight and feed consumption were reduced at 1000 and 2000 mg/kg/day doses in males but not females. The reductions in body weight were not considered clinically adverse since they were less than 10% relative to controls. The reductions in feed consumption were considered a test article related non-adverse finding as the lower feed consumption did not result in any clinical signs during the in-life phase of the treatment period. Most importantly, no test article-related effect was seen on feed efficiency (Table 10). Therefore, the reductions in body weight and feed consumption may be due to poor acceptability rather than less efficient utilization of the diets.

Table 10. Mean Feed Efficiency (Body Weight Gain/Feed Consumed) of Male and Female Rats

Days	G1, 0 mg/kg/day		G2, 500 mg/kg/day		G3, 1000 mg/kg/day		G4, 2000 mg/kg/day	
	Males n=10	Females n=10	Males n=10	Females n=10	Males n=10	Females n=10	Males n=10	Females n=10
1-8	0.20 ± 0.022	0.12 ± 0.018	0.21 ± 0.015	0.13 ± 0.029	0.20 ± 0.034	0.13 ± 0.026	0.18 ± 0.009	0.15 ± 0.037
8-15	0.15 ± 0.018	0.10 ± 0.014	0.17 ± 0.015	0.11 ± 0.018	0.15 ± 0.032	0.11 ± 0.046	0.16 ± 0.022	0.11 ± 0.018
15-22	0.15 ± 0.005	0.08 ± 0.032	0.13 ± 0.019[a]	0.08 ± 0.031	0.13 ± 0.014[a]	0.10 ± 0.046	0.12 ± 0.008[a]	0.09 ± 0.015
22-29	0.14 ± 0.019	0.09 ± 0.027	0.13 ± 0.029	0.07 ± 0.015	0.12 ± 0.022	0.06 ± 0.035	0.11 ± 0.030	0.07 ± 0.045
29-36	0.07 ± 0.015	0.03 ± 0.077	0.08 ± 0.013	0.06 ± 0.019	0.06 ± 0.021	0.07 ± 0.025	0.08 ± 0.019	0.05 ± 0.030
36-43	0.09 ± 0.015	0.05 ± 0.037	0.08 ± 0.007	0.06 ± 0.036	0.06 ± 0.013	0.04 ± 0.024	0.07 ± 0.022	0.04 ± 0.027
43-50	0.08 ± 0.008	0.08 ± 0.012	0.08 ± 0.015	0.07 ± 0.039	0.09 ± 0.010	0.06 ± 0.030	0.06 ± 0.027	0.05 ± 0.037
50-57	0.07 ± 0.008	0.01 ± 0.037	0.02 ± 0.025[a]	0.00 ± 0.042	0.06 ± 0.015	0.04 ± 0.023	0.07 ± 0.025	0.03 ± 0.020
57-64	0.06 ± 0.010	0.04 ± 0.047	0.08 ± 0.021	0.05 ± 0.034	0.06 ± 0.020	0.03 ± 0.040	0.03 ± 0.030	0.06 ± 0.037
64-71	0.05 ± 0.013	0.07 ± 0.031	0.05 ± 0.020	0.05 ± 0.014	0.05 ± 0.010	0.05 ± 0.019	0.05 ± 0.018	0.03 ± 0.020
71-78	0.06 ± 0.015	0.02 ± 0.027	0.05 ± 0.020	0.03 ± 0.033	0.04 ± 0.013	0.04 ± 0.033	0.04 ± 0.024	0.03 ± 0.025
78-85	0.01 ± 0.013	0.01 ± 0.019	0.02 ± 0.010	0.00 ± 0.046	0.01 ± 0.023	0.00 ± 0.023	-0.02 ± 0.022	0.01 ± 0.032
85-90	0.04 ± 0.029	0.03 ± 0.026	0.04 ± 0.012	0.04 ± 0.037	0.04 ±0.043	0.02 ± 0.031	0.07 ± 0.022	0.02 ± 0.060

[a]Significantly lower than the control group (G1) at $p < 0.05$

There were no test article-related adverse changes reported in hematology, coagulation, serum clinical chemistry, or urine parameters in either sex in test groups compared with controls. In hematology (Table 11), decreased absolute neutrophil count in all treated groups in males was considered incidental and likely due to random biological variation as there was no dose correlation and it only occurred in male animals. In coagulation parameters, changes of PT values in all treated males and APTT values in all treated females were considered as incidental because there was no clear dose correlation, no consistency between sexes, and no related changes in the related hematology parameter platelet counts. In the clinical chemistry (Table 12), an increased concentration of blood urea nitrogen in males at 1000 and 2000 mg/kg/day and in females at 1000 mg/kg/day was noted, but the levels of blood urea nitrogen were within historical values obtained from control rats of this age and strain (3.84-8.85 mmol/L, n=99). These changes were not consistent between sexes. In addition, the differences in plasma calcium levels and sodium levels in treated rats compared to controls were considered a non-adverse incidental effect because the findings were not dose-related and were well within historical values obtained from the rats of this age and strain (calcium historical control range: 1.47-6.48 mmol/L, n =99) (sodium historical control range: 134.60-151.10 mEq/L, n=99).

Table 11. Mean Hematology and Coagulation Values

Parameter	G1, 0 mg/kg/day		G2, 500 mg/kg/day		G3, 1000 mg/kg/day		G4, 2000 mg/kg/day	
	Males n=10	Females n=10	Males n=10	Females n=10	Males n=10	Females n=10	Males n=10	Females n=10
Red blood cells (10^{12}/L)	9.30 ± 0.39	8.35 ± 0.16	9.31 ± 0.30	8.28 ± 0.41	9.36 ± 0.28	8.26 ± 0.22	9.49 ± 0.28	8.44 ± 0.25
Hemoglobin (g/L)	157 ± 2	149 ± 3	156 ± 2	152 ± 4	160 ± 4	150 ± 4	159 ± 5	151 ± 5
Hematocrit (L/L)	0.509 ± 0.013	0.480 ± 0.012	0.508 ± 0.015	0.479 ± 0.021	0.521 ± 0.015	0.480 ± 0.017	0.518 ± 0.018	0.486 ± 0.013
Mean corpuscular volume (fL)	54.8 ± 2.6	57.35 ± 1.4	54.6 ± 1.7	57.9 ± 1.3	55.6 ± 2.1	58.2 ± 1.8	54.7 ± 1.5	57.5 ± 1.7
Mean corpuscular hemoglobin (pg)	16.9 ± 0.6	17.9 ± 0.4	16.8 ± 0.5	18.4 ± 0.7	17.1 ± 0.5	18.1 ± 0.6	16.8 ± 0.4	17.9 ± 0.6
Mean corpuscular hemoglobin concentration (g/L)	309 ± 6	311 ± 3	308 ± 7	317 ± 13	307 ± 4	312 ± 6	307 ± 3	311 ± 5
Reticulocytes (10^{12}/L)	0.189 ± 0.045	0.202 ±0.055	0.184 ± 0.025	0.195 ± 0.051	0.158 ± 0.019	0.129 ± 0.045[a]	0.185 ± 0.023	0.175 ± 0.059
Reticulocytes (%)	2.04 ± 0.49	2.42 ± 0.65	1.97 ± 0.26	2.36 ± 0.65	1.69 ± 0.23	1.57 ± 0.55[a]	1.96 ± 0.28	2.09 ± 0.74
Platelets (10^9/L)	820 ± 79	1007 ± 157	898 ± 138	977 ± 109	907 ± 93	969 ± 141	916 ± 127	1045 ± 118
Mean platelet volume (fL)	10.0 ± 0.8	9.8 ± 0.3	9.9 ± 0.2	9.8 ± 0.6	10.2 ± 0.3	10.1 ± 0.6	10.4 ± 0.3	10.4 ± 0.3[b]
White blood corpuscles (10^9/L)	8.45 ± 1.00	6.19 ± 1.31	6.92 ± 1.07[a]	6.89 ± 1.21	7.78 ± 0.99	5.4 ± 0.79	8.13 ± 1.11	6.16 ± 1.03
Neutrophils (10^9/L)	2.07 ± 0.52	0.98 ± 0.35	1.45 ± 0.23[a]	1.00 ± 0.29	1.41 ± 0.45[a]	0.78 ± 0.14	1.54 ± 0.48[a]	0.97 ± 0.46
Lymphocytes (10^9/L)	5.93 ± 0.60	4.92 ± 1.07	5.15 ± 0.98	5.6 ± 0.95	6.05 ± 0.75	4.37 ± 0.65	6.17 ± 0.68	4.92 ± 0.94
Monocytes (10^9/L)	0.23 ± 0.07	0.15 ± 0.03	0.17 ± 0.04	0.15 ± 0.03	0.17 ± 0.04	0.12 ± 0.04	0.20 ± 0.05	0.12 ± 0.03
Basophils (10^9/L)	0.01 ± 0.00	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.00 ± 0.00	0.01 ± 0.01	0.00 ± 0.00
Eosinophils (10^9/L)	0.18 ± 0.14	0.11 ± 0.02	0.11 ± 0.06	0.09 ± 0.03	0.10 ± 0.04	0.09 ± 0.04	0.15 ± 0.10	0.10 ± 0.03
Prothrombin time (PT; (seconds)	17.7 ± 0.6	17.8 ± 0.5	16.6 ± 1.0[a]	17.4 ± 1.2	16.7 ± 0.6[a]	17.3 ± 0.5	16.6 ± 0.9[a]	16.5 ± 0.6[a]
Activated partial thromboplastin time (APTT; seconds)	14.9 ± 3.2	10.1 ± 1.6	13.3 ± 2.8	14.5 ± 3.1[b]	14.7 ± 2.8	13.8 ± 3.6[b]	13.1 ± 1.7	14.5 ± 2.6[b]

[a]Significantly lower than the control group G1 at $p < 0.05$
[b]Significantly higher than the control group G1 at $p < 0.05$

Table 12. Values of Clinical Chemistry								
	G1, 0 mg/kg/day		G2, 500 mg/kg/day		G3, 1000 mg/kg/day		G4, 2000 mg/kg/day	
Parameter	Males n=10	Females n=10	Males n=10	Females n=10	Males n=10	Females n=10	Males n=10	Females n=10
Glucose (mmol/L)	5.36 ± 0.65	5.26 ± 0.55	5.24 ± 0.48	5.57 ± 0.55	5.03 ± 0.40	5.37 ± 0.44	4.97 ± 0.50	5.32 ± 0.49
Blood urea nitrogen (mmol/L)	5.10 ± 0.56	6.00 ± 0.46	5.60 ± 0.44	5.66 ± 0.72	5.86 ± 0.85[a]	7.21 ± 0.66[a]	6.30 ± 0.58[a]	6.61 ± 0.59
Creatinine (µmol/L)	36 ± 4	46 ± 4	40 ± 5	43 ± 3	37 ± 5	47 ± 4	37 ± 4	42 ± 5
Aspartate amino transferase (U/L)	94 ± 13	95 ± 6	92 ± 9	95 ± 8	93 ± 6	94 ± 7	101 ± 8	101 ± 18
Alanine amino transferase (U/L)	59 ± 7	44 ± 3	56 ± 9	41 ± 4	57 ± 8	43 ± 7	63 ± 9	48 ± 8
Gamma glutamyl transpeptidase (U/L)	4 ± 1	4 ± 0	4 ± 1	4 ± 1	4 ± 1	4 ± 0	4 ± 1	4 ± 1
Alkaline phosphatase (U/L)	77 ± 11	55 ± 6	77 ± 6	60 ± 12	82 ± 9	55 ± 9	86 ± 14	53 ± 7
Creatinine kinase (U/L)	211 ± 65	236 ± 27	219 ± 64	221 ± 56	198 ± 41	179 ± 40[b]	203 ± 29	220 ± 86
Total bilirubin (µmol/L)	2.97 ± 0.58	3.38 ± 0.75	2.31 ± 0.98[b]	2.58 ± 0.64[b]	2.27 ± 0.48	2.51 ± 0.63[b]	2.36 ± 0.39	2.48 ± 0.61[b]
Total cholesterol (mmol/L)	2.99 ± 0.36	3.33 ± 0.27	2.85 ± 0.33	3.18 ± 0.29	2.66 ± 0.28	3.51 ± 0.37	2.47 ± 0.24[b]	3.16 ± 0.32
Triglyceride (mmol/L)	0.66 ± 0.17	0.54 ± 0.16	0.74 ± 0.28	0.36 ± 0.22[b]	0.54 ± 0.14	0.52 ± 0.11	0.47 ± 0.08[b]	0.43 ± 0.12
Total plasma protein (g/L)	71.2 ± 1.3	71.1 ± 2.1	72.4 ± 2.0	70.5 ± 2.2	72.7 ± 1.8	73.3 ± 2.4	71.5 ± 2.7	72.4 ± 3.7
Albumin (g/L)	31.7 ± 0.7	33.7 ± 1.1	32.2 ± 0.8	33.1 ± 1.2	32.8 ± 0.8[b]	34.9 ± 1.2	32.8 ± 1.2[b]	33.9 ± 1.7
Globulin (g/L)	39.5 ± 1.1	37.4 ± 1.5	40.2 ± 1.3	37.4 ± 1.4	39.9 ± 1.3	38.5 ± 1.4	38.7 ± 1.6	38.4 ± 2.2
Albumin/Globulin (ratio)	0.80 ± 0.03	0.90 ± 0.04	0.80 ± 0.02	0.89 ± 0.03	0.82 ± 0.02	0.91 ± 0.02	[a]0.85 ± 0.02	0.88 ± 0.03
Inorganic phosphorous (mmol/L)	1.94 ± 0.17	1.79 ± 0.25	1.82 ± 0.09	1.89 ± 0.20	1.94 ± 0.19	1.78 ± 0.16	2.02 ± 0.09	1.76 ± 0.18
Calcium (mmol/L)	2.47 ± 0.06	2.39 ± 0.04	2.30 ± 0.08[b]	2.29 ± 0.09[b]	2.14 ± 0.12[b]	2.13 ± 0.08[b]	2.15 ± 0.07[b]	2.13 ± 0.12[b]
Sodium (mEq/L)	141.6 ± 1.2	141.4 ± 1.3	143.5 ± 1.1[a]	142.1 ± 1.7	144.0 ± 1.7[a]	143.0 ± 1.5[a]	145.4 ± 1.4[a]	144.3 ± 1.0[a]
Potassium (mEq/L)	3.73 ± 0.13	3.26 ± 0.27	3.71 ± 0.17	3.40 ± 0.21	3.52 ± 0.21	3.40 ± 0.21	3.65 ± 0.20	3.51 ± 0.21
Chloride (mEq/L)	108.8 ± 1.1	108.3 ± 1.1	109.2 ± 0.9	190.0 ± 1.7	109.0 ± 1.6	108.7 ± 1.6	110.5 ± 1.2[a]	111.3 ± 1.4[a]

[a]Significantly higher than the control group at $p < 0.05$.
[b]Significantly lower than the control group at $p < 0.05$.

No adverse effects were seen on absolute or relative organ weights and no changes in gross or histopathology were seen except for increased absolute and relative weights of the caecum with and without contents at the highest dose only in males and females (Table 13). This change was considered test article-related as it was microscopically associated with mucosal hypertrophy/hyperplasia (high dose males, 8/10 hypertrophy/hyperplasia with 5 minimal, 3 mild; high dose females, 7/10 hypertrophy/hyperplasia with 4 minimal, 3 mild). It was noted that the brain weight relative to body weight in 1000 and 2000 mg/kg/day groups of males were significantly increased as compared to the control group, but the increase was not dose-dependent. Because the absolute weight of brains in these two groups were not different from the control group, the reduced terminal body weight of these two groups may have been the cause of the increase in the brain relative to body weight. Thus, this observation was considered an incidental change and not associated with any microscopic changes in the histopathological examination. In addition, absolute and relative to brain weights of liver in males at 1000 mg/kg/day and absolute and relative to body and brain weights of liver in males at 2000 mg/kg/day were significantly reduced as compared to the control. No histopathological findings were seen in the livers of males except one minimal necrosis in the control and one minimal inflammatory focus in the 2000 mg/kg/day group. In addition, no adverse findings were noted in these two groups of male rats in terms of clinical chemistry parameters that are related to compromised liver function, such as T. Bil, ALT, AST, ALP, and GGT (Gowda et al., 2009). No changes in liver weights were noted in treated females. Incidental reduction in liver weight was seen in females given 2500 mg GOS/kg/day in a study of another GOS product (Anthony et al. 2006). Thus, the reductions of liver weight in males at 1000 and 2000 mg/kg/day dose from this study were not considered to be of toxicological significance. Other statistically significant changes in organ weights were not considered to be test article-related adverse effects because they were single incidences.

Because the caecum is an area of significant bacterial fermentation, cecal hypertrophy/hyperplasia is thought to occur because of the increased amounts of short chain fatty acids that are produced by bacterial fermentation after large amounts of non-adsorbed carbohydrate and dietary fiber enter the caecum and colon (Levine, 1991; Demigne and Remesy 1985; Lupton and Kurtz 1993). Increased concentrations of short chain fatty acids and enhanced ion absorption due to decreased pH can alter the osmotic balance of the GI tract and enhance the fluid volume of enterocytes. Therefore, in the current study, the histological change seen in the cecum of high dose animals, although related to test article administration, is considered an adaptive rather than toxic response.

Table 13. Organ Weight of Male Rats

	G1, 0 mg/kg/day			G2, 500 mg/kg/day			G3, 1000 mg/kg/day			G4, 2000 mg/kg/day		
Organ	Abs	Relative to Body Weight	Relative to Brain	Abs	Relative to Body Weight	Relative to Brain	Abs	Relative to Body Weight	Relative to Brain	Abs	Relative to Body Weight	Relative to Brain
Terminal fasting body weight (g)	410.35 ± 21.17	-	-	404.99 ± 25.30	-	-	379.82 ± 21.09[b]	-	-	375.45 ± 24.13[b]	-	-
Adrenals (g)	0.0533 ± 0.0057	0.0130 ± 0.0015	2.6879 ± 0.3251	0.0526 ± 0.0054	0.0130 ± 0.0012	2.7211 ± 0.2910	0.0542 ± 0.0061	0.0143 ± 0.0015	2.7397 ± 0.2689	0.0496 ± 0.0039	0.0132 ± 0.0007	2.5406 ± 0.1715
Brain (g)	1.9856 ± 0.0668	0.4846 ± 0.0214	-	1.9346 ± 0.0947	0.4783 ± 0.0187	-	1.9774 ± 0.0593	0.5220 ± 0.0314[a]	-	1.9543 ± 0.0879	0.5216 ± 0.0258[a]	-
Cecum with content (g)	3.8003 ± 0.7685	0.932 ± 0.215	192.205 ± 42.587	3.7213 ± 0.9320	0.921 ± 0.229	192.922 ± 49.343	4.6803 ± 0.5799	1.235 ± 0.162[a]	236.806 ± 29.825	5.1308 ± 1.2295[a]	1.372 ± 0.336[a]	262.866 ± 63.005[a]
Cecum without content (g)	1.0938 ± 0.1264	0.266 ± 0.024	55.077 ± 5.999	1.1419 ± 0.1563	0.282 ± 0.032	59.053 ± 7.775	1.2433 ± 0.1897	0.328 ± 0.049[a]	63.031 ± 10.446	1.3541 ± 0.1385[a]	0.361 ± 0.029[a]	69.357 ± 7.074[a]
Epididymides (g)	1.3643 ± 0.1264	0.3328 ± 0.0299	68.7173 ± 6.1035	1.3339 ± 0.1485	0.3298 ± 0.0362	68.9905 ± 7.4278	1.2990 ± 0.1704	0.3417 ± 0.0393	65.6413 ± 7.9133	1.3224 ± 0.0760	0.3529 ± 0.0202	67.7201 ± 3.8274
Heart (g)	1.3026 ± 0.0650	0.3177 ± 0.0140	65.6208 ± 2.9566	1.2680 ± 0.0900	0.3132 ± 0.0153	65.5345 ± 3.3326	1.1975 ± 0.1062[b]	0.3156 ± 0.0249	60.5204 ± 4.4850[b]	1.2107 ± 0.1000	0.3223 ± 0.0127	61.9179 ± 3.8052
Kidneys (g)	2.4511 ± 0.2902	0.5964 ± 0.0544	123.3483 ± 13.2567	2.4100 ± 0.2004	0.5950 ± 0.0322	124.4552 ± 6.2574	2.2962 ± 0.1880	0.6048 ± 0.0396	116.1132 ± 8.6270	2.2462 ± 0.2058	0.5980 ± 0.0334	114.7811 ± 6.6965
Liver (g)	10.3398 ± 0.7257	2.5192 ± 0.1039	520.8712 ± 34.8687	10.1220 ± 1.0965	2.4973 ± 0.1820	522.6148 ± 41.4108	9.1957 ± 0.7975[b]	2.4202 ± 0.1452	465.0234 ± 37.1198[b]	8.7667 ± 0.9153[b]	2.3332 ± 0.1627[b]	448.0115 ± 35.0781[b]
Lungs (g)	1.8906 ± 0.1254	0.4623 ± 0.0266	95.6305 ± 5.7684	1.8822 ± 0.1771	0.4650 ± 0.0380	97.3380 ± 8.5463	1.7779 ± 0.1154	0.4686 ± 0.0277	89.8684 ± 4.2526	1.7717 ± 0.1256	0.4720 ± 0.0181	90.5892 ± 3.4710
Pituitary (g)	0.0138 ± 0.0014	0.0034 ± 0.0003	0.6942 ± 0.0755	0.0129 ± 0.0022	0.0032 ± 0.0005	0.6643 ± 0.1009	0.0129 ± 0.0015	0.0034 ± 0.0003	0.6542 ± 0.0760	0.0133 ± 0.0021	0.0035 ± 0.0005	0.6814 ± 0.1137
Prostate (g)	1.1110 ± 0.1875	0.2714 ± 0.0472	55.9817 ± 9.3903	1.1338 ± 0.1386	0.2804 ± 0.0344	58.6542 ± 7.1842	1.1332 ± 0.1308	0.2987 ± 0.0348	57.2497 ± 5.8199	1.0961 ± 0.1179	0.2935 ± 0.0403	56.2409 ± 7.0662
Seminal vesicles and coagulating glands (g)	1.8898 ± 0.2393	0.4601 ± 0.0493	95.0919 ± 10.7924	1.7950 ± 0.2785	0.4421 ± 0.0560	92.8364 ± 13.9992	1.9087 ± 0.2025	0.5031 ± 0.0516	96.5170 ± 9.7606	1.8562 ± 0.2769	0.4948 ± 0.0705	95.1747 ± 14.9460
Spleen (g)	0.7646 ± 0.0674	0.1863 ± 0.0125	38.5305 ± 3.4031	0.7233 ± 0.0828	0.1791 ± 0.0221	37.4411 ± 4.4660	0.6675 ± 0.0915	0.1755 ± 0.0192	33.7264 ± 4.1850[b]	0.6714 ± 0.1010	0.1786 ± 0.0214	34.2927 ± 4.2832
Testes (g)	3.9364 ± 0.2414	0.9611 ± 0.0700	198.3755 ± 12.7308	3.7731 ± 0.5431	0.9332 ± 0.1383	195.2016 ± 28.1322	3.6621 ± 0.6412	0.9629 ± 0.1649	185.1202 ± 31.6020	3.9015 ± 0.2850	1.0404 ± 0.0643	199.7008 ± 13.0502
Thymus (g)	0.2566 ± 0.0465	0.0626 ± 0.0108	12.9702 ± 2.5722	0.2665 ± 0.0563	0.0661 ± 0.0155	13.8596 ± 3.3186	0.2316 ± 0.0490	0.0611 ± 0.0128	11.6819 ± 2.3178	0.2057 ± 0.0337	0.0549 ± 0.0091	10.5278 ± 1.6995

Table 13. Organ Weight of Male Rats

	G1, 0 mg/kg/day			G2, 500 mg/kg/day			G3, 1000 mg/kg/day			G4, 2000 mg/kg/day		
Organ	Abs	Relative to Body Weight	Relative to Brain	Abs	Relative to Body Weight	Relative to Brain	Abs	Relative to Body Weight	Relative to Brain	Abs	Relative to Body Weight	Relative to Brain
Thyroid with parathyroids (g)	0.0300 ± 0.0059	0.0073 ± 0.0013	1.5089 ± 0.2780	0.0271 ± 0.0046	0.0067 ± 0.0012	1.4030 ± 0.2366	0.0326 ± 0.0063	0.0086 ± 0.0017	1.6523 ± 0.3406	0.0300 ± 0.0059	0.0080 ± 0.0016	1.5410 ± 0.3156

[a]Significantly higher than the control group at $p < 0.05$.
[b]Significantly lower than the control group at $p < 0.05$.

Cecal enlargement is a common finding in toxicology studies of GOS products. An oral dose of 2000 mg Oligomate (55% GOS)/kg/day (825 mg GOS/kg/d) in rats increased cecum weight over 90 days (Kobayashi et al., 2009). Cecal enlargement was also seen in male and female Wistar rats when fed GOS syrup in diets at levels of 1600, 3200, and 6100 mg/kg/day (684, 1368, and 2608 mg GOS/kg/day), and 1800, 3600, and 6900 mg/kg/day (770, 1539, and 2959 mg GOS/kg/day) in female and male rats, respectively (Lina 1995 in GRN 236). In general, feeding GOS at levels from 2.96-20% in the diet of rats will result in an increased cecum weight (Kobayashi et al. 2009; Kawakami et al. 2005; Chonan et al. 2001; Djouzi and Andrieux 1997; Kikuchi-Hayakawa et al. 1997; Chonan and Watanuki 1996; Kikuchi et al. 1996; Chonan et al. 1995; Chonan and Watanuki 1995; Hayashi et al. 1991; Ohtsuka et al. 1990). Chonan and Watanuki (1996) also found cecal hypertrophy after administering approximately 650 mg GOS/kg/day for 30 days. Feeding a diet containing 2% GOS has a similar effect on cecal weight increase in pigs (Houdijk et al. 2002). GOS-related effects reported in studies of GOS products (cecal weight increase) 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 cecum is not found in humans, although in rats, it is the site of fermentation for non-digestible substances. Therefore, cecal enlargement along with mucosal hypertrophy and hyperplasia has been observed as a response in several rodent species to feed ingredients other than GOS such as modified starches, polyols, some fibers, and lactose; these ingredients share the feature of being poorly absorbed and osmotically active (Haschek et al., 2010). Many studies have demonstrated that consumption of pectin (Adam et al., 2015), maltitols, glucomannan, cellulose (Konishi et al., 1984; Oku, 1997; Oku, 1998), fructans (Nzeusseu, 2006), and wheat bran (Jacobs and Schneeman, 1981) can cause mucosal hyperplasia/hypertrophy of caecum/colon in rats. It is noted that enhanced colonic mucosal growth (hypertrophy/hyperplasia) was found in rats fed dietary fiber cellulose and wheat bran that have been previously shown to inhibit the development of genotoxin-induced colonic neoplasia in rats (Whiteley et al., 1996). It has also been reported that mucosal hypertrophy in rodents represents a physiological adaptation to increased osmotic forces when high doses of indigestible substances are consumed; the effect is reversible after test article is withdrawn from the diet (Greaves, 2012; Haschek et al., 2010; Newberne et al., 1988).

The histopathlogic features of the cecal enlargement noted in the current study are indicative of an adaptive response. Mucosal hypertrophy/hyperplasia were characterized by an increase in cell density and mitotic activity within the crypts involving diffuse areas of the mucosa and/or by the presence of elongated mucosal glands with increased height of surface columnar cells. The cytoplasm of epithelial cells had increased basophilia with slightly elongated

or vesicular nuclei. Importantly, there were no polyps observed in the caecum. These pathologic features are produced by other non-digestible substances which produce characteristic increases in cecal crypt depth, circumference, number of crypts and number of cells per crypt in the cecum (Lupton and Kurtz 1993).

Therefore, hypertrophy/hyperplasia without atypical cellular features represents a compensatory and adaptive response to a large amount of GOS, consistent with the effects seen with other poorly absorbable carbohydrates (Greaves, 2012). Thus, the observed cecal hypertrophy/hyperplasia, without evidence of polyps, is considered compensatory and not pre-neoplastic and, although test article-related, is not considered to be a toxic response.

In conclusion, the NOAEL for VITAGOS™ following oral gavage is 4,082 mg/kg body weight/day (2,000 mg GOS/kg/day) under the test conditions employed.

2. Corroborative Subchronic Toxicology Studies

In GRN 236, a corroborative 90-day toxicology study conducted by Anthony et al. (2006) using a GOS product manufactured with β-galactosidase derived from *Bacillus circulans* was reviewed. In this study, male and female Sprague Dawley rats were gavaged with either water, 2500, or 5000 mg GOS syrup/kg/day containing approximately 45% GOS (% of total weight), resulting in daily intake of 1125 and 2250 mg GOS/kg/day. Although there was a significant decrease in feed intake in the rats that had been treated with 5000 mg GOS syrup/kg/day compared to the group receiving water, the NOAEL was set at 5000 mg/kg/day of the GOS product (equivalent to 2250 mg GOS/kg/day) due to the lack of toxicologically relevant effects on other parameters such as clinical observations, gross necropsies, organ weights, and histological examinations.

In GRN 334, another corroborative 90-day toxicology conducted by Kobayashi et al. (2009) using a GOS product manufactured with β-galactosidases derived from *S. singularis* and *K. lactis* was reviewed. Male and female Sprague Dawley rats were gavaged with water, 500, 1000, or 2000 mg/kg/day of a syrup containing approximately 55% GOS (% dry matter), resulting in a daily intake of 206.25, 412.5, 825 mg GOS/kg. There were no GOS-related changes in clinical signs, body weight, water intake, feed intake, urinalysis, ophthalmology, hematology, blood chemistry, organ weights or cecum weight, gross pathology, or histopathology. The relative and absolute weight of the cecum was higher in the 2000 mg/kg/day male group compared to control. The NOAEL was set to 2000 mg/kg/day for the GOS-containing product equivalent to 825 mg GOS/kg/day.

In GRN 620, an unpublished 30-day study in adult rats using a GOS product manufactured with a β-galactosidase derived from *A. oryzae* and 42-day study in juvenile rats conducted by Kobayashi et al. (2014a) using a GOS product manufactured with β-galactosidases derived from *S. singularis* and *K. lactis* were summarized. In the unpublished 30-day study, male and female rats were gavaged with 0, 500, 1000, or 2000 mg/kg/day of a GOS-containing product (46% oligosaccharides), resulting in a daily intake of 0, 230, 450, 900 mg GOS/kg/day. There were no deaths, relevant clinical signs, or GOS-related ophthalmological findings reported during the study. There were also no differences in body weight, food consumption, organ weight, macroscopic, histopathological changes, hematology, coagulation, serum clinical chemistry, or urine parameters between groups. The NOAEL was determined to be 2000 mg GOS product/kg/day equivalent to 900 mg GOS/kg/day.

As summarized in GRNs 484, 495, 518, and 569, Desbuards et al. (2012) evaluated the effects of perinatal GOS in pregnant mice and their offspring. Pregnant BALB/cj mice were fed a control diet or a diet supplemented with a prebiotic mixture containing approximately 1620 mg GOS and 400 mg inulin/kg body weight/day, which was calculated based on reported maternal feed intake, body weight values, and GOS content of the diet (approximately 45%), during gestation and lactation. Although the study was not conducted in accordance with good laboratory practices (GLP) and deviated from internationally accepted guidelines, there were no significant differences in maternal body weight gain or feed intake during pregnancy between the two groups. There were also no differences in the number of offspring per dam between the two groups. In the pups, the body weights of the GOS/inulin-treated male pups were significantly higher at weaning and at days 2, 40, and 48 post-weaning compared to the male pups receiving the control diet. Body length, colon length, and relative thigh muscle weight were also significantly higher in the GOS/inulin-treated male pups. No other developmental or reproductive toxicological endpoints were examined.

3. Corroborative Reproductive and Developmental Toxicology Studies

Additional toxicology studies that corroborate the safety of VITAGOS™ include a neonatal rodent toxicity study conducted in juvenile rats and a one-generation reproductive and developmental toxicity study (Kobayashi et al., 2014a,b).

In the study by Kobayashi et al. (2014a), juvenile Sprague-Dawley rats were administered the GOS product that was the subject of GRN 334 by gavage for 42 days starting on post-natal day 4 at 0, 500, 1000, or 2000 mg/kg/day of a syrup containing approximately 56.9 % GOS (% dry matter), resulting in a daily intake of 213.4, 5426.8, and 853.5 mg GOS/kg. 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 equivalent to 853.5 mg GOS/kg/day (Kobayashi et al., 2014a).

Kobayashi et al. (2014b) evaluated the developmental and reproductive effects of the GOS product that was the subject of GRN 334 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 of a syrup containing approximately 56.9 % GOS (% dry matter), resulting in a daily intake of 213.4, 426.8, and 853.5 mg GOS/kg/day. 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 GOS per kg/day equivalent to 853.5 mg GOS/kg/day (Kobayashi et al., 2014b).

4. Other Studies

Three additional published animal studies were summarized in GRN 620 and corroborate the safe ingestion of GOS (Verheijden et al., 2015; Hogenkamp et al., 2015; and Morel et al., 2015).

Verheijden et al. (2015) investigated the effect of GOS on the development of allergy in adult mice. Mice were maintained on a control diet or a diet containing 1% GOS and after two weeks all mice were sensitized to house dust mite with an intranasal administration of 1 µg house dust mite. Seven to 10 days later the mice were challenged intranasally with either phosphate buffered saline or 10 µg house dust mite, and airway hyperresponsiveness was quantified using EMKA invasive measurement of dynamic resistance (EMKA Technologies), and bronchoalveolar lavage fluid cell counts and cytokine and chemokine levels. Compared to control diet, the GOS-containing diet reduced airway hyperresponsiveness, bronchoalveolar fluid proinflammatory cytokine levels, and eosinophil cell counts, indicating that GOS may reduce the risk of developing allergy.

Hogenkamp et al. (2015) mated parental female mice that had been maintained on either a control diet or 3% GOS/FOS-supplemented diet (9 parts GOS, 1 part FOS) with male mice fed the control diet and continued the dietary intervention during gestation. At 6 weeks post-delivery, the male offspring were then sensitized to ovalbumin (OVA)-induced allergy with an intraperitoneal injection of 10 µg ovalbumin adsorbed into 22.5% aluminum hydroxide. The allergic response was then provoked 8 wk later with a subcutaneous injection of 12.5 µg OVA in the pinnae of one ear. The other ear was injected with saline. The reaction was quantified by measuring ear swelling. One week later, the offspring were challenged 3 times with 10 g/L of nebulized OVA and airway responsiveness (lung resistance) was quantified. Importantly, the offspring of the GOS/FOS-treated dams showed significantly reduced increases in ear swelling compared to the offspring derived from the females that received the control diet. In addition, although there were not enough control mice in the study to determine the significance of the GOS/FOS diet on airway responsiveness, the airway hyperresponsiveness was less in the offspring derived from the GOS/FOS-treated dams.

Morel et al. (2015) characterized the cecal microbiota of suckling rats that had been gavaged with vehicle or a GOS/FOS mixture (9:1 ratio), which delivered approximately 2.25 g GOS/kg/day, from post-natal day 5 to 14. All rats were weaned to normal chow on day 21. Although no safety or tolerance endpoints were assessed, the GOS/FOS mixture increased bifidobacteria and decreased firmicutes counts at day 14. By day 131 the distribution of the microbiota in the GOS/FOS group resembled that of the suckling rats that received the vehicle control.

D. CLINICAL STUDIES

Numerous clinical studies have been conducted in infants and adults to support the safe use of GOS in infant formulas and conventional foods. Importantly, these studies included endpoints that evaluated the effects of GOS on fecal microflora, gastrointestinal physiology, the immune system, and tolerance. Although some of the studies are unpublished, all have been extensively summarized in GRAS Notifications 236, 285, 286, 334, 484, 489, 495, 518, and 620. In general, GOS are well tolerated, and have been reported to increase the abundance of bifidobacteria and lactobacilli in the gastrointestinal tract in infants and adults, increase fecal short-chain fatty acid concentrations in infants and adults, improve stool consistency in infants, reduce the incidence of atopic dermatitis in infants, and alleviate the symptoms of irritable bowel syndrome in adults (Silk et al., 2009; Vulevic et al., 2008; Depeint et al., 2008; Bouhnik et al., 2004; Ito et al., 1990; Gopal et al., 2003; Fanaro et al., 2009; Walton et al., 2012; Moro et al., 2006; Schmelzle et al., 2003).

1. Studies Conducted in Infants

The use level of 7.2 g GOS/L in infant formulas was proposed by GTC Nutrition in GRN 286, which received a "no questions" letter from the FDA in 2009. To support the safety of GOS for the intended use, GTC summarized published clinical studies conducted in infants and showed the 7.2 g/L GOS in combination with 0.8g/L FOS had no adverse effects. Since then, the use level of GOS in infant formulas has been the subject of five additional GRAS notifications and the use levels have been increased to 7.8 g/L (GRN 334, GRN 489, GRN 495, GRN 569, GRN 620). All GRNs provided updates of the published studies the support the intended use of GOS in infant formulas and received "no questions" letters from FDA.

Since GRN 620 was filed with the FDA, two additional studies in healthy term infants, one study in term infants with a positive history of allergy, and one study in healthy children have been published (Matsuki et al., 2016; Civardi et al., 2015; Boženský et al., 2015; Pontes et al., 2016) The experimental details and results from these newly published studies are summarized in Table 14. Consistent with the results reported in the clinical trials summarized in previous GRAS Notifications, GOS-containing infant and follow-on formulas are well-tolerated.

Table 14. Recent Studies of GOS Ingestion in Infants

Reference	Study Design and Population	Treatments (Numbers of Subjects)	Duration	Safety Parameters
Civardi et al., 2015	Randomized, double-blind, placebo- controlled trial in healthy full-term infants (<12 months of age)	Group 1 (Control): Formula; n=59 Group 2: Formula enriched with 7g GOS/L, β-palmitate, and acidified milk; n=51	135 days	Withdrawals: • 62 subjects were enrolled in Group 1 • 55 subjects were enrolled in Group 2 • Compliance was similar for both groups • Three subjects were lost to follow-up in Group 1 • Four subjects were lost to follow-up in Group 2 Adverse Events: • Only gastrointestinal adverse events were evaluated; those that were reported were mild and there were no differences between the two groups. • No drop-outs due to adverse events occurred. • No severe adverse events were reported. Tolerance: • Mean number of stools/day was similar between the two groups. • Frequency of intestinal gas and bowel cramps were similar in both groups. Growth Parameters: • Weight change was similar in the two groups. • Length change was similar in the two groups. • Head circumference was similar in the two groups. Other Parameters: • Clostridia counts (determined by quantitative PCR) were similar in the two groups. • Bifidobacteria counts (determined by quantitative PCR) were significantly increased ($p < 0.05$) in Group 2.
Matsuki et al., 2016	Randomized, double-blind, placebo- controlled trial in healthy full-term infants (<12 months of age)	Group 1 (Control): Formula with dextrins; n=14 Group 2: Formula with 3 g GOS/L; n=16 Note: Supplementation with up to 20% breast milk was permitted	14 days	Withdrawals: • Eighteen subjects were enrolled Group 1 • Seventeen were enrolled in Group 2 • Compliance was similar for both groups. • Four subjects withdrew in Group 1. • One subject withdrew in Group 2. • Withdrawals were due to introduction of probiotics, change in feeding, or a parental decision. Adverse Events: • Adverse side effects were monitored and none were reported.

Table 14. Recent Studies of GOS Ingestion in Infants

Reference	Study Design and Population	Treatments (Numbers of Subjects)	Duration	Safety Parameters
				Tolerance: • There were no significant differences between the groups in fecal short-chain fatty acid levels, pH, or stool frequency. Other Parameters: • Abundance of bifidobacteria significantly increased ($p<0.05$) in the GOS-treated group compared to the control group. • At the species level, there were no significant chances in the bifidobacteria.
Bozensky et al., 2015	Randomized, placebo-controlled trial in term infants with a positive history of allergy (atopic eczema, allergic rhinitis, and/or asthma) in their parents or siblings (<12 months of age)	Group 1 (Control): Formula with hydrolyzed protein; n= 51 Group 2: Formula with hydrolyzed protein supplemented with 5g GOS/L; n=52	6 months	Withdrawals: • 60 subjects were enrolled in each group. • Compliance was similar for both groups. • Nine subjects withdrew in Group 1 (four did not adhere to the protocol; five discontinued due to intolerance. • Eight subjects withdrew in Group 2 (four did not adhere to the protocol; one was excluded due to another serious illness; three did not tolerate the product. • Withdrawals were due to introduction of probiotics, change in feeding, or a parental decision. Adverse Events: • None were reported. Tolerance: • There were no significant differences between the groups in stool, vomiting, or crying frequency. • GOS significantly reduced ($p<0.05$) stool consistency (watery, runny, and mushy). Other Parameters: • There was no significant difference in atopic symptoms as judged by the SCORAD (Scoring Atopic Dermatitis) values.

Table 14. Recent Studies of GOS Ingestion in Infants

Reference	Study Design and Population	Treatments (Numbers of Subjects)	Duration	Safety Parameters
Pontes et al., 2016	Randomized, double-blind, placebo-controlled study in healthy children (1 to 4 years old)	Group 1 (Control): Cow's milk-based beverage; n=131 Group 2: Cow's milk-based beverage containing 30 mg of docosahexanoic acid, 1.2 g blend of polydextrose and GOS (1:1) ratio, and 8.7 mg of yeast β-glucan per serving; n=125 Children were to consume the products 3x/day; GOS intake = 1.8 g/day	28 days	Withdrawals: • Two subjects withdrew in Group 1 • Five subjects withdrew in Group 2 • Reasons for withdrawal were not reported. Adverse Events: • Ninety-nine types of adverse events were reported; only the occurrence of thrush was significantly increased in Group 2. • Ten subjects in the Group 1 experienced at least on serious adverse event • Two subjects in Group 2 reported at least one serious adverse event Tolerance: • Average daily intake of the products during the study was not significantly different between the groups. • There was no difference in the incidence of acute respiratory infections or diarrheal disease (≥ liquid or semi-liquid stools in 24 hr with fever and/or vomiting and/or dehydration and compromised general status) between the groups. • Group 2 had significantly less allergic manifestations (allergic rhinitis or conjunctivitis, wheezing, allergic cough, eczema, and urticaria) than Group 1. • Group 2 had significantly softer stools ($p<0.05$) compared to Group 1 in the first 3 months of the study. • Eight of the 98 subjects that were 12- 24 month were constipated; all were in the control group; five remained constipated at the end of the trial. • In the children 25 to 48 months old, there was no significant difference in the percentage of children who remained constipated at the end of the study. Other Parameters: • In both groups there was significant increase from baseline to end of study in weight- and length/height-for-age z scores; there was no difference between the groups. • There were no differences between the two groups in fecal IgA, serum IL-10, TGF-β1, TGF- β2, IL-4, and IFNγ, and stool parasites. • There were no significant differences in serum zinc and ferritin, hemoglobin, hematocrit, red blood cells, white blood cells, and platelets.

2. Studies Conducted in Adults

New Francisco Biotechnology Corporation proposed increasing the use level to 11 g/serving in conventional foods in GRN 518. GRN 518 subsequently received a "no questions" letter from FDA in 2014. To support the safety of GOS for the intended use and at the intended use level, New Francisco Biotechnology Corporation incorporated by reference the information summarized in GRNs 236, 285, 286, and 334, and summarized the clinical studies conducted in adults that were published from 2010 to 2014. Since GRN 518, GRN 569 and 620 were filed with the FDA. Both GRN 569 and 620 provided updates of published studies. GRN 569 received a "no questions" letter in 2015 whereas GRN 620 is still in review. Importantly, no additional clinical studies involving adults have been published since the filing of GRN 620.

E. ALLERGENICITY

The basic requirement of an allergic reaction is the antigen-mediated cross-linking of antigen-specific IgE antibodies bound to F_c receptors expressed on the surface of mast cells and basophils. The crosslinking then induces mast cells and basophils to degranulate, releasing histamine and leukotrienes into the extracellular matrix, causing capillary venule dilation, endothelium activation, and increased vascular permeability, redness and swelling. If the antigen is systemic or rapidly absorbed, histamine and leukotriene release is widespread and can result in anaphylaxis and potentially death. Importantly, antigen-specific IgE antibodies are generated during a primary immune response to the antigen and only when the antigen-specific IgE reencounters the sensitizing antigen or an antigen that mimics the sensitizing antigen, does an allergic response develop. It is also important to note that environmental stimuli and genetics are currently believed to be contributing factors to the development of allergy (reviewed in Wang and Sampson, 2011).

The allergenicity of GOS has been summarized in an amendment to GRN 236 and in GRN 620. Two case series documenting 21 cases of allergic reactions associated with the ingestion of GOS are publicly available, Vo et al. (2012) and Chiang et al. (2012), and were reviewed in an amendment to GRN 236. More recently two additional studies have been published and are summarized in GRN 620 (Kaneko et al., 2014; Soh et al., 2015).

Vo et al. (2012) identified 17 cases of allergic reactions to ingested milk products in Vietnam from October 9 to October 28, 2009. All subjects were Vietnamese and 16 of the 17 cases had consumed milk containing GOS. The median age of the 17 subjects was 10 years old (range 2 to 15 years old). Four subjects were allergic to various foods, but not milk. Twelve subjects developed symptoms within 20 minutes and 3 subjects developed symptoms 1, 6, and 49

hr after consuming the milk product. The most frequent symptom was an itchy maculo-papular skin rash (94%). Three subjects experienced difficult breathing. The source of the GOS in the supplemented milk was not disclosed. Importantly, a case-by-case description of the reactions was not provided, making it difficult to interpret the results. Moreover, in a case-control study of 50 neighborhood controls, the methods were not reported and the specific allergens and/or provoking substances were not identified.

Chiang et al. (2012) identified five cases (four subjects were Chinese and one was Malaysian) in Singapore from December 2007 to January 2012. The age of the subjects ranged from 5 year to 38 years old. All the subjects were tolerant to cow's milk, had no known previous exposure to GOS, and reacted to a cow's milk product supplemented with Vivinal® GOS, which is the subject of GRN 236 and manufactured using a β-galactosidase derived from *B. circulans* by FrieslandCampina Domo. There were no deaths and all subjects were treated with different combinations of antihistamines, steroids, β2-agonists, adrenaline, and/or oxygen. Subsequent testing of the five cases showed that they were all reactive to GOS with a degree of polymerization (DP) of three and greater via skin prick testing. *In vitro* studies also revealed that basophils harvested from each of the subjects increased cell surface expression of the activation marker CD203c in a GOS- and dose-dependent manner. Subsequent *in vitro* studies using different GOS fractions showed that the basophil reactivity increased in a DP-dependent manner with GOS of DP greater than 4 being more potent than GOS with a DP less than 4. Although serum levels of GOS-specific IgE levels were not determined directly, indirect basophil activation studies showed that the sera of two of three GOS-reactive subjects conferred GOS-reactivity to otherwise GOS-tolerant basophils, suggesting that the allergic responses induced following the ingestion of GOS were IgE-mediated.

Soh et al. (2015) conducted a clinical study to evaluate the prevalence of allergy to two different formulations of GOS, Vivinal® GOS and Oligomate GOS. For reference, Vivinal® GOS is manufactured a β-galactosidases derived from *B. circulans* and Oligomate GOS is manufactured using β-galactosidases derived from *S. singularis* and *K. lactis* (GRN 236; GRN 334). Four hundred eighty-seven individuals, 5 years and older and with no history of cow's milk or GOS allergy were recruited from allergy clinics in Singapore with eczema, asthma, allergic rhinitis, and food allergies and enrolled in the study. Reactivity to GOS was determined using skin prick tests, basophil activation assays, and open oral challenges. Skin prick testing identified thirty subjects that were reactive to Vivinal® GOS. In subsequent basophil activation testing, 15 of the 30 had positive skin prick tests to Vivinal® GOS. Reactivity to Oligomate GOS via skin prick and basophil activation testing was not determined. Thirteen of the 30 subjects that had positive skin prick tests also consented to oral challenges with Vivinal® GOS and Oligomate

GOS. Six of these 13 subjects reacted to Vivinal® GOS in oral challenges (five in the basophil activation test (BAT) positive group and one in the BAT negative group) whereas none reacted to Oligomate GOS. Thus, Soh et al. estimated the prevalence of Vivinal® GOS allergy to be as great as 3.5 % in the Singapore atopic population. Moreover, Soh et al. speculated that the variable reactivity to GOS products may be due to structural differences in GOS products.

To identify the allergenic epitopes in GOS and a potentially hypoallergenic form of GOS, Kaneko et al. (2014) compared the allergenicity of six different GOS preparations manufactured with different β-galactosidases and manufacturing conditions, in blood harvested from three Japanese adults reported to have anaphylactic reactions to GOS, and histamine-release assays. Two of the adults were reported to be reactive to GOS manufactured with a β-galactosidase derived from *B. circulans* and the other adult was reported to be reactive to GOS manufactured with a β-galactosidase derived from *A. oryzae*. The GOS preparations included a GOS product manufactured using a combination of β-galactosidases derived from *A. oryzae* and *Streptococcus thermophilus*, three different GOS products manufactured using a β-galactosidase derived from *B. circulans,* one GOS product manufactured with a β-galactosidase derived from *Sporobolomyces singularis*, and one GOS product manufactured with a combination of β-galactosidases derived from *S. singularis* and *K. lactis*. A comparison of the histamine release induced by a GOS product manufactured with β-galactosidases derived from *A. oryzae* and *S. thermophilus* and GOS product manufactured with β-galactosidase derived from *B. circulans* using blood harvested from the two subjects that were reported to be sensitive to GOS manufactured using a β-galactosidase derived from *B. circulans* showed that the histamine release induced by GOS product manufactured with β-galactosidase derived from *B. circulans* required more than 100-fold less than the GOS product manufactured with *A. oryzae* and *S. thermophilus.* Importantly, the histamine releases induced with the GOS product manufactured with β-galactosidases derived *A. oryzae* and *S. thermophilus* were deemed to be either negative (less than 10%) or pseudonegative (10-15%). In addition, the GOS produced using a combination of β-galactosidases derived from *A. oryzae* and *S. thermophilus* failed to induce a positive histamine release when combined with blood harvested from the one patient that was reported to be sensitive to a GOS manufactured with β-galactosidases derived from *A. oryzae* and *S. thermophilus.* Subsequent histamine release assays using tri- and tetra-saccharides obtained from one of the GOS products manufactured with the β-galactosidase derived from *B. circulans* revealed: 1) that trisaccharides of Gal-β(1-4)-Gal-β(1-3)-Glc and Gal-β(1-4)-(Gal-β(1-6))-Glc were either negative (less than 10%) or pseudonegative (10-15%) for inducing histamine release at all concentrations tested (0.1 to 100 μg/ml); 2) the trisaccharide Gal-β(1-4)-Gal-β(1-4)-Glc induced approximately 20% histamine release in only one of the *B. circulans* β-galactosidase-manufactured GOS-sensitive subjects patients at the highest dose tested (100

μg/ml); and 3) tetrasaccharides were at least 10-fold more potent than the most potent trisaccharide (Gal-β(1-4)-Gal-β(1-4)-Glc) at inducing positive histamine release in the *B. circulans* β-galactosidase-manufactured GOS-sensitive subjects. Additionally, a linear tetrasaccharide GOS with the structure of Gal-β(1-4)-Gal-β(1-4)-Gal-β(1-3)-Glc and a branched tetrasaccharide GOS with a structure of Gal-β(1-4)-(Gal-β(1-4)-Gal-β(1-6))-Glc were more potent than a linear GOS tetrasaccharide with a structure of Gal-β(1-4)-Gal-β(1-4)-Gal-β(1-4)-Glc. No other tetrasaccharides were tested and similar studies using tri- and tetra-saccharide fractions of GOS manufactured with the combination of β-galactosidases derived from *A. oryzae* and *S. thermophilus* were not conducted. These results are consistent with the results reported by Chiang et al. (2012) and that suggest that GOS manufactured with a β-galactosidase derived from *B. circulans* may be at least 100-fold more potent at inducing allergenic responses in patients sensitized to GOS manufactured using a β-galactosidase derived from *B. circulans* than the GOS manufactured with a combination of β-galactosidases derived *A. oryzae* and *S. thermophiles*. Moreover, the linear and branched tetrasaccharide GOS with the structures of Gal-β(1-4)-Gal-β(1-4)-Gal-β(1-3)-Glc and Gal-β(1-4)-(Gal-β(1-4)-Gal-β(1-6)-Glc in the GOS product manufactured using a β-galactosidase derived from *B. circulans* appear to be potent inducers of histamine release in allergic subjects.

Importantly compositional studies have shown that the type of β-glycosidic bonds present in GOS is dependent on the type of β-galactosidases used during production (Yanahira et al., 1995; Greenberg and Mahoney, 1983; Martinez-Villaluenga et al., 2008; Rodriguez-Colinas et al., 2011; Rodriguez-Colinas et al., 2012; Rodriguez-Colinas et al., 2014; Urrutia et al., 2013; Kaneko et al., 2014). Specifically, GOS manufactured with β-galactosidase derived from *B. circulans, S. singularis,* or a combination of *S. singularis* and *K. lactis* contain predominantly oligosaccharides with 1-4 β-glycosidic bonds (Rodriguez-Colinas et al., 2011; Rodriguez-Colinas et al., 2012; Kaneko et al., 2014; Yanahira et al., 1995) whereas GOS manufactured with β-galactosidases derived from *K. lactis*, *A. oryzae*, *S. thermophilus,* or a combination of β-galactosidases derived from *A. oryzae* and *S. thermophilus* contain predominantly oligosaccharide chains with 1-6 β-glycosidic bonds (Greenberg and Mahoney, 1983; Martinez-Villaluenga et al., 2008; Rodriguez-Colinas et al., 2011; Rodriguez-Colinas et al., 2012; Rodriguez-Colinas et al., 2014; Kaneko et al., 2014; Urrutia et al., 2013). To understand the types of β-glycosidic bonds present in VITAGOS™, the structures and relative percentages of the different saccharide species were confirmed by high performance size exclusion chromatography (HPSEC), high performance anion exchange chromatography (HPAEC)-PD, methylation analysis and 1D and 2D nuclear magnetic resonance (NMR). Although VITAGOS™ contains glucose, galactose, and lactose; the bulk of the saccharide fraction is GOS (Table 15). Trisaccharide GOS are the predominant GOS species followed by tetrasaccharide

GOS. GOS species with a DP greater than 5 constitute less than 3.3%. The GOS species in the DP2 fraction contain 1-2, 1-3, 1-6 β-glycosidic linkages, whereas the three species in the DP3 contain either a combination of 1-6, 1-4, and 1-3 β-glycosidic linkages, or only 1-4 linkages. In contrast, the DP4 fraction contains three GOS species with combinations of 1-6, 1-4, and 1-3 linkages. Importantly, the structures of almost all oligosaccharides in the different DP fractions were characterized and VITAGOS™ does not contain linear tetrasaccharides with either only 1-4 linkages or branched tetrasaccharides with a combination of 1-4 and 1-6 linkages, which were proposed by Kaneko et al. (2014) to be the species responsible for inducing the GOS-related allergic reactions. Additionally, the linear trisaccharide GOS containing only 1-4 linkages represents only a minor fraction of the overall saccharide content of VITAGOS™.

Table 15. Saccharide Species in VITAGOS™

Saccharide (DP)	Percent of the DP Fraction in the Saccharide Fraction of VITAGOS™[2]	Saccharide Species	Percent of Each Species in Each DP Fraction of VITAGOS™[1]	Percent of Each Species in the Saccharide Fraction of VITAGOS™[3]
Monosaccharide (DP1)	19.9	Galactose	10.6	2.1
		Glucose	89.4	17.8
		Total	100	19.9
Disaccharides (DP2)	32.1	βGal(1→4)Glc (lactose)	34.2	11.0
		βGal(1→6)Glc[a] (GOS)	30.5	9.8
		βGal(1→6)Gal (GOS)	11.1	3.6
		βGal(1→3)Glc (GOS)	9.7	3.1
		βGal(1→3)Gal (GOS)	9.7	3.1
		βGal(1→2)Glc (GOS)	4.7	1.5
		Other	Trace	Trace
		Total	99.9	32.1
Trisaccharides (DP3)	33.5	βGal(1→6)βGal(1→4)Glc[1] (GOS)	63.2	21.2
		βGal(1→3)βGal(1→4)Glc (GOS)	25	8.4
		βGal(1→4)βGal(1→4)Glc (GOS)	11.8	4.0
		Others	Trace	Trace
		Total	100	33.5
Tetrasaccharides (DP4)	11.2	βGal(1→6)βGal(1→6)βGal(1→4)Glc[a] (GOS)	40.9	4.6
		βGal(1→6)βGal(1→3)βGal(1→4)Glc (GOS)	37	4.1
		βGal(1→6)βGal(1→4)βGal(1→4)Glc (GOS)	22.2	2.5
		Others	Trace	Trace
		Total	100.1	11.2
Pentasaccharides (≥ DP5)	3.3	ND (GOS)	-[c]	3.3

GOS = galacto-oligosaccharides; DP = Degree of Polymerization; ND = Not Determined.
[1]Obtained from the results of methylation analysis and confirmed by peak integration of ^{1}H NMR spectroscopy performed on one batch of VITAGOS™.
[2]Deduced from HPSEC analysis based on relative peak area.
[3]Values were calculated by dividing the percent of each species in each DP fraction of VITAGOS™ by 100 and multiplying the quotient by the percent of the DP fraction in the saccharide fraction of VITAGOS™.

Taken together the characterization studies of VITAGOS™ confirm the results from other published studies showing that GOS manufactured with a β-galactosidase derived from *A. oryzae* contains oligosaccharide chains having predominantly with 1-6 β-glycosidic bonds. The studies also show that VITAGOS™ contains small amounts of Gal-β(1-4)-Gal-β(1-4)-Glc and does not contain Gal-β(1-4)-Gal-β(1-4)-Gal-β(1-3)-Glc, Gal-β(1-4)-Gal-β(1-4)-Gal-β(1-4)-Glc, or Gal-β(1-4)-(Gal-β(1-4)-Gal-β(1-6))-Glc, all of which have been implicated in provoking the allergic reactions to GOS in Southeast Asia. Importantly, Gal-β(1-4)-Gal-β(1-4)-Glc is present in other GOS products that have not been associated with allergic reactions and is not a potent inducer of histamine release in *B. circulans* β-galactosidase-manufactured GOS-sensitive subjects (Kaneko et al., 2014). These data therefore show that VITAGOS™ does not contain the GOS species that are believed to be responsible for inducing the GOS-mediated allergic reactions.

In summary, GOS-containing products have been available worldwide for over a decade and only a small number of allergic reactions have been reported in subjects 5 years and older residing in Southeast Asia. Moreover, there is no evidence indicating that GOS itself is a sensitizing antigen because all cases of allergic reactions were in subjects' who had no known reactivity to GOS (Vo et al., 2012; Chiang et al., 2012; Kaneko et al., 2014; Soh et al. 2015). In addition, GOS allergenicity is confined only to those products containing GOS that has been manufactured using a β-galactosidase derived from *B. circulans* and in vitro studies suggest that tetrasaccharides with structures of Gal-β(1-4)-Gal-β(1-4)-Gal-β(1-3)-Glc and Gal-β(1-4)-(Gal-β(1-4)-Gal-β(1-6)-Glc may be responsible for these reactions. VITAGOS™ is manufactured with *A. oryzae* and *K. lactis* and biochemical studies conducted by Vitalus Nutrition Inc. confirm that the product does not contain these species (Table 15). Therefore, it is unlikely that VITAGOS™ in general will provoke GOS allergic responses.

F. REGULATORY APPROVALS ACROSS THE WORLD

In the United States, a total of seven GOS-containing products have been determined GRAS for use in infant formulas and selected conventional foods at levels up to 7.8 g/L and 11 g/serving, respectively, and resulted in ten GRAS Notifications (GRN 236, 285, 286, 334, 484, 489, 495, 518, 569, and 620). All GRAS Notifications have received "no questions" letters from the FDA.

In the European Union, GOS is approved for use in infant and follow-on formulas in combination with fructo-oligosaccharides (FOS) at levels up to 8 g (90% GOS and 10% FOS)/L (7.2 g GOS and 0.8 g FOS/L) (Commission Delegated Regulation (EU) 2016/127).

In Australia and New Zealand, GOS is permitted in infant and follow-on formulas at levels up to 290 mg/100 kJ, or approximately 8 g/L (Australia New Zealand Food Standards Code – Standard 2.9.1 – 7).

In Japan, GOS have been commercially available since 1995 and are considered as Food for Specified Health Uses (FOSHU).

VII. SUPPORTING DATA AND INFORMATION

A. REFERENCES

All information included in the following list of references is generally available.

Adam, C. L., Williams, P. A., Garden, K. E., Thomson, L. M., Ross, A. W. (2015). Dose-dependent effects of a soluble dietary fibre (pectin) on food intake, adiposity, gut hypertrophy and gut satiety hormone secretion in rats. Plos One 10, e0115438.

Anthony, J.C., Merriman, T.N., and Heimbach, J.T. (2006). 90-day oral (gavage) study in rats with galacto-oligosaccharides syrup. Food Chem Toxicol 44, 819–826.

Armanian, A.M., Sadeghnia, A., Hoseinzadeh, M., Mirlohi, M., Feizi, A., Salehimehr, N., Saee, N., and Nazari, J. (2014). The Effect of Neutral Oligosaccharides on Reducing the Incidence of Necrotizing Enterocolitis in Preterm infants: A Randomized Clinical Trial. Int J Prev Med 5, 1387–1395.

Australia New Zealand Food Standards Code - Standard 2.9.1 - Infant formula Products (https://www.legislation.gov.au/Details/F2015L00409 - accessed on 1/4/17).

Bouhnik, Y., Raskine, L., Simoneau, G., Vicaut, E., Neut, C., Flourié, B., Brouns, F., and Bornet, F.R. (2004). The capacity of nondigestible carbohydrates to stimulate fecal bifidobacteria in healthy humans: a double-blind, randomized, placebo-controlled, parallel-group, dose-response relation study. Am J Clin Nutr 80, 1658–1664.

Boženský, J., Hill, M., Zelenka, R., and Skýba, T. (2015). Prebiotics Do Not Influence the Severity of Atopic Dermatitis in Infants: A Randomised Controlled Trial. PLoS One 10, e0142897.

Chatchatee, P., Lee, W.S., Carrilho, E., Kosuwon, P., Simakachorn, N., Yavuz, Y., Schouten, B., Graaff, P.L., and Szajewska, H. (2014). Effects of growing-up milk supplemented with prebiotics and LCPUFAs on infections in young children. J Pediatr Gastroenterol Nutr 58, 428–437.

Chiang, W.C., Huang, C.H., Llanora, G.V., Gerez, I., Goh, S.H., Shek, L.P., Nauta, A.J., Van Doorn, W.A., Bindels, J., Ulfman, L.H., Knipping, K., Delsing, D.J., Knol, E.F., and Lee, B.W. (2012). Anaphylaxis to cow's milk formula containing short-chain galacto-oligosaccharide. J Allergy Clin Immunol 130, 1361–1367.

Chonan O, Watanuki M. Effect of galacto-oligosaccharides on calcium absorption in rats. J Nutr Sci Vitaminol (Tokyo). 1995 Feb;41(1):95-104.

Chonan O, Matsumoto K, Watanuki M. Effect of galacto-oligosaccharides on calcium absorption and preventing bone loss in ovariectomized rats. Biosci Biotechnol Biochem. 1995 Feb;59(2):236-9.

Chonan O, Watanuki M. The effect of 6'-galacto-oligosaccharides on bone mineralization of rats adapted to different levels of dietary calcium. Int J Vitam Nutr Res. 1996;66(3):244-9.

Chonan O, Takahashi R, Watanuki M. Role of activity of gastrointestinal microflora in absorption of calcium and magnesium in rats fed beta1-4 linked galacto-oligosaccharides. Biosci Biotechnol Biochem. 2001 Aug;65(8):1872-5.

Chonan, O., Shibahara Sone, H., Takahashi, R., Ikeda, M., Kikuchi Hayakawa, H., Ishikawa, F., Kimura, K., and Matsumoto, K. (2004). Undigestibility of galacto-oligosaccharides. Journal of the Japanese Society for Food Science and Technology (Japan).

Civardi, E., Garofoli, F., Longo, S., Mongini, M.E., Grenci, B., Mazzucchelli, I., Angelini, M., Castellazzi, A., Fasano, F., Grinzato, A., Fanos, V., Budelli, A., and Stronati, M. (2015). Safety, growth, and support to healthy gut microbiota by an infant formula enriched with functional compounds. Clin Nutr

Coppa, G.V., Gabrielli, O., Pierani, P., Zampini, L., Rottoli, A., Carlucci, A., and Giorgi, P.L. (1991). Qualitative and quantitative studies of carbohydrates of human colostrum and mature milk. RIVISTA ITALIANA DI PEDIATRIA-ITALIAN JOURNAL OF PEDIATRICS 17, 303–307.

Coppa, G.V., Pierani, P., Zampini, L., Gabrielli, O., Carlucci, A., Catassi, C., and Giorgi, P.L. (1997). Lactose, oligosaccharide and monosaccharide content of milk from mothers delivering preterm newborns over the first month of lactation. Minerva pediatrica 49, 471–475.

Cui, SW., (2005). Structural Analysis of Polysaccharides. In: Cui SW (ed) Food Carbohydrates: Chemistry, Physical Properties and Applications. CRC Press, Florida, 115-122.

da Costa Ribeiro, H., Ribeiro, T.C., de Mattos, A.P., Pontes, M., Sarni, R.O., Cruz, M.L., Nogueira-de-Almeida, C.A., Mussi-Pinhata, M.M., de Carvalho Norton, R., and Steenhout, P. (2015). Normal Growth of Healthy Infants Born from HIV+ Mothers Fed a

Reduced Protein Infant Formula Containing the Prebiotics Galacto-Oligosaccharides and Fructo-Oligosaccharides: A Randomized Controlled Trial. Clin Med Insights Pediatr 9, 37–47.

Dasopoulou, M., Briana, D.D., Boutsikou, T., Karakasidou, E., Roma, E., Costalos, C., and Malamitsi-Puchner, A. (2015). Motilin and gastrin secretion and lipid profile in preterm neonates following prebiotics supplementation: a double-blind randomized controlled study. JPEN J Parenter Enteral Nutr 39, 359–368.

Demigné C, Rémésy C, Rayssiguier Y. Effect of fermentable carbohydrates on volatile fatty acids, ammonia and mineral absorption in the rat caecum. Reproduction Nutrition Développement, 1980, 20 (4B), pp.1351-1359. HAL Id: hal-00897739, https://hal.archives-ouvertes.fr/hal-00897739.

Demigné C, Rémésy C. Stimulation of absorption of volatile fatty acids and minerals in the cecum of rats adapted to a very high fiber diet. J Nutr. 1985 Jan;115(1):53-60.

Depeint, F., Tzortzis, G., Vulevic, J., I'anson, K., and Gibson, G.R. (2008). Prebiotic evaluation of a novel galacto-oligosaccharide mixture produced by the enzymatic activity of Bifidobacterium bifidum NCIMB 41171, in healthy humans: a randomized, double-blind, crossover, placebo-controlled intervention study. Am J Clin Nutr 87, 785–791.

Desbuards, N., Gourbeyre, P., Haure-Mirande, V., Darmaun, D., Champ, M., and Bodinier, M. (2012). Impact of perinatal prebiotic consumption on gestating mice and their offspring: a preliminary report. Br J Nutr 107, 1245–1248.

Djouzi Z, Andrieux C. Compared effects of three oligosaccharides on metabolism of intestinal microflora in rats inoculated with a human faecal flora. Br J Nutr. 1997 Aug;78(2):313-24.

Fanaro, S., Marten, B., Bagna, R., Vigi, V., Fabris, C., Peña-Quintana, L., Argüelles, F., Scholz-Ahrens, K.E., Sawatzki, G., Zelenka, R., Schrezenmeir, J., de Vrese, M., and Bertino, E. (2009). Galacto-oligosaccharides are bifidogenic and safe at weaning: a double-blind randomized multicenter study. J Pediatr Gastroenterol Nutr 48, 82–88.

FSANZ (2008). Final Assessment Report: Proposal P306. Addition of Inulin/FOS & GOS to Food.

Giovannini, M., Verduci, E., Gregori, D., Ballali, S., Soldi, S., Ghisleni, D., Riva, E., and PLAGOS, T.S.G. (2014). Prebiotic effect of an infant formula supplemented with galacto-oligosaccharides: randomized multicenter trial. J Am Coll Nutr 33, 385–393.

Gopal, P.K., Prasad, J., and Gill, H.S. (2003). Effects of the consumption of Bifidobacterium lactis HN019 (DR10 TM) and galacto-oligosaccharides on the microflora of the gastrointestinal tract in human subjects. Nutrition Research 23, 1313–1328.

Gowda S, Desai PB, Hull VV, Math AA, Vernekar SN, Kulkarni SS. A review on laboratory liver function tests. Pan Afr Med J. 2009 Nov 22;3:17.

Greaves, P. (2012). Digestive system. In Histopathology of Preclinical Toxicity Studies, Fourth Edition (Oxford, UK: Academic Press), pp. 325-431.

Greenberg NA, Mahoney RR. Formation of oligosaccharides by β-galactosidase from Streptococcus thermophiles. Food Chemistry, Volume 10, Issue 3, March 1983, Pages 195–204.

GRN 90 (2002). Enzyme Technical Association.

GRN 236 (2008). Friesland Foods Domo.

GRN 285 (2009). GTC Nutrition.

GRN 286 (2009). GTC Nutrition.

GRN 334 (2010). Yakult Pharmaceutical Industry Co., Ltd.

GRN 484 (2014). Clasado, Inc.

GRN 489 (2014). International Foods Focus Ltd.

GRN 495 (2014). Clasado, Inc.

GRN 518 (2014). New Francisco Biotechnology Corporation.

GRN 569 (2015). New Francisco Biotechnology Corporation.

GRN 620 (2016). Nestle.

Haschek, W. M., Rousseaux C. G., and Wallig M. A. (2010). Gastrointestinal Tract. In Fundamentals of Toxicologic Pathology, Second Edition (Oxford, UK: Academic Press), pp. 163-196.

Hayashi M, Taki S, Ueda F, Shimazaki Y, Ito O, Kamata S, Kakiichi N, Shitara A. 4'-galacto-oligosaccharide affects sodium and potassium metabolism in rats. J Nutr. 1991 Oct;121(10):1606-12.

Hogenkamp, A., Thijssen, S., van Vlies, N., and Garssen, J. (2015). Supplementing pregnant mice with a specific mixture of nondigestible oligosaccharides reduces symptoms of allergic asthma in male offspring. J Nutr 145, 640–646.

Houdijk JGM, Verstegena MWA, Boscha MW, van Laere KJM. Dietary fructo-oligosaccharides and transgalacto-oligosaccharides can affect fermentation characteristics in gut contents and portal plasma of growing pigs. Livestock Production Science 73 (2002) 175–184.

Institute of Medicine (US) Panel on Macronutrients, and Institute of Medicine (US) Standing Committee on the Scientific Evaluation of Dietary Reference Intakes (2005). Energy. In Dietary reference intakes for energy, carbohydrate, fiber, fat, fatty acids, cholesterol, protein, and amino acids, eds. (Washington, D.C.: National Academies Press), pp. 107–264.

Ito, M., Deguchi, Y., Miyamori, A., Matsumoto, K., Kikuchi, H., Matsumoto, K., Kobayashi, Y., Yajima, T., and Kan, T. (1990). Effects of administration of galacto-oligosaccharides on the human faecal microflora, stool weight and abdominal sensation. Microbial Ecology in Health and Disease 3, 285–292.

Jacobs LR, Schneeman BO. Effects of dietary wheat bran on rat colonic structure and mucosal cell growth. J Nutr. 1981 May;111(5):798-803.

Kaneko, K., Watanabe, Y., Kimura, K., Matsumoto, K., Mizobuchi, T., and Onoue, M. (2014). Development of hypoallergenic galacto-oligosaccharides on the basis of allergen analysis. Biosci Biotechnol Biochem 78, 100–108.

Kawakami K, Makino I, Asahara T, Kato I, Onoue M. Dietary galacto-oligosaccharides mixture can suppress serum phenol and p-cresol levels in rats fed tyrosine diet. J Nutr Sci Vitaminol (Tokyo). 2005 Jun;51(3):182-6.

Kikuchi H, Andrieux C, Riottot M, Bensaada M, Popot F, Beaumatin P, Szylit O. Effect of two levels of transgalactosylated oligosaccharide intake in rats associated with human faecal microflora on bacterial glycolytic activity, end-products of fermentation and bacterial steroid transformation. J Appl Bacteriol. 1996 Apr;80(4):439-46.

Kikuchi-Hayakawa H1, Kimura M, Watanuki M. Adaptation of rate of organic acid production of hindgut bacteria to chronic intake of galacto-oligosaccharide in the rat. J Nutr Sci Vitaminol (Tokyo). 1997 Jun;43(3):357-68.

Kobayashi, T., Yasutake, N., Uchida, K., Ohyama, W., Kaneko, K., and Onoue, M. (2009). Safety of a novel galacto-oligosaccharide: Genotoxicity and repeated oral dose studies. Hum Exp Toxicol 28, 619–630.

Kobayashi, T., Ishida, S., Kaneko, K., and Onoue, M. (2014a). A 6-week oral gavage toxicity study of a novel galacto-oligosaccharide in juvenile rats. Hum Exp Toxicol 33, 722–728.

Kobayashi, T., Takano, M., Kaneko, K., and Onoue, M. (2014b). A one-generation reproduction toxicity study in rats treated orally with a novel galacto-oligosaccharide. Hum Exp Toxicol 33, 814–821.

Konishi F, Shidoji Y, Oku T, Hosoya N. Mode of rat cecal enlargement induced by a short-term feeding on glucomannan. Jpn J Exp Med. 1984 Jun;54(3):139-42.

Kunz, C., Rudloff, S., Baier, W., Klein, N., and Strobel, S. (2000). Oligosaccharides in human milk: structural, functional, and metabolic aspects. Annu Rev Nutr 20, 699–722.

Lee, L.Y., Bharani, R., Biswas, A., Lee, J., Tran, L.A., Pecquet, S., and Steenhout, P. (2015). Normal growth of infants receiving an infant formula containing Lactobacillus reuteri, galacto-oligosaccharides, and fructo-oligosaccharide: a randomized controlled trial. Matern Health Neonatol Perinatol 1, 9.

Levine, G. M. (1991). Regulation of intestinal mucosal growth. In Growth of the Gastrointestinal Tract: Gastrointestinal Hormones and Growth Factors, Morisset, J. and Solomon, T. E. (eds) (Boca Raton, FL: CRC Press), pp. 176-188.

Lupton JR, Kurtz PP. Relationship of colonic luminal short-chain fatty acids and pH to in vivo cell proliferation in rats. J Nutr. 1993 Sep;123(9):1522-30.

Macfarlane, G.T., Steed, H., and Macfarlane, S. (2008). Bacterial metabolism and health-related effects of galacto-oligosaccharides and other prebiotics. J Appl Microbiol 104, 305–344.

Martinez, J.A., and Ballew, M.P. (2011). Infant formulas. Pediatr Rev 32, 179–89; quiz 189.

Martínez-Villaluenga C, Cardelle-Cobas A, Corzo N, Olano A, Villamiel M. Optimization of conditions for galacto-oligosaccharide synthesis during lactose hydrolysis by β-galactosidase from Kluyveromyces lactis (Lactozym 3000 L HP G). Food Chemistry, Volume 107, Issue 1, 1 March 2008, Pages 258–264.

Matsuki, T., Tajima, S., Hara, T., Yahagi, K., Ogawa, E., and Kodama, H. (2016). Infant formula with galacto-oligosaccharides (OM55N) stimulates the growth of indigenous bifidobacteria in healthy term infants. Benef Microbes 1–10.

Meli, F., Puccio, G., Cajozzo, C., Ricottone, G.L., Pecquet, S., Sprenger, N., and Steenhout, P. (2014). Growth and safety evaluation of infant formulae containing oligosaccharides derived from bovine milk: a randomized, double-blind, noninferiority trial. BMC Pediatr 14, 306.

Morel, F.B., Oozeer, R., Piloquet, H., Moyon, T., Pagniez, A., Knol, J., Darmaun, D., and Michel, C. (2015). Preweaning modulation of intestinal microbiota by oligosaccharides or amoxicillin can contribute to programming of adult microbiota in rats. Nutrition 31, 515–522.

Moro, G., Arslanoglu, S., Stahl, B., Jelinek, J., Wahn, U., and Boehm, G. (2006). A mixture of prebiotic oligosaccharides reduces the incidence of atopic dermatitis during the first six months of age. Arch Dis Child 91, 814–819.

Narumi, K., Takasawa, H., Ohyama, W., and Kaneko, K. (2014). In vivo comet assay of a novel galacto-oligosaccharide in rats. Hum Exp Toxicol 33, 488–495.

Newberne, P.M., Conner, M.W., and Estes, P. C. (1988). The influence of food additives and related materials on lower bowel structure and function. Toxicologic Pathology 16,184-197.

Nzeusseu A, Dienst D, Haufroid V, Depresseux G, Devogelaer JP, Manicourt DH. Inulin and fructo-oligosaccharides differ in their ability to enhance the density of cancellous and cortical bone in the axial and peripheral skeleton of growing rats. Bone. 2006 Mar;38(3):394-9. Epub 2005 Oct 24.

Ohtsuka, K., Tsuji, K., Nakagawa, Y., Ueda, H., Ozawa, O., Uchida, T., and Ichikawa, T. (1990). Availability of 4'galactosyllactose (O-beta-D-galactopyranosyl-(1→4)-O-beta-D-

galactopyranosyl-(1→4)- D-glucopyranose) in rat. J Nutr Sci Vitaminol (Tokyo) 36, 265–276.

Ohtsuka, K., Tsuji, K., Nakagawa, Y., Ueda, H., Ozawa, O., Uchida, T., and Ichikawa, T. (1991). Utilization and metabolism of [U-14C]4' galactosyllactose (O-beta-D-galactopyranosyl-(1→4)-O-beta-D-galactopyranosyl-(1→4)- D-glucopyranose) in rats. J Nutr Sci Vitaminol (Tokyo) 37, 173–184.

Oku, T. (1997). Large bowel enlargement induced by dietary fiber. In Food Factors for Cancer Prevention, Ohigashi, H, Osawa, T., Terao, J., Watanabe, S., and Yoshikawa, T. (eds) (Tokyo, Japan: Springer), pp. 654-659.

Oku T, Kwon S. Disaccharidase activity in rat cecum and colon with hyperplasia induced by maltitol or glucomannan. J Nutr Sci Vitaminol (Tokyo). 1998 Feb;44(1):69-78.

Pontes, M.V., Ribeiro, T.C., Ribeiro, H., de Mattos, A.P., Almeida, I.R., Leal, V.M., Cabral, G.N., Stolz, S., Zhuang, W., and Scalabrin, D.M. (2016). Cow's milk-based beverage consumption in 1- to 4-year-olds and allergic manifestations: an RCT. Nutr J 15, 19.

Rodriguez-Colinas B, de Abreu MA, Fernandez-Arrojo L, de Beer R, Poveda A, Jimenez-Barbero J, Haltrich D, Ballesteros Olmo AO, Fernandez-Lobato M, Plou FJ. Production of Galacto-oligosaccharides by the β-Galactosidase from Kluyveromyces lactis : comparative analysis of permeabilized cells versus soluble enzyme. J Agric Food Chem. 2011 Oct 12;59(19):10477-84. doi: 10.1021/jf2022012. Epub 2011 Sep 8.

Rodriguez-Colinas B, Poveda A, Jimenez-Barbero J, Ballesteros AO, Plou FJ. Galacto-oligosaccharide synthesis from lactose solution or skim milk using the β-galactosidase from Bacillus circulans. J Agric Food Chem. 2012 Jun 27;60(25):6391-8. doi: 10.1021/jf301156v. Epub 2012 Jun 19.

Rodriguez-Colinas B, Fernandez-Arrojo L, Ballesteros AO, Plou FJ. Galacto-oligosaccharides formation during enzymatic hydrolysis of lactose: towards a prebiotic-enriched milk. Food Chem. 2014 Feb 15;145:388-94. doi: 10.1016/j.foodchem.2013.08.060. Epub 2013 Aug 28.

Saito, T., Itoh, T., and Adachi, S. (1987). Chemical structure of three neutral trisaccharides isolated in free form from bovine colostrum. Carbohydr Res 165, 43–51.

Sako T, Matsumoto K, Tanaka R. Recent progress on research and applications of non-digestible galacto-oligosaccharides. International Dairy Journal, Volume 9, Issue 1, January 1999, Pages 69–80.

Sangwan, V., Tomar, S.K., Singh, R.R., Singh, A.K., and Ali, B. (2011). Galacto-oligosaccharides: novel components of designer foods. J Food Sci 76, R103–11.

Scientific Committee on Food, 2003 (EU 2016/127). Commission Delegated Regulation (EU) 2016/127 of 25 September 2015 supplementing Regulation (EU) 609/2013 of the European Parliament and of the Council as regards the specific compositional and information requirements for infant formula and follow-on formula and as regards requirements on information relating to infant and young child feeding. Official Journal of the European Communities L25/1, 2.2.2016.

Scientifc Committee on Food. (2001). Statement on the Use of Resistant Short Chain Carbohydrates (Oligofructose and Oligogalactose) in Infant Formulae and in Follow-on Formulae. SCF/CS/NUT/IF/35 Final

Schmelzle, H., Wirth, S., Skopnik, H., Radke, M., Knol, J., Böckler, H.M., Brönstrup, A., Wells, J., and Fusch, C. (2003). Randomized double-blind study of the nutritional efficacy and bifidogenicity of a new infant formula containing partially hydrolyzed protein, a high beta-palmitic acid level, and nondigestible oligosaccharides. J Pediatr Gastroenterol Nutr 36, 343–351.

Silk, D.B., Davis, A., Vulevic, J., Tzortzis, G., and Gibson, G.R. (2009). Clinical trial: the effects of a trans-galacto-oligosaccharide prebiotic on faecal microbiota and symptoms in irritable bowel syndrome. Aliment Pharmacol Ther 29, 508–518.

Simeoni, U., Berger, B., Junick, J., Blaut, M., Pecquet, S., Rezzonico, E., Grathwohl, D., Sprenger, N., Brüssow, H., Study, T., and Szajewska, H. (2015). Gut microbiota analysis reveals a marked shift to bifidobacteria by a starter infant formula containing a synbiotic of bovine milk-derived oligosaccharides and Bifidobacterium animalis subsp. lactis CNCM I-3446. Environ Microbiol

Slavin, J. (2013). Fiber and prebiotics: mechanisms and health benefits. Nutrients 5, 1417–1435.

Smiricky-Tjardes, M.R., Flickinger, E.A., Grieshop, C.M., Bauer, L.L., Murphy, M.R., and Fahey, G.C. (2003). In vitro fermentation characteristics of selected oligosaccharides by swine fecal microflora. J Anim Sci 81, 2505–2514.

Soh, J.Y., Huang, C.H., Chiang, W.C., Llanora, G.V., Lee, A.J., Loh, W., Chin, Y.L., Tay, V.Y., Chan, Y.H., Dianne, D., and Lee, B.W. (2015). Anaphylaxis to galacto-oligosaccharides--an evaluation in an atopic population in Singapore. Allergy 70, 1020–1023.

Suarez, F.L., Springfield, J., Furne, J.K., Lohrmann, T.T., Kerr, P.S., and Levitt, M.D. (1999). Gas production in human ingesting a soybean flour derived from beans naturally low in oligosaccharides. Am J Clin Nutr 69, 135–139.

Toba, T., Watanabe, A., and Adachi, S. (1982). Allolactose and 6-0- -D-Galactopyranosyl-D-Galactose in Commercial Yogurt. Journal of Dairy Science 65, 702–706.

Torres, D.P.M., Goncalves, M.D.P.F., Teixeira, J. A., and Rodrigues, L.R. (2010). Galacto-oligosacchaides: production, properties, applications, and significance as prebiotics. Comprehensive Reviews in Food Science and Food Safety 9, 438-454.

Tzortzis, G., and Vulveic, J. (2009) Galacto-oligosaccharide prebiotics. In Prebiotics and probiotics science and technology, Charalampipoulos D., and R.A. Rastall, eds. (Reading, UK: Springer), 207-244.

Underwood, M.A., Kalanetra, K.M., Bokulich, N.A., Mirmiran, M., Barile, D., Tancredi, D.J., German, J.B., Lebrilla, C.B., and Mills, D.A. (2014). Prebiotic oligosaccharides in premature infants. J Pediatr Gastroenterol Nutr 58, 352–360.

Urrutia P, Rodriguez-Colinas B, Fernandez-Arrojo L, Ballesteros AO, Wilson L, Illanes A, Plou FJ. Detailed analysis of galacto-oligosaccharides synthesis with β-galactosidase from Aspergillus oryzae. J Agric Food Chem. 2013 Feb 6;61(5):1081-7. doi: 10.1021/jf304354u. Epub 2013 Jan 29.

U.S. FDA. (2013) Enzyme Preparations Used in Food (Partial List). Silver Spring (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN) Available at: http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/EnzymePreparations/default.htm [Page Last Updated: 08/21/2013

Verheijden, K.A., Willemsen, L.E., Braber, S., Leusink-Muis, T., Delsing, D.J., Garssen, J., Kraneveld, A.D., and Folkerts, G. (2015). Dietary galacto-oligosaccharides prevent airway eosinophilia and hyperresponsiveness in a murine house dust mite-induced asthma model. Respir Res 16, 17.

Vo, T.H., Le, N.H., Patel, M.S., Phan, L.T., and Tran Minh, N.N. (2012). Acute allergic reactions in Vietnamese children after drinking a new milk product. Foodborne Pathog Dis 9, 156–159.

Vulevic, J., Drakoularakou, A., Yaqoob, P., Tzortzis, G., and Gibson, G.R. (2008). Modulation of the fecal microflora profile and immune function by a novel trans-galacto-oligosaccharide mixture (B-GOS) in healthy elderly volunteers. Am J Clin Nutr 88, 1438–1446.

Walton, G.E., van den Heuvel, E.G., Kosters, M.H., Rastall, R.A., Tuohy, K.M., and Gibson, G.R. (2012). A randomised crossover study investigating the effects of galacto-oligosaccharides on the faecal microbiota in men and women over 50 years of age. Br J Nutr 107, 1466–1475.

Wang, J., and Sampson, H.A. (2011). Food allergy. J Clin Invest 121, 827–835.

Whiteley LO, Higgins JM, Purdon MP, Ridder GM, Bertram TA. Evaluation in rats of the dose-response relationship among colonic mucosal growth, colonic fermentation, and dietary fiber. Dig Dis Sci. 1996 Jul;41(7):1458-67.

WHO (1987). Toxicological versus physiological responses. In: Principles for the Safety Assessment of Food Additives and Contaminants in Food. (Environmental Health Criteria, no 70). Geneva, Switz.: World Health Organization (WHO), International Programme on Chemical Safety (IPCS).

Williams, T., Choe, Y., Price, P., Katz, G., Suarez, F., Paule, C., and Mackey, A. (2014). Tolerance of formulas containing prebiotics in healthy, term infants. J Pediatr Gastroenterol Nutr 59, 653–658.

Wisker, E., Feldheim, W., Pomeranz, Y., and Meuser, F. (1985). Dietary fiber in cereals. Advances in cereal science and technology (USA).

Yanahira S, Kobayashi T, Suguri T, Nakakoshi M, Miura S, Ishikawa H, Nakajima I. Formation of oligosaccharides from lactose by Bacillus circulans beta-galactosidase. Biosci Biotechnol Biochem. 1995 Jun;59(6):1021-6.

Zhou Y, Kruger C, Ravi GS, Santhosh Kumar DP, Vijayasarathi SK, Lavingia M, Chen X, Ambriz P. Safety Evaluation of Galacto-Oligosaccharides: Sub-Chronic Oral Toxicity Study in Sprague-Dawley Rats. Toxicology Research and Application, 2017, 1, 1-12.

B. EXPERT PANEL STATEMENT

We, the members of the Expert Panel, qualified by scientific training and experience to evaluate the safety of substances directly or indirectly added to food, have performed a comprehensive and critical review of available information and data on the safety and Generally Recognized As Safe (GRAS) status of the use of VITAGOS™ as an ingredient in powdered, ready-to-feed and concentrated liquid versions of milk-based non-exempt term infant formulas, and selected conventional foods. This GRAS determination for the use of GOS for the intended uses specified above has been shown to be safe and GRAS, using scientific procedures, under the Federal Food, Drug, and Cosmetic Act (FFDCA), as described under 21 CFR §170.30(b). The safety of the intake of VITAGOS™ has been determined to be GRAS by demonstrating that the safety of this level of intake is generally recognized by experts qualified by both scientific training and experience to evaluate the safety of substances directly added to food, and is based on generally available and accepted information.

The proposed use of VITAGOS™ as an ingredient for the intended uses in foods and infant formulas has been determined to be safe through scientific procedures set forth under 21 CFR §170.30(b) based on the following:

1. GOS are non-digestible oligosaccharides consisting of 1 to 7 galactose units linked via β(1-2), β(1-3), β(1-4), or β(1-6) glycosidic bonds to either a terminal glucose or galactose. Although tri- to hexa-saccharides with 2 to 5 galactose units (degree of polymerization (DP) of 3 to 6) tend to be the main components of GOS-containing products, disaccharides (DP2) consisting of galactose and glucose with different β-glycoside bonds from lactose are also present and defined as GOS because they have physiological characteristics that are similar to longer GOS.
    a. VITAGOS™ is a GOS-containing product manufactured using lactose and β-galactosidases derived from *Aspergillus oryzae* and *Kluyveromyces lactis* in a manner similar to other GOS-containing products that have received "no questions" letters from the United States Food and Drug Administration.
    b. All processing aids used to produce VITAGOS™ comply with appropriate federal regulations.
    c. A comparison of the manufacturing processes and product specifications for VITAGOS™ and other GOS-containing products shows that VITAGOS™ is essentially equivalent to the other GOS-containing products currently marketed in the United States for use in infant formulas and conventional foods.

2. GOS are transported through the upper gastrointestinal tract to the colon where they are fermented by the resident microbiota into short-chain fatty acids, carbon dioxide, methane, and hydrogen.
3. GOS present in food are either naturally occurring in human milk and colostrum, bovine colostrum, and fermented milk products or synthetic, which are then added to the food during processing and formulation.
4. Synthetic GOS have a long history of use worldwide.
    a. In Japan, GOS have been commercially available since 1995 and are considered as Food for Specified Health Uses (FOSHU).
    b. In the United States, the first GOS product was determined GRAS for use in term infant formula and selected conventional foods, and received a "no questions" letter from the FDA in 2008 (GRN 236). Since then, six additional GOS-containing products have been determined GRAS for use in infant formulas and selected conventional foods at levels up to 7.8 g/L and 11 g/serving, respectively, resulting in ten GRAS Notifications (GRN) to the FDA (GRN 236, 285, 286, 334, 484, 489, 495, 518, 569, and 620).
    c. In the European Union, the safety of GOS was reviewed by the Scientific Committee on Food (SCF) in 2003 and was approved for use in infant and follow-on formulas in combination with fructo-oligosaccharides (FOS) at levels up to 8 g (90% GOS and 10% FOS)/L (7.2 g GOS and 0.8 g FOS/L) (Select Committee on Food EU 2016/127).
    d. In Australia and New Zealand, the safety of GOS was reviewed by the Food Standards of Australia and New Zealand (FSANZ) in 2008 and is permitted in infant and follow-on formulas at levels up to 290 mg/100 kJ, or approximately 8 g/L (Australia New Zealand Food Standards Code – Standard 2.9.1 – 7).
5. The safety of VITAGOS™ was determined in a pivotal published 90-day toxicology study that identified a no observed adverse effect level (NOAEL) of 4082 mg/kg/day (2000 mg GOS/kg/day), which was the highest dose tested.
6. The safety of VITAGOS™ was corroborated by multiple subchronic, developmental, reproductive, and genotoxicology studies conducted on other GOS-containing products.
7. GOS-containing products are well tolerated in humans and have been reported to increase the abundance of bifidobacteria and lactobacilli in the gastrointestinal tract in infants and adults, increase fecal short-chain fatty acid concentrations in infants and adults, improve stool consistency in infants, reduce the incidence of atopic

dermatitis in infants, and alleviate the symptoms of irritable bowel syndrome in adults.

8. GOS is available worldwide and, although GOS-containing products have been reported to provoke allergic reactions in sensitized individuals living in Vietnam and Singapore, there have been no reported reactions to GOS-containing products outside of Vietnam and Singapore. Thus, the reported cases likely represent unique, rare, geographically localized allergic reactions to GOS-containing products.
9. The addition of VITAGOS™ to infant formula at 7.2 g GOS/L is the same use level of other GOS products in infant formula that have been determined GRAS and received "no questions" letters from the FDA (GRN 286, 334, 569) and will result in intakes of approximately 5.1 and 6.9 g GOS/day for one-month-old and six-month-old infants, respectively.
10. VITAGOS™ will be used as an alternative source of GOS in selected conventional foods at the intended use levels specified for Oligomate in GRN 334. Thus, the dietary exposure to VITAGOS™ from the intended uses will not increase GOS-intake in the United States. The estimated mean and 90th percentile exposure to VITAGOS™ from the intended uses in selected conventional foods are 12.2 and 25.3 g per person per day (g/p/d), respectively.

Determination of the GRAS status of VITAGOS™ under the intended conditions of use has been made through the deliberations of Roger Clemens, DrPH, CNS, CFS, FACN, FIFT, A. Wallace Hayes, PhD, DABT, FATS, ERT, CNS, FACN, and Thomas Sox PhD, JD. These individuals are qualified by scientific training and experience to evaluate the safety of food and food ingredients. These experts have carefully reviewed and evaluated the publicly available information summarized in this document, including the safety of VITAGOS™ and the human exposure to VITAGOS™ resulting from its intended use as an ingredient in powdered non-exempt term infant formula and selected conventional foods:

> *There is no evidence in the available information on VITAGOS™ that demonstrates, or suggests reasonable grounds to suspect, a hazard to the public when VITAGOS™ is used at levels that might reasonably be expected from the proposed applications of VITAGOS™ for use in powdered non-exempt term infant formulas and selected conventional food as proposed by Vitalus Nutrition Inc.*

Therefore, VITAGOS™ is safe and GRAS at the proposed levels of addition to the intended foods. VITAGOS™ is, therefore, excluded from the definition of a food additive, and may be used in the U.S. without the promulgation of a food additive regulation by the FDA under 21 CFR.

Roger Clemens, DrPH, CNS, FACN, FIFT
GRAS Expert Panel Member
School of Pharmacy
University of Southern California

Signature: (b) (6)

Date: June 8, 2017

A. Wallace Hayes, PhD, DABT, FATS, ERT
GRAS Expert Panel Member
Harvard School of Public Health

Signature: (b) (6)

Date: June 8, 2017

Thomas E. Sox, PhD, JD
GRAS Expert Panel Member
Principal, Pondview Consulting LLC

Signature: (b) (6)

Date: June 8, 2017

Claire Kruger, PhD, DABT
Scientific Advisor to the Panel
ChromaDex Spherix Consulting

Signature: (b) (6)

Date: June 8, 2017

From:	Claire Kruger
To:	Morissette, Rachel
Cc:	Dietrich Conze; Kathy Brailer
Subject:	RE: question regarding submitted GRAS notice on galacto-oligosaccharides
Date:	Monday, August 21, 2017 12:06:32 PM
Attachments:	image007.png image008.png image010.png

Dear Rachel:

None of the intended uses involve products under the jurisdiction of the U.S. Department of Agriculture (meat, poultry, egg products, catfish).

Best regards,

Claire

Claire Kruger, PhD, DABT, CFS
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Senior Director Regulatory Affairs

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From: Morissette, Rachel [mailto:Rachel.Morissette@fda.hhs.gov]
Sent: Monday, August 21, 2017 11:39 AM
To: Claire Kruger <ClaireK@chromadex.com>
Subject: question regarding submitted GRAS notice on galacto-oligosaccharides

Dear Dr. Kruger,

My name is Dr. Rachel Morissette and I am the Consumer Safety Officer assigned to your recently submitted notice on behalf of Vitalus Nutrition Inc. for the intended use of galacto-oligosaccharides in infant formula and other conventional foods. Before I can issue your filing letter, can you please confirm if any of your intended uses involve products under the jurisdiction of the U.S. Department of Agriculture (meat, poultry, egg products, catfish)? Thank you for your attention to this matter.

Best regards,

Rachel Morissette, Ph.D.
Consumer Safety Officer

Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration
rachel.morissette@fda.hhs.gov

From:	Kathy Brailer on behalf of Claire Kruger
To:	Morissette, Rachel
Cc:	Dietrich Conze; Claire Kruger; Kathy Brailer
Subject:	RE: Questions for GRAS Notice GRN 000721
Date:	Tuesday, October 31, 2017 12:00:07 PM
Attachments:	image007.png image016.png image018.png image024.png image025.png GRN 721 Response to FDA 10-31-17.pdf

Dear Rachel,

Attached please find the requested clarifications to GRN 721. Should you need anything else, just let us know.

Best regards,

Claire Kruger, PhD, DABT, CFS
President
Senior Director Regulatory Affairs

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From: Morissette, Rachel [mailto:Rachel.Morissette@fda.hhs.gov]
Sent: Friday, October 27, 2017 7:42 AM
To: Claire Kruger <ClaireK@chromadex.com>
Cc: Dietrich Conze <DietrichC@chromadex.com>; Kathy Brailer <KathyB@chromadex.com>
Subject: RE: Questions for GRAS Notice GRN 000721

Thank you.

Best,

Rachel

Rachel Morissette, Ph.D.
Consumer Safety Officer

Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration

rachel.morissette@fda.hhs.gov

U.S. FOOD & DRUG ADMINISTRATION

From: Claire Kruger [mailto:ClaireK@chromadex.com]
Sent: Thursday, October 26, 2017 3:54 PM
To: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Cc: Dietrich Conze <DietrichC@chromadex.com>; Kathy Brailer <KathyB@chromadex.com>
Subject: RE: Questions for GRAS Notice GRN 000721

Dear Rachel:
I am confirming that we received the letter with requested clarifications to GRN 721 and will respond within 10 business days.
Best regards,
Claire

Claire Kruger, PhD, DABT, CFS
President
Senior Director Regulatory Affairs

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From: Morissette, Rachel [mailto:Rachel.Morissette@fda.hhs.gov]
Sent: Monday, October 23, 2017 9:31 AM
To: Claire Kruger <ClaireK@chromadex.com>
Subject: Questions for GRAS Notice GRN 000721

Dear Claire,
Please find attached a letter requesting clarification on a few issues regarding GRN 000721 (GOS).

Best regards,

Rachel

Rachel Morissette, Ph.D.

Consumer Safety Officer

Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration
rachel.morissette@fda.hhs.gov

ChromaDex
SPHERIX CONSULTING

October 31, 2017

Rachel Morissette, Ph.D.
Consumer Safety Officer
U.S. Food and Drug Administration
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
Division of Biotechnology and GRAS Notice Review
5001 Campus Drive, HFS-255
College Park, MD 20740

Dear Dr. Morissette:

In response to your e-mail of October 23, 2017, following are our responses to FDA's questions on GRN 721. FDA's questions are in italics and our responses are in plain text.

1) *On page 33 of the notice, Vitalus cites the study by Kobayashi et al. (2014a) stating that the animals were gavaged "at 0, 500, 1000, or 2000 mg/kg/day of a syrup containing approximately 56.9% GOS (% dry matter), resulting in a daily intake of 213.4, 5426.8, and 853.5 mg GOS/kg". The value of 5426.8 is incorrect. Please confirm that the correct dose level is 426.8 mg GOS/kg.*

   5426.8 was a typographical error. The correct dose level is 426.8 mg GOS/kg.

2) *On page 34 of the notice, Vitalus states that "The NOAEL for reproductive function of male and female parent animals was 2,000 mg GOS per kg/day equivalent to 853.5 mg GOS/kg/day (Kobayashi et al., 2014b)." Please confirm that the sentence should read as follows: "The NOAEL for reproductive function of male and female parent animals was 2,000 mg GOS* product *per kg/day equivalent to 853.5 mg GOS/kg/day (Kobayashi et al., 2014b)." or alternatively "The NOAEL for reproductive function of male and female parent animals was 2,000 mg GOS <u>syrup</u> per kg/day equivalent to 853.5 mg GOS/kg/day (Kobayashi et al., 2014b)."*

   The sentence on page 34, should read, "The NOAEL for reproductive function of male and female parent animals was 2,000 mg GOS syrup/kg/day, which is equivalent to 853.5 mg GOS/kg/day (Kobayashi et al., 2014b)."

3) *On page 23 Section B (Genotoxicity Studies) of the notice, Vitalus summarizes genotoxicity studies that were conducted with GOS products other than VITAGOS (the subject of GRN 000721). Nonetheless, the notifier concludes that "Therefore, VITAGOS™ is not genotoxic." Please elaborate on the basis for this conclusion (e.g. very similar composition of one or more of the tested GOS products to VITAGOS, etc.). Furthermore, please explain if these studies provide a basis for Vitalus to conclude that VITAGOS is not genotoxic or if it only provides a foundation to state that VITAGOS is not <u>expected</u> to be genotoxic.*

The conclusion that VITAGOS™ is not genotoxic is based on the facts that GOS products contain common nutrients (oligosaccharides, galactose, lactose, glucose, minerals), which do not possess functional groups that are associated with DNA reactivity, and the genotoxicity studies conducted by Kobayashi et al. (2009) and Narumi et al. (2014), which were performed using a GOS product that is manufactured in a manner and has a compositional profile similar to VITAGOS™. Both Kobayashi et al. and Narumi et al. used a product, which is the subject for GRN 334, manufactured using a combination of β-galactosidases derived from *Sporobolomyces singularis* and *Kluyveromyces lactis*, ion exchange and evaporation, and has a GOS content of no less than 55%. Because Vitalus' conclusion is based on the manufacturing and compositional similarities between VITAGOS™ and the GOS product that was used in the other studies, it is more appropriate to state that VITAGOS™ is not expected to be genotoxic.

4) *On page 31 the notifier states that "The cecum is not found in humans". This statement is incorrect. Please revise this statement and explain how this correction may alter, or not, Vitalus' discussion and conclusion regarding the toxicological relevance of cecal enlargement in rodents to humans.*

The statement "The cecum is not found in humans" is not correct and the sentence should be revised to, "Unlike the human cecum, the rat cecum is a site where non-digestible substances are fermented." Although both humans and rats have cecums, there are known anatomical and functional differences between the rat and human cecum that explain why cecal enlargement in rats following the ingestion of diets containing high amounts of non-digestible carbohydrates is not toxicologically relevant to humans. Specifically, the rat cecum is a large, curved kidney-shaped blind pouch where peristaltic and anti-peristaltic movements occur, facilitating the digestion and fermentation of non-digestible carbohydrates by resident bacteria. The bacteria in turn produce short-chain fatty acids that are then absorbed. Thus, following ingestion of diets containing high amounts of non-digestible substances, fermentation products such as organic acids may have an adverse impact on the rat cecum because of the prolonged period of exposure. The human cecum, in contrast, does not contain these resident bacteria and therefore is not exposed to the same non-digestible carbohydrate metabolites and conditions as the rat cecum (Greaves et al., 2012). Importantly, correcting the statement "the cecum is not found in humans" does not change the conclusion regarding the toxicological relevance of cecal enlargement in rodents to humans.

5) *In Table 2 of the notice, the **Regulatory Status** entry for **β-galactosidase (derived from Aspergillus oryzae)** refers to a series of GRNs and the FDA Partial List of Enzyme Preparations Used in Food rather than discussing the safety of the production organism itself. Please discuss the safety of Aspergillus oryzae as a production organism and not the enzyme preparation (e.g., as shown in GRNs 103, 106, 113, 122, 142, 201). Additionally, please discuss the known safety of the wild-type β-galactosidase enzyme from A. oryzae (such as shown in GRN 510 where the β-galactosidase from A. oryzae is produced in A. niger).*

In the United States, a variety of enzyme preparations derived from *Aspergillus oryzae*, including β-galactosidase, are Generally Recognized As Safe (GRAS) (21 CFR §137.105; GRN 8; GRN 34; GRN 75; GRN 90; GRN 103; GRN 113; GRN 122; GRN 142; GRN 201). *Aspergillus oryzae* is a member of the *Aspergillus flavi* group of *Aspergillus* species and has long history of safe use in the production of fermented foods (for review see Chang and Ehrlich, 2010). Although *A. oryzae* is almost genetically identical to its relative *A. flavus*, which is a known to produce mycotoxins, namely aflatoxin (Payne et al., 2006), genotypic and phenotypic studies have shown that multiple strains of *A. oryzae* possess non-functional aflatoxin-producing genes and do not produce aflatoxin (Tao and Chung, 2014).

The GOS present in VITAGOS™ are synthesized using the β-galactosidase derived *A. oryzae* strain GL 470, which is same strain used to prepare the β-galactosidase used to manufacture the GOS that is the subject of GRN 620. Importantly, in GRN 620 Nestle Nutrition determined that *A. oryzae* GL 470 does not produce any mycotoxins or aflatoxin, confirming the results of Tao and Chung (2014). Thus, the use of *A. oryzae* GL 470 β-galactosidase is safe and suitable for use in the production of food and VITAGOS™ is, therefore, not a potential source of mycotoxins.

As a protein, the β-galactosidase used to produce VITAGOS™ has the potential to cause food allergic reactions. However, proteins are ubiquitous and only a small percentage of them are food allergens. Moreover, the enzymes used to manufacture VITAGOS™ are heat inactivated and removed during manufacturing though filtration and resin adsorption processes. For reference, the protein specification for the finished product is no more than 0.2%, the limit of detection for the assay is 0.16 %, and data from the five batches presented in GRN 721 show that protein was not detected. To confirm that a β-galactosidase derived from *A. oryzae* is not a food allergen, an extensive literature search using PubMed and Google Scholar was conducted using the key words *Aspergillus oryzae*, galactosidase, and allergy. Although the *A. oryzae* β-galactosidase has been reported to be an aeroallergen (Bernstein et al., 1999; Stöcker et al., 2016), no reports of the enzyme being a food allergen were found. These results indicate that allergic reactions resulting from the ingestion of a β-galactosidase derived from A. oryzae or a product manufactured with a β-galactosidase derived from *A. oryzae* are not expected and, thus, meet the GRAS standard of reasonable certainty of no harm.

Should you have additional questions, please let us know.

Sincerely,

(b) (6)

Claire L, Kruger, Ph.D., D.A.B.T.
President

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References

Bernstein, J.A., Bernstein, D.I., Stauder, T., Lummus, Z., and Bernstein, I.L. (1999). A cross-sectional survey of sensitization to Aspergillus oryzae-derived lactase in pharmaceutical workers. J Allergy Clin Immunol 103, 1153–1157.

Chang, P.K., and Ehrlich, K.C. (2010). What does genetic diversity of Aspergillus flavus tell us about Aspergillus oryzae. Int J Food Microbiol 138, 189–199.

Greaves, P. (2012). Digestive System, Large Intestine. In Histopathology of Preclinical Toxicity Studies, Fourth Edition (Oxford, UK: Academic Press), Chapter 8, pp. 391-405.

GRN 8 (1999). Novo Nordisk.

GRN 34 (2000). Novo Nordisk.

GRN 75 (2001). Novozymes North America Inc.

GRN 90 (2002). Enzyme Technical Association.

GRN 103 (2002). Novozymes North America, Inc.

GRN 113 (2003). Enzyme Technical Association.

GRN 122 (2003). Novozymes North America, Inc.

GRN 142 (2004). Novozymes North America Inc.

GRN 201 (2006). Novozymes North America Inc.

GRN 620 (2016). Nestle.

Kobayashi, T., Yasutake, N., Uchida, K., Ohyama, W., Kaneko, K., and Onoue, M. (2009). Safety of a novel galacto-oligosaccharide: Genotoxicity and repeated oral dose studies. Hum Exp Toxicol 28, 619–630.

Kobayashi, T., Takano, M., Kaneko, K., and Onoue, M. (2014b). A one-generation reproduction toxicity study in rats treated orally with a novel galacto-oligosaccharide. Hum Exp Toxicol 33, 814–821.

Narumi, K., Takasawa, H., Ohyama, W., and Kaneko, K. (2014). In vivo comet assay of a novel galacto-oligosaccharide in rats. Hum Exp Toxicol 33, 488–495.

Payne, G.A., Nierman, W.C., Wortman, J.R., Pritchard, B.L., Brown, D., Dean, R.A., Bhatnagar, D., Cleveland, T.E., Machida, M., and Yu, J. (2006). Whole genome comparison of Aspergillus flavus and A. oryzae. Medical Mycology 44, S9–S11.

Stöcker, B., Grundmann, S., Mosters, P., Nitzsche, P., and Brehler, R. (2016). Occupational sensitization to lactase in the dietary supplement industry. Arch Environ Occup Health 71, 259–267.

Tao, L., and Chung, S.H. (2014). Non-aflatoxigenicity of commercial Aspergillus oryzae strains due to genetic defects compared to aflatoxigenic Aspergillus flavus. J Microbiol Biotechnol 24, 1081–1087.

From:	Claire Kruger
To:	Morissette, Rachel
Cc:	Dietrich Conze; Kathy Brailer
Subject:	RE: literature search for GRN 721
Date:	Wednesday, November 01, 2017 3:51:46 PM
Attachments:	image007.png image008.png image010.png

Hi Rachel:

The literature search noted in our response was conducted on October 26, 2017.

Best regards,

Claire

Claire Kruger, PhD, DABT, CFS
President
Senior Director Regulatory Affairs

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From: Morissette, Rachel [mailto:Rachel.Morissette@fda.hhs.gov]
Sent: Wednesday, November 1, 2017 2:41 PM
To: Claire Kruger <ClaireK@chromadex.com>
Subject: literature search for GRN 721

Hi Claire,

Can you please confirm the date through which an updated literature search was done for GRN 721?

Thanks,

Rachel

Rachel Morissette, Ph.D.
Consumer Safety Officer

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