GENERALLY RECOGNIZED AS SAFE (GRAS) NOTIFICATION FOR ALPHA-GALACTO-OLIGOSACCHARIDES (ALPHAGOS®) IN CONVENTIONAL FOODS AND BEVERAGES AND NON-EXEMPT INFANT FORMULAS

Prepared for:

Olygose
Parc Technologique des Rives de l'Oise
BP 50149, F-60201 Compiegne Cedex
France

Prepared by:

Spherix Consulting Group, Inc.
11821 Parklawn Drive, Suite 310
Rockville, MD 20852
USA

November 13, 2019

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LIST OF ABBREVIATIONS

Alanine Aminotransferase (ALT)

Alkaline Phosphatase (ALP)

Alpha-Galacto-Oligosaccharides (ALPHA-GOS)

Aspartate aminotransferase (ASAT)

Centers for Disease Control (CDC)

Code of Federal Regulations (CFR)

Colony forming units (CFU)

Council on the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH)

Cyclophosphamide (CPA)

Degrees of polymerization (DP)

Dry matter (DM)

European Feed Ingredients Safety Certification (EFISC)

Federal Food, Drug, and Cosmetic Act (FFDCA)

Food and Nutrition Database for Dietary Studies (FNDDS)

Foundation for Food Safety System Certification (FSSC)

Freedom of Information Act (FOIA)

Functional Observational Battery (FOB)

Galacto-oligosaccharides (GOSs)

Generally Recognized As Safe (GRAS)

Genetically modified organism (GMO)

Good Laboratory Practices (GLP)

GRAS Notification (GRN)

Hematocrit (HCT)

Hemoglobin (HGB)

High-Performance Anion-Exchange Chromatography (HPAEC)

Limit of quantitation (LOQ)

Mean Corpuscular Hemoglobin Concentration (MCHC)

Mitomycin C (MMC)

Mobile Examination Center (MEC)

National Center for Health Statistics (NCHS)

National Health and Nutrition Examination Surveys (NHANES)

No Observed Adverse Effect Level (NOAEL)

Not detected (ND)

Organization for Economic Cooperation and Development (OECD)

Parts per million (PPM)

Polychlorinated biphenyls (PCBs)

Primary Sampling Units (PSUs)

Pulsed Amperometeric Detection (PAD)

Rafinose family oligosaccharides (RFO)

Red blood cell count (RBC)

United States Department of Agriculture (USDA)

US Environmental Protection Agency (EPA)

US Food and Drug Administration (FDA)

White blood cell count (WBC)

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

A. SUBMISSION OF GRAS NOTICE

Olygose is hereby submitting a GRAS notice in accordance with subpart E of part 170.

B. NAME AND ADDRESS OF THE SPONSOR

Olygose
Parc Technologique des Rives de l'Oise
BP 50149, F-60201 Compiegne Cedex
France

C. COMMON OR USUAL NAME

Alpha-galacto-oligosaccharides; alpha-GOS

D. TRADE SECRET OR CONFIDENTIAL INFORMATION

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

E. INTENDED USE

AlphaGOS® will be added to beverages (carbonated/non-carbonated, juice, flavored water), ready to drink iced coffee and teas, sports drinks, energy drinks, soups, meal-replacement drinks, processed fruits and vegetable juices, dairy products analogs, dairy and analogs, sugars and sweets, coffee and tea, non-exempt infant formulas, baby cereals, baby foods, and toddler foods.

F. BASIS FOR GRAS DETERMINATION

This GRAS determination for the use of AlphaGOS® 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 AlphaGOS® 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 uses of AlphaGOS® as an ingredient for the intended uses in foods and beverages, as listed above, has been determined to be safe through scientific procedures set forth under 21 CFR §170.30(b) based on the following:

1. AlphaGOS® is an alpha-linked galacto-oligosaccharide (alpha-GOS) manufactured using pea solubles derived from *Pisum sativum*.

2. All raw materials and processing aids used to produce AlphaGOS® comply with appropriate US federal regulations. The production process takes place in a facility operating under Good Manufacturing Practice (GMP) and is controlled to ensure a consistently reproducible product, free of known contaminants.

3. AlphaGOS® consists of 1 to 3 glucose units linked via α (1→6) glycosidic bonds to a terminal galactose and is a non-digestible oligosaccharide.

    a. Alpha-GOS pass 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, similar to other fermentation products following the ingestion of other non-digestible materials.

    b. Alpha-GOS is naturally present in certain foods, such as legumes, from which AlphaGOS® is derived. Raffinose and stachyose (both alpha-GOS) are the main short chain carbohydrates in legumes, particularly lima beans, red beans, lentils and chick peas, and are found in other foods like Jerusalem artichokes.

    c. AlphaGOS® has a history of consumption in many countries including France, Germany, United Kingdom, Italy, Netherlands, Canada, Spain, Czech Republic, South Korea, and Australia.

4. The pivotal toxicology studies supporting the GRAS status of AlphaGOS® are a 90-day subchronic toxicity study in rats, an Ames test, an *in vitro* chromosome aberration study and a 3-week neonatal piglet study (Kruger et al., 2017a; Kruger et al., 2017b).

    a. AlphaGOS® was not genotoxic in an Ames test or in an *in vitro* chromosome aberration study.

    b. The subchronic toxicity study established a no observed adverse effect level (NOAEL) of at least 2000 mg AlphaGOS®/kg body weight/day, the highest dose tested.

    c. Safety and tolerance in an infant population was addressed in a neonatal piglet study using piglet formula supplemented with 8 mg/mL (8 g/L) of AlphaGOS®, the proposed intake for infant formula in human infants. The neonatal piglet model is frequently used to test ingredients at levels consistent with intended infant exposure (Constable et al. 2017).

5. Two published clinical trials of AlphaGOS® (Morel et al. 2015) reported that AlphaGOS® is well tolerated in adult human subjects with no test-article related adverse events recorded at levels up to 12 g/day for 14 days.

Therefore, AlphaGOS® is safe and GRAS at the proposed levels of addition to the intended foods and beverages and infant formulas listed in this GRAS document. AlphaGOS® is 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. Together, these results confirm safety of the proposed EDI of AlphaGOS® in foods and beverages of 65 and 128 mg/kg/day or 4.3 g/day and 8.5 g/day for the mean and 90th percentile consumers (age 2 and up) and the safety of intake from infant formulas at a level of addition of 8 g/L.

G. PREMARKET APPROVAL

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

H. AVAILABILITY OF INFORMATION

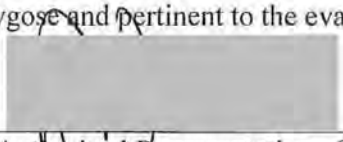
The data and information that serve as the basis for this GRAS determination will be available for review and copying at reasonable times at the office of Claire L. Kruger, PhD, DABT, Managing Partner, Spherix Consulting Group, Inc., at 11821 Parklawn Drive, Suite 310, Rockville, MD 20852. Telephone: 301-775-9476; Email: ckruger@spherixgroup.com, or be sent to FDA upon request.

I. FREEDOM OF INFORMATION ACT

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 Olygose and pertinent to the evaluation of the safety and GRAS status of the use of this substance.



November 13, 2019

Signature of Authorized Representative of Olygose

François DELBAERE, CEO

Date

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

A. COMMON OR USUAL NAME

Alpha-galacto-oligosaccharides; alpha-GOS; α-GOS

B. TRADE NAME

Alpha-galacto-oligosaccharides produced by Olygose are currently marketed under three brand names: Cravingz'Gone™, AlphaGOS® and P-GOS™. The P-GOS™ is the brand marketed for use in non-exempt infant formulas. The product is referred to as AlphaGOS® in this GRAS notice.

C. DESCRIPTION OF ALPHAGOS®

AlphaGOS® consists of alpha-galacto-oligosaccharides (alpha-GOS) in a syrup or powder. The powder is the spray dried syrup, with no additional excipients.

1. Identity

Alpha-GOS are non-digestible saccharides, present in many natural sources such as legumes (Gawłowska et al., 2017). Alpha-GOS consists of α-1,6-linked chains of D-galactose attached to the 6-position of D-glucose. AlphaGOS® is a mix of bi-, tri-and tetrasaccharides, respectively named melibiose (CAS# 5340-95-4), manninotriose (CAS# 13382-86-0), and verbascotetraose (CAS# 1111-08-6). These three alpha-GOS molecules are derived from the raffinose family oligosaccharides (raffinose, stachyose and verbascose) (Figure 1). During the production process, sulfuric acid hydrolyzes the terminal fructose from the raffinose family oligosaccharides to produce the oligosaccharides found in AlphaGOS®.

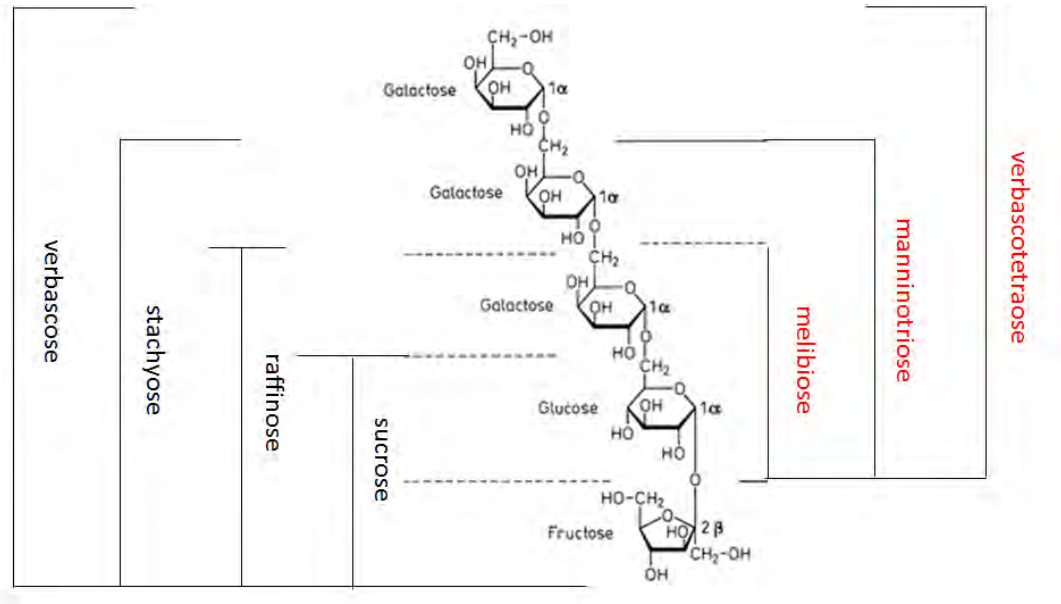


Figure 1. Alpha-GOS Chemical Structure

2. Biochemical Characterization

AlphaGOS® is a mixture of alpha-linked melibiose, manninotriose, and verbascotetraose (Table 1), generated through the hydrolysis of raffinose, stachyose, and verbascose, respectively. Olygose uses High-Performance Anion-Exchange Chromatography (HPAEC) with Pulsed Amperometeric Detection (PAD) for the quantitation of melibiose, manninotriose, and verbascotetraose. This is a validated method derived from the AOAC 997.08 method for detection of fructans in food products. The apparatus used (HPAEC chain, chromatography column, reagents) is identical to those described in AOAC 997.08, but the concentration of each sugar is measured against solutions of pure melibiose, manninotriose, and verbascotetracose instead of concentration determined by hydrolysis-released sugars.

Olygose have validated their method for the detection of melibiose, manninotriose, and verbascotetraose within their product. The proportions of the oligosaccharides described in Table 1 are based on historical data from multiple batches of AlphaGOS® batches.

Table 1. Alpha-Galacto-Oligosaccharide Content of AlphaGOS®

Oligosaccharide	CAS#	Content in AlphaGOS®
Maltose, Sucrose (DP1)	Various	< 0.5%
Melbiose (DP2)	5340-95-4	1-10%
Manninotriose (DP3)	13382-86-0	38-50%
Verbascotetraose (DP4)	1111-08-6	40-55%
DP: Degrees of Polymerization		

D. PRODUCTION PROCESS

1. Production of AlphaGOS®

The manufacturing process of AlphaGOS® takes place at Olygose, Venette, France , and is described in Figure 2. Briefly, AlphaGOS® is manufactured from pea (*Pisum sativum*) solubles by a series of filtration steps, sugar hydrolysis by sulfuric acid, nanofiltration to remove monosaccharides, polishing and evaporation. Pea solubles obtained from starch and protein wet extraction from peas and must contain 60 – 85% moisture, as defined in "Commission Regulation (EU) 575/2011" (2011). It is mostly composed of soluble proteins and oligosaccharides. The final AlphaGOS® product can be either in the form of a thick syrup or a spray dried powder (no additional excipients).

The pea solubles raw material is supplied by Roquette, Lestrem, France and is inspected for pH, color, odor, and temperature before use in the production process (quality control 1, QC1). The pea solubles are initially centrifuged at 60°C to remove any solids. After centrifugation, the non-soluble matter weight should be < 0.2% (QC2). The pea solubles supernatant fraction then undergoes a series of filtration steps to remove protein. QC3-6 assesses the saccharide content of the retentate at each filtration step. The filtrate is demineralized with resins to remove ash, with the conductivity of the filtrate < 200 µS/cm (QC7) before the sulfuric acid hydrolysis reaction. Sulfuric acid is added to the demineralized liquid and incubated for 10 hours at 80°C and pH of 2.3 to hydrolyze the terminal fructose from the oligosaccharides. The reaction is stopped by the addition of sodium hydroxide until a pH of 4-5 is reached. The solution is then cooled to < 25°C, and the sum of all terminal fructose-linked sugars at this stage must be less than 1% (QC8). The solution is then filtered at 25°C to remove remaining fructose and glucose monosaccharides. The monosaccharide content after this filtration step must be less than 0.5% (QC9). The salts from the neutralized acids in the previous step are removed (referred to as polishing in Figure 2) from the oligosaccharide solution at 25°C, with the conductivity of the solution less than 100 uS/cm (QC10). Activated carbon is then added to the oligosaccharide solution for 2 hours and heated to 60°C to further remove any impurities. The oligosaccharide solution then undergoes a heating step at 72°C for at least 30 minutes and a final filtration step. The filtrate is then evaporated until the dry matter of the oligosaccharide syrup is greater than 78% (QC11).

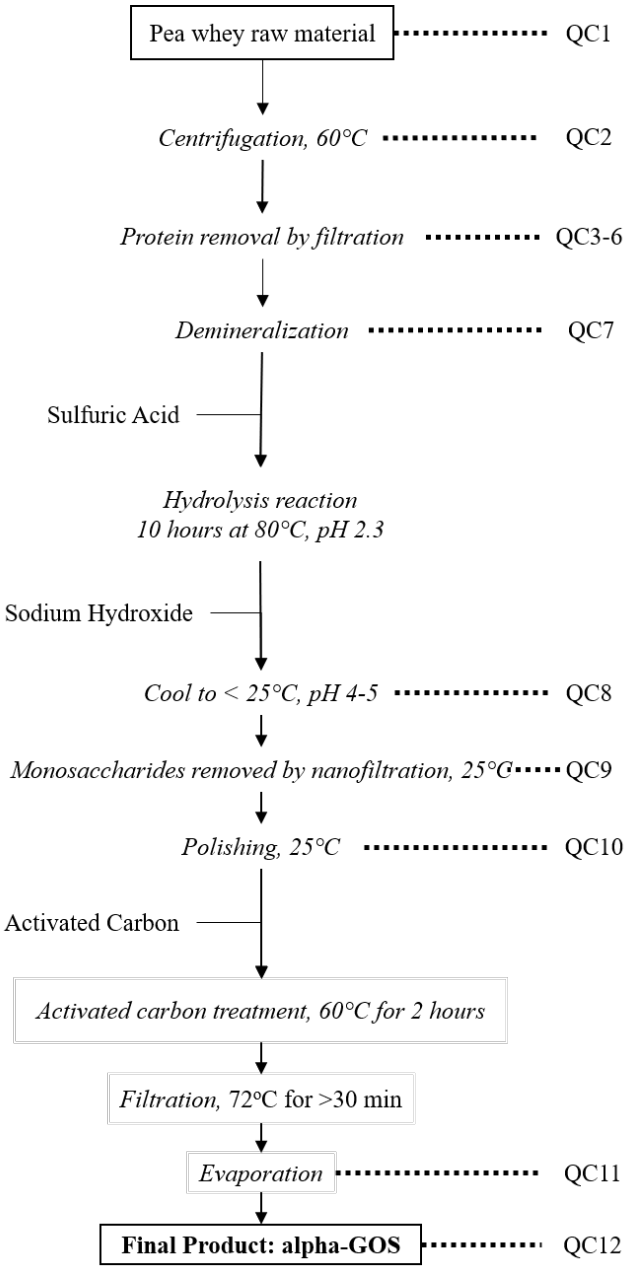


Figure 2. Production of AlphaGOS®

AlphaGOS® is manufactured from pea solubles. The pea solubles is assessed for quality (QC1) concentrated (Q2) and protein removed through a series of filtration steps. The sugar content of the pea solubles is assessed at each filtration step (QC3-6). The pea solubles is then demineralized, as assessed by ash content (QC7) before sulfuric acid is added for the hydrolysis reaction, digesting the starch in pea solubles to oligosaccharides. The reaction is cooled and halted by the addition of sodium hydroxide until the pH is between 4 – 5 (QC8). The resulting oligosaccharides are filtered to exclude monosaccharides (QC9), and the oligosaccharide solution is further polished to remove remaining ash (QC10), filtered and evaporated to yield a syrup with at least 78% sugar (QC11). The syrup is then assessed for compliance with product specifications (QC12).

After the evaporation step, the resulting syrup is considered the final AlphaGOS® product and compliance with the product specifications (Tables 3 and 4) is assessed (QC12). The water activity present in the syrup is low enough (< 0.8 a_w) to prevent most microbial contaminants. If the AlphaGOS® syrup fails any of the product specifications assessed in QC12, the product is reworked at the appropriate step in the process. AlphaGOS® syrup is filled under microbiologically controlled environment into 1000-liter IBC tanks manufactured from food grade high density polyethylene (HDPE). The syrup may then be spray dried to generate AlphaGOS® powder at Lesaffre Ingredient Services, Cérences, France. AlphaGOS® powder is packed under a microbiologically controlled environment (filtered air with over pressured packing room) into 20 kg bags with sealed inner food-grade polyethylene (PE) liner. Once packed both the syrup and powder grades are stored at room temperature in a dry environment.

2. Processing Facilities

The oligosaccharides (raffinose family of oligosaccharides) used in the production of AlphaGOS® are found in pea solubles. Pea solubles are obtained during the refining of pea starch and pea protein. Roquette (Lestrem, France) supplies the raw ingredient, pea solubles. Roquette is a GMP facility with European Feed Ingredients Safety Certification (EFISC), accredited with Foundation for Food Safety System Certification (FSSC) 22000. The pea solubles are derived from conventional non-GMO peas *(Pisum sativum*) sourced from France, Belgium and Germany and received in FSSC certified factories.

Olygose (Venette, France) processes the raw ingredient, pea solubles, into AlphaGOS® syrup. It is currently in the process of ISO 22000 certification.

Lesaffre Ingredients Services (Cérences, France) produces AlphaGOS® powder from AlphaGOS® syrup via spray drying. The manufacturing plant has been accredited by the Foundation for Food Safety System Certification, which combine ISO 22000:2005, ISO/TS 22002-1 and additional FSSC 22000 regulatory requirements.

3. Raw Materials, Processing Aids, and Food Contact Substances

All processing aids (ingredients, enzymes, resins etc.), raw material and food contact surfaces comply with 21 US Code of Federal Regulations (CFR) and/or Food and Chemical Codex standards. Table 2 lists all processing aids used in the production of AlphaGOS®.

Table 2. AlphaGOS® Processing Aids' Regulatory Compliance	
Processing Aid	**21CFR Regulation**
Sulfuric Acid	§184.1095
Sodium Hydroxide	§184.1763
Bentonorit CA1 Activated Carbon	GRAS, see Appendix
Membranes, Resins & Filters	
Suez Water Technologies Dairy PW Series membrane	§175, 176, 177, 178, 182
GE DK Series membrane	§175, 176, 177, 178, 182
Suez Water Technologies Dairy DK8038C30	§175, 176, 177 178, 182
GE Duracon NF2 Series membrane	§175, 176, 177, 178, 182
DIAION PK208LH Resin	§173.25
A-103S Resin	§177.2710
A 510 Resin	§173.25
C-150S Resin	§173.25
BECO K1, KD10, KDS15 & S80 Filters	§177.2260

E. FINISHED PRODUCT SPECIFICATIONS AND OTHER QUALITY ATTRIBUTES

1. Product Specifications

To ensure a consistent food-grade product, Olygose tests each batch of AlphaGOS® powder and syrup for compliance with a defined set of product specifications using compendial methods (Tables 3 and 4). Data from three batches of AlphaGOS® powder and syrup demonstrate control of the production process and compliance with the product specifications.

Table 3. Specifications and Batch Analysis for AlphaGOS® Powder						
Parameter	**Method**	**Specification**	**LOQ**	**AlphaGOS® Batch**		
				2014 009 12	**2015 01 01**	**2017 01 01**
Appearance	Visual	White	-	White	White	White
Dry matter (%)	Karl Fisher – Based on ISO 8534	> 95	-	97.0	96.5	96.5
Protein (%)*	Internal method based on Reg CE No. 152/2009 Kjeldhal	< 0.5	0.2	< 0.2	0.20	< 0.2
Ash (%)*	Internal Method Based on Reg CE No. 152/2009	< 0.5	0.1	< 0.2	< 0.1	< 0.2
Alpha-GOS (DP2-DP4 with Gal(n) alpha 1-4 Glc structure) (%)*	Internal method based on AOAC 997.08	96 ± 3	-	96.09	97.76	96.51
Digestible sugars (%)*	Internal method based on AOAC 997.08	< 5	0.2	< 0.2	< 0.3	< 0.2
Microbials *(cfu/g unless otherwise stated)*						
Aerobic plate count	NF EN ISO 4833-1 (A)	< 1000	-	500	< 40	<40
Escherichia. coli	NF ISO 16649-2 modified	< 1	-	< 1	< 1	<1
Enterobacteria	NF ISO 21528-2	< 1	-	< 1	< 1	<1
Coliforms	NF ISO 4832	< 1	-	< 1	< 1	<1
Staphylococcus aureus	NF EN ISO 6888-3 (A)	ND in 1 g	-	ND	ND	ND
Pseudomonas aeruginosa	Ph. Eur.2.6.13	ND in 1 g	-	ND	ND	ND
Yeast and Molds	NF V08-059 (A)	≤ 10	-	< 10	< 10	<10
Additional Microbials for Infant formula ingredients *(cfu/g unless otherwise stated)*						
Cronobacter spp.	ISO TS 22964 (A)	ND in 25 g	-	ND	ND	ND
Bacillus cereus	NF EN ISO 7932 (A)	50-500/g	-	< 10	< 10	< 10
Heavy Metals *(ppm)*						
Arsenic	ISO 11885/ISO 17294-2(A)	≤ 1 ppm	-	0.058	< 0.05	0.061
Cadmium	ISO 11885/ISO 17294-2(A)	<1 ppm	-	< 0.01	< 0.01	< 0.01
Lead	ISO 11885/ISO 17294-2(A)	<1 ppm	-	< 0.02	< 0.02	< 0.02
Mercury	DIN EN 13806; ASU L 00.00-19/4(A)	<1 ppm	-	< 0.005	< 0.005	< 0.005

*Specification as percent of dry matter
Abbreviations: LOQ: limit of quantitation, ISO: International Organization for Standardization, DP: degree of polymerization; Gal: galactose; Glc: glucose, AOAC: Association of Official Analytical Chemists, NF:Norme Française, EN: European Norm, Ph. Eur.: European Pharmacopaeia, cfu: colony forming units, ND: not detected, TS: technical specifications, DIN: Deutsches Institut für Normung (German Institute for Standardization), ASU: Official Collection of Testing Methods according to §64 German Food and Feed Code(LFGB) , ppm: parts per million

Table 4. Specifications and Batch Analysis for AlphaGOS® Syrup

Parameter	Method	Specification	LOQ	AlphaGOS® Batch		
				IBC 2014 02	IBC 2014 10	IBC 2016 01
Appearance	Visual	Clear	-	Clear	Clear	Clear
Dry matter (%)	Karl Fisher – Based on ISO 8534	> 72	-	79.32	72.92	73.77
Protein (%) *	Internal method based on Reg CE No. 152/2009 Kjeldhal	< 0.5	0.2	< 0.2	< 0.2	< 0.2
Ash (%) *	Internal Method Based on Req CE No. 152/2009	< 0.5	0.1	< 0.1	< 0.1	< 0.1
Alpha-GOS (DP2-DP4 with Gal(n) alpha 1-4 Glc structure) (%) *	Internal method based on AOAC 997.08	96 ± 3	-	97.75	96.87	96.20
Digestible sugars (%) *	Internal method based on AOAC 997.08	< 5	0.2	0.2	0.37	0.22
Microbials *(cfu/g unless otherwise state)*						
General bacterial count	NF EN ISO 4833-1	< 1000	-	< 10	< 10	<10
E. coli	NF ISO 16649 2	< 1	-	< 1	< 1	<1
Enterobacteria	NF V 08 054	< 1	-	< 1	< 1	<1
Coliforms	NF V 08 050	< 1	-	< 1	< 1	<1
Staphylococcus aureus	EN ISO 6888-1:1999ce	ND in 1 g	-	ND	ND	ND
Pseudomonas aeruginosa	Ph. Eur.2.6.13	ND in 1 g	-	ND	ND	ND
Yeast and Molds	NF V 08-059	≤ 10	-	< 10	< 10	<10
Additional Microbials for Infant formula ingredients *(cfu/g unless otherwise stated)*						
Cronobacter spp.	ISO TS 22964 (A)	ND in 25 g	-	ND	ND	ND
B. cereus	NF EN ISO 7932 (A)	50-500/g	-	< 10	< 10	< 10
Heavy Metals *(ppm)*						
Arsenic	ISO 11885/ISO 17294-2(A)	≤ 1 ppm	-	< 0.05	< 0.05	< 0.05
Cadmium	ISO 11885/ISO 17294-2(A)	<1 ppm	-	< 0.01	< 0.01	< 0.01
Lead	ISO 11885/ISO 17294-2(A)	<1 ppm	-	< 0.02	< 0.02	< 0.02
Mercury	DIN EN 13806; ASU L 00.00-19/4(A)	<1 ppm	-	< 0.005	< 0.005	< 0.005

*Specification as percent of dry matter
Abbreviations: LOQ: limit of quantitation, ISO: International Organization for Standardization, DP: degree of polymerization; Gal: galactose; Glc: glucose, AOAC: Association of Official Analytical Chemists, NF:Norme Française, EN: European Norm, Ph. Eur.: European Pharmacopaeia, cfu: colony forming units, ND: not detected, TS: technical specifications, DIN: Deutsches Institut für Normung (German Institute for Standardization), ASU: Official Collection of Testing Methods according to §64 German Food and Feed Code(LFGB) , ppm: parts per million

2. Other Quality Attributes

In addition to ensuring compliance with product specifications, the quality of AlphaGOS® is assessed by oligosaccharide content analysis, allergen screening, mycotoxin screening and screening for additional contaminants.

a. Oligosaccharide Content in AlphaGOS® Powder and Syrup

AlphaGOS® is a mix of bi-, tri-and tetrasaccharides, respectively named melibiose (DP2), manninotriose (DP3), and verbascotetraose (DP4). These saccharides are detected by the previously discussed method based on AOAC 997.08 for both the powder and syrup forms of AlphaGOS®. Batches of AlphaGOS® typically contain a range of individual oligosaccharides, with melibiose contributing 1-10%, manninotriose 38–50% and verbascotetraose 40-55% of the alpha-GOS content (Tables 5 and 6). The ranges in specific oligosaccharides reflect the inherent biological variability from the raw material, derived from *Pisum sativum*.

Table 5. Specific Oligosaccharide Content in AlphaGOS® Powder

	AlphaGOS® Powder Batch		
Parameter	**2014 009 12**	**2015 01 01**	**2017 01 01**
alpha-GOS (DP2-DP4 with Gal(n) alpha 1-4 Glc structure) %	96.09	97.76	96.51
Melibiose (DP2) %	3.96	5.88	2.67
Manninotriose (DP3) %	48.92	41.77	39.72
Verbascotetraose (DP4) %	43.21	50.11	54.12
Method: Internal method based on AOAC 997.08			

Table 6. Specific Oligosaccharide Content in AlphaGOS® Syrup

	AlphaGOS® Syrup Batch		
Parameter	**IBC 2014 02**	**IBC 2014 10**	**IBC 2016 01**
alpha-GOS (DP2-DP4 with Gal(n) alpha 1-4 Glc structure) %	97.75	96.87	96.20
Melibiose (DP2)	3.80	4.66	1.60
Manninotriose (DP3)	47.66	47.74	39.52
Verbascotetraose (DP4)	46.29	44.47	55.08
Method: Internal method based on AOAC 997.08			

b. Allergens Screened in AlphaGOS®

AlphaGOS® is annually screened for allergens. Three non-consecutive batches of AlphaGOS® were shown to be free of both soy and gluten proteins (Table 7). Additionally, the production facilities do not process any of the following known allergens: wheat, cereals, crustaceans, eggs, fish, peanuts, soy, milk, nuts, celery, mustard, sulphur dioxide and sulphites in concentration higher than 10 ppm, sesame seeds, lupin, mollusks, corn (maize), potato, cocoa, coconut, fruits, other legumes, latex, liliaceae, and mushrooms. Since the AlphaGOS® production process is an environment in which it is unlikely for proteins to survive and the protein specification for both the powder and syrup is very low (0.5%), the risk of allergens in AlphaGOS® is also very low.

Table 7. Wheat and Soy Proteins in AlphaGOS®			
	AlphaGOS® Batch		
Protein	**2014 009 012***	**2015 01 01***	**2017 06 01***
Soy	ND (< 2.5 mg/kg)	ND (< 2.5 mg/kg)	ND (< 5 mg/kg)
Gluten	ND (< 10 ppm)	ND (< 10 ppm)	ND (< 2.5 mg/kg)
*Method: ELISA. Batches 2014 009 12 and 2015 01 01 performed by Eurofins, Batch 2017 06 01 by Wessling			

c. *Mycotoxins, Dioxins and Polychlorinated biphenyls, Pesticides, Phenoxy Carboxylic Acids and Perchlorate in AlphaGOS®*

AlphaGOS® is monitored for various contaminants, such as fungal toxins and chemical contaminants from water or soil, pesticides, and polychlorinated biphenyls (PCBs). These contaminants are analyzed in 10 – 15 batches of AlphaGOS® powder and 12 – 24 batches of syrup every 12 months. The data shown in this section are from the powder form of AlphaGOS®. Since the powder form is generated from AlphaGOS® syrup, these data also describe the quality attributes of the syrup.

Mycotoxins toxins were screened in three non-consecutive batches of AlphaGOS® powder. No toxins were detected above the limit of detection in any of the batches of AlphaGOS® powder (Table 8). These contaminants are analyzed in batches of AlphaGOS® every 12 months.

Table 8. Mycotoxin Screen in AlphaGOS® Powder			
Mycotoxin	**AlphaGOS® Batch**		
	2014 009 12*	**2015 01 01***	**2017 06 01****
Aflatoxins, µg/kg. Method: EN 15851, modified			
Aflatoxin B1	< 0.01	< 0.01	< 0.01
Aflatoxin B2	< 0.01	< 0.01	< 0.01
Aflatoxin G1	< 0.01	< 0.01	< 0.01
Aflatoxin G2	< 0.01	< 0.01	< 0.01
Fumonisins, µg/kg, method: Internal LC/MSMS			
Fumonisin B1	< 20	< 20	< 10
Fumonisin B2	< 20	< 20	< 10
Fusarial toxins, µg/kg *Food Addit. Contam.* 2005 Aug; 22(80):752-60			
Deoxynivalenol (vomitoxin)	<20	<20	< 40
Ochratoxin A	< 0.1	< 0.1	< 0.5
Zearalenone	< 5	< 5	< 10
T-2 toxin	< 1	< 1	< 10
HT-2 toxin	< 3	< 3	<10

*Batches 2014 009 12 and 2015 01 01 were screened for mycotoxins at Eurofins. Their methods are indicated in the table.
**Batch 2017 06 01 was screened at Wessling. The methods used for screening the indicated parameters in this batch were:
Aflatoxins: ASU L 15.00-2(A)
Fumonisins: DIN EN 14352(A)
Deoxynivalenol (vomitoxin): WES 072(A)
Ochratoxin A: A. Thellmann, W. Weber: DLR 93 (1), 1997, S. 1-3(A)
Zearalenone: WES 128(A)
T-2 toxin: J. Agric. Food Chem., 2008, (56) pp. 4968-4975(A)

Three non-consecutive batches of AlphaGOS® powder were screened for dioxins and polychlorinated biphenyls (PCBs). No dioxin or PCB species were detected at the limit of detection in AlphaGOS® powder (Table 9). This screen is performed every 12 months.

Table 9. Dioxins and PCBs Screen in AlphaGOS® Powder			
	AlphaGOS® Batch		
Dioxins and PCBs	**2015 01 01***	**2014 009 12***	**2017 06 01****
PCDD/F (WHO 17): *Method: Regl. EU 2017/644 (food) (pg/g)*			
2,3,7,8-TCDD	<0.0193	<0.0190	<0.02
1,2,3,7,8-PeCDD	<0.0193	<0.0190	<0.04
1,2,3,4,7,8-HxCDD	<0.0193	<0.0190	<0.06
1,2,3,6,7,8-HxCDD	<0.0193	<0.0190	<0.06
1,2,3,7,8,9-HxCDD	<0.0193	<0.0190	<0.06
1,2,3,4,6,7,8-HpCDD	<0.0193	<0.0190	<0.3
OCDD	<0.0386	<0.0381	<1.00
2,3,7,8-TCDF	<0.0193	<0.0190	<0.04
1,2,3,7,8-PeCDF	<0.0193	<0.0190	<0.04
2,3,4,7,8-PeCDF	<0.0193	<0.0190	<0.04
1,2,3,4,7,8-HxCDF	<0.0193	<0.0190	<0.06
1,2,3,6,7,8-HxCDF	<0.0193	<0.0190	<0.06
1,2,3,7,8,9-HxCDF	<0.0193	<0.0190	<0.06
2,3,4,6,7,8-HxCDF	<0.0193	<0.0190	NS
1,2,3,4,6,7,8-HpCDF	<0.0193	<0.0190	<0.3
1,2,3,4,7,8,9-HpCDF	<0.0193	<0.0190	<0.3
OCDF	<0.0386	<0.0381	<1.0
PCB (WHO 12) *Method: Regl. EU 2017/644 (food) (pg/g, unless otherwise specified)*			
PCB 77	<0.242	<0.238	<2.00
PCB 81	<0.242	<0.238	<1.00
PCB 105	<4.83	<4.76	<10.0
PCB 114	<4.83	<4.76	<20.0
PCB 118	<4.83	<4.76	<40.0
PCB 123	<4.83	<4.76	<2.00
PCB 126	<0.242	<0.238	<0.25
PCB 156	<4.83	<4.76	<10.0
PCB 157	<4.83	<4.76	<2.00
PCB 167	<4.83	<4.76	<4.00
PCB 169	<0.242	<0.238	<1.00
PCB 189	<4.83	<4.76	<4.00
PCB 28	<0.00966 ng/g	<0.00952 ng/g	<40.0
PCB 52	<0.00966 ng/g	<0.00952 ng/g	<40.0
PCB 101	<0.00966 ng/g	<0.00952 ng/g	<40.0
PCB 138	<0.00966 ng/g	<0.00952 ng/g	<80.0
PCB 153	<0.00966 ng/g	<0.00952 ng/g	<80.0
PCB 180	<0.00966 ng/g	<0.00952 ng/g	<80.0
*Analysis of Batches 2014 009 12 and 2015 01 01 performed by Eurofins **Analysis of Batch 2017 06 01 performed by Wessling NS: Not Specified			

Pesticide residues were screened for in two non-consecutive batches of AlphaGOS® powder by Eurofins (Table 10). No pesticides were observed in these batches at the specified limits of quantitation. This analysis is performed every 12 months.

Table 10. Pesticides Not Detected in Two Batches of AlphaGOS® Powder		
Pesticide residue (Limit of quantitation in mg/kg)		
4,4-Dibromobenzophenone (0.005)	Aclonifen (0.01)	Acrinathrine (0.01)
Aldrin (0.001)	Benfluraline (0.001)	Benzoylprop-ethyl (0.005)
Beta-endosulfan (0.001)	Bifénox (0.01)	Bifenthrine (0.01)
Binapacryl (0.005)	Bromocyclen (0.005)	Bromoxynil-octanoate (0.005)
Butraline (0.01)	Cadusaphos (0.001)	Chlordane-cis (0.001)
Chlordane-gamma (beta, trans) (0.001)	Chlorfenapyr (0.005)	Chlorfenprop-méthyl (0.005)
Chlorfenson (0.005)	Chloroneb (0.01)	Chlorothalonil (0.005)
Chlorthal diméthyle (0.001)	Cyfluthrine (0.01)	Cyperméthrine (0.01)
Cypermethrine-alpha (0.01)	Cyphenothrine (0.01)	Cyhexatin (TCyT) (0.01)
DDD, o,p (0.002)	DDD, p,p (0.002)	DDE, o,p' (0.002)
DDE,p,p' (0.001)	DDT,o,p' (0.001)	DDT,p,p (0.002)
Deltamethrine (0.01)	Demeton (0.001)	Demeton-S-methyl (0.001)
Demeton-S-methyl-sulfone (0.001)	Diallat (0.05)	Dichlobénil (0.002)
Dichlone (0.01)	Dichloran (0.001)	Dichlorobenzophenone, o,p (0.005)
Dichlorobenzophenone, p,p (degradation dicofol) (0.005)	Dicofol, o,p (0.005)	Dicofol, p,p (0.005)
Dieldrine (0.001)	Dienochlor (0.005)	Dinitramine (0.002)
Dinobuton (0.005)	Dinocap (0.05)	Disulfoton (0.001)
Disulfoton sulfone (0.001)	Disulfoton sulfoxide (0.001)	Endosulfan alpha (0.001)
Endosulfan sulfate (0.002)	Endrine (0.002)	Esfenvalerate (0.005)
Ethalfluraline (0.002)	Ethoprophos (0.001)	Etridiazole (0.005)
ETU (Ethylene thiourea) (0.003)	Fenbutatin oxide (0.01)	Fenfluthrine (0.005)
Fenpropathrine (0.005)	Fenson(0.005)	Fensulfothion (0.001)
Fensulfothione sulfone (0.001)	Fensulfothion-PO-sulfon (0.001)	Fensulfothion-PO-sulfoxide (0.001)
Fentine (salts included) (0.003)	Fenvalerate (RR-/SS-Isomer) (0.005)	Fenvalerate (RS-/SR-Isomer) (0.005)
Fipronil (0.003)	Fipronil desulfinyl (0.003)	Fipronil sulfite (0.003)
Fipronil sulfon (0.01)	Flubenzimine (0.002)	Fluchloraline (0.002)
Flucythrinate (0.01)	Flumetraline (0.002)	Fluorodifen (0.005)
Fluoroimide (0.01)	HCH Alpha (0.001)	HCH Beta (0.002)
HCH Delta (0.002)	HCH, gamma – Lindane (0.001)	HCH epsilon (0.002)
Heptachlore (0.001)	Heptachlore epoxyde, cis (0.001)	Heptachlore epoxyde, trans (0.001)
Hexachlorobenzène (HCB) (0.001)	Ioxynil-Octanoate (0.005)	Isobenzane (0.001)
Isodrine (0.001)	Isopropalin (0.002)	Kétoendrin-delta (0.005)
Lambda cyhalothrine (0.005)	Méthoxychlore (0.005)	Mirex (0.001)
Nitrapyrine (0.005)	Nitrofene (0.002)	Nonachlor, trans (0.002)
Nonachlor, cis (0.001)	Octachlorostyrène (0.005)	Omethoate (0.001)
Oxychlordane (0.001)	Oxydéméton methyl (0.001)	Oxyfluorfene (0.002)
Pendiméthaline (0.002)	Pentachloroaniline (0.001)	Pentachloroanisole (PCA) (0.001)
Pentachlorobenzène (0.002)	Pentachlorothioanisole (0.002)	Perméthrine (0.01)
Plifenate (0.005)	Profluraline (0.001)	PTU (Propylène Thiourea) (0.003)
Quintozène (0.001)	S 421 (0.005)	Tau-fluvalinate (0.01)
Tecnazéne (0.001)	Téfluthrine (0.005)	Terbufos (0.001)
Terbufos-sulfon (0.001)	Terbufos-sulfoxide (0.001)	Tétradifon (0.002)
Tétrasul (0.005)	Toxaphène (camphéchlor) (0.04)	Tralomethrin (0.01)
Transfluthrin (0.005)	Triallate (0.005)	Trichloronate (0.002)
Trifluraline (0.001)		
Method: Liquid Chromatography/Mass Spectrometry		

Phenoxy carboxylic acids were screened for in two non-consecutive lots of AlphaGOS® powder. This analysis is performed every 12 months to demonstrate that no phenoxy herbicides are present in AlphaGOS®. This pesticide screen is required for infant formula ingredients in the European Union, Commission Directive 2006/141/EC (2006). No phenoxy carboxylic acids were found in two batches of AlphaGOS® (Table 11).

Table 11. Phenoxy Carboxylic Acids Screen in AlphaGOS® Powder, Shown as Totals After Hydrolysis

Parameter	LOQ (mg/kg)	AlphaGOS® Batch	
		2014 0009 12	2015 01 01
1-acid naphthylacetic	0.005	N.D.	N.D.
2,4,5-T	0.01	N.D.	N.D.
2,4-D	0.005	N.D.	N.D.
2,4-DB	0.005	N.D.	N.D.
2-Naphthoxyacetic acid.	0.005	N.D.	N.D.
4-CPA	0.005	N.D.	N.D.
Benazolin	0.01	N.D.	N.D.
Bentazone	0.005	N.D.	N.D.
Bromoxynil	0.005	N.D.	N.D.
Chloramben	0.01	N.D.	N.D.
Clodinafop	0.01	N.D.	N.D.
Cloprop	0.01	N.D.	N.D.
Clopyralid	0.01	N.D.	N.D.
Cyclanilide	0.02	N.D.	N.D.
Dicamba	0.005	N.D.	N.D.
Dichlorprop	0.005	N.D.	N.D.
Diclofop	0.01	N.D.	N.D.
Fenoprop	0.005	N.D.	N.D.
Fluazifop	0.005	N.D.	N.D.
Fluroxypyr	0.005	N.D.	N.D.
Haloxyfop	0.003	N.D.	N.D.
Ioxynil	0.005	N.D.	N.D.
MCPA	0.005	N.D.	N.D.
MCPB	0.005	N.D.	N.D.
Mecoprop	0.005	N.D.	N.D.
Picloram	0.01	N.D.	N.D.
Quinclorac	0.005	N.D.	N.D.
Quinmerac	0.01	N.D.	N.D.
Quizalofop	0.02	N.D.	N.D.
Triclopyr	0.01	N.D.	N.D.

LOQ: limit of quantitation (mg/kg)
N.D.: not detected

Previous water testing results described 9.1 µg/L perchlorate in the water used during the production process. Perchlorate is a chemical commonly used as an oxidizer in rocket propellants, munitions, fireworks, and some fertilizers (Kucharzyk et al., 2009). It has been recognized as a contaminant by the EPA as of February 2011 (EPA (2011-02-11). "Drinking Water: Regulatory Determination on Perchlorate." 76 FR 7762). EPA calculated a tap water screening level of 11 µg/L perchlorate and perchlorate salts. Therefore, perchlorate is monitored

annually in the finished product to ensure that processing removes potential perchlorate contamination from water used in processing. Three non-consecutive batches of AlphaGOS® had no detectable perchlorate at the limit of detection (Table 12).

Table 12. Perchlorate Screen in AlphaGOS® Powder

	2014 009 12*	2017 01 01*	2017 06 01**
Perchlorates (ClO_4)[a] (mg/kg)	N.D.	N.D.	N.D.

[a]Method: LC/MS/MS
N.D.: not detected
*Analysis of Batches 2014 009 12 and 2015 01 01 performed by Eurofins, limit of detection 0.2 mg/kg
**Analysis of Batch 2017 06 01 performed by Wessling, limit of detection 0.01 mg/kg

F. STABILITY OF ALPHAGOS®

1. Stability of AlphaGOS® Powder and Syrup

The stability of AlphaGOS® powder and syrup stored under ambient warehouse conditions (between 50°F and 85°F and a relative humidity not exceeding 75%) is described below (Tables 13 and 14). Percentage dry matter, individual oligo saccharides and microbial content were evaluated in three non-consecutive batches of both AlphaGOS® powder and syrup. The dry matter content of AlphaGOS® powder batch 2014 009 12 was 94.94% after 70 months, failing to meet the specification of > 95%. These results support the one-year shelf life of the product, as the batch failed during the fifth year of the study. The stability of the finished product will continue to be monitored to support the intended shelf life of the product. The AlphaGOS® powder and syrup comply with the described product specifications up to one year in storage.

Table 13. Stability of AlphaGOS® Syrup

Parameter	Specification	AlphaGOS® Syrup Batch								
		IBC 2014 02			IBC 2014 10				IBC 2016 01	
		9 mo	41 mo	53 mo	1 mo	13 mo	27 mo	33 mo	12 mo	31 mo
Dry matter (%)	>72	79.3	74.7	75.5	79.5	72.9	76.5	76.3	73.8	76.0
alpha-GOS (% on DM)	96 ± 3	97.8	96.2	96.0	97.2	96.9	96.1	96.1	96.2	96.1
DP2: Melibiose (%)	-	3.8	3.8	3.9	4.6	4.7	4.6	4.7	1.6	1.4
DP3: Manninotriose (%)	-	47.7	46.4	46.3	48.1	47.7	47.6	47.4	39.5	39.5
DP4: Verbascotetraose (%)	-	46.3	46.0	45.8	44.5	44.5	43.9	44.0	55.1	55.3
Microbial Parameters	**Specification**	**IBC 2014 02**			**IBC 2014 10**				**IBC 2016 01**	
		0 mo		**43 mo**	**0 mo**		**35 mo**		**0 mo**	**20 mo**
General bacteria count (cfu/g)	<1000	<10		<10	<10		<10		<10	<10
Escherichia coli (cfu/g)	<1	<1		<1	<1		<1		<1	<1
Enterobacteria (cfu/g)	<1	<1		<1	<1		<1		<1	<1
Coliforms (cfu/g)	<1	<1		<1	<1		<1		<1	<1
Staphylococcus aureus	ND in 1 g	ND		ND	ND		ND		ND	ND
Pseudomonas aeuginosa	ND in 1 g	ND		ND	ND		ND		ND	ND
Yeast and mold (cfu/g)	≤10	<10		<10	<10		<10		<10	<10

-: not specified
ND: not detected
Syrup stored under ambient warehouse conditions, between 50°F and 85°F and a relative humidity not exceeding 75% in a 20 kg bag with sealed inner food-grade polyethylene (PE) liner.

Table 14. Stability of AlphaGOS® Powder

Parameter	Specification	AlphaGOS® Powder Batch																
		2014 009 12							2015 01 01						2017 01 01		
		24 mo			38 mo			70 mo	0 mo	10 mo			21 mo		1 mo		6 mo
Dry matter (%)	>95	97.0			96.3			94.9	96.9	96.5			95.3		96.5		95.6
alpha-GOS (% on DM)	96 ± 3	96.1			96.1			95.5	97.6	97.8			97.6		96.5		96.3
DP2: Melibiose (%)	-	4.0			3.9			3.8	5.8	5.9			5.6		2.7		2.6
DP3: Manninotriose (%)	-	48.9			49.2			48.4	41.5	41.8			41.7		39.7		39.7
DP4: Verbascotetraose (%)	-	43.2			43.0			43.3	50.3	50.1			50.3		54.1		54.0
Microbials	**Specification**	**0 mo**	**14 mo**	**18 mo**	**22 mo**	**27 mo**	**29 mo**	**36 mo**	**10 mo**	**13 mo**	**17 mo**	**20 mo**	**24 mo**	**31 mo**	**1 mo**	**8 mo**	**11 mo**
General bacteria count (cfu/g)	<1000	<10	8	<40	<4	5	40	500	240	<10	<1	<1	<40	<40	<10	<40	<400
E. coli (cfu/g)	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	-
Enterobacteria (cfu/g)	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Coliforms (cfu/G)	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	-
*Staphylococcus aureus**	ND in 1 g	-	-	ND	-	-	ND	ND	-	ND	-	-	ND	ND	ND	-	-
*Pseudomonas aeuginosa**	ND in 1 g	-	-	-	-	-	-	ND	-	-	-	-	-	ND	-	-	ND
Yeast and mold (cfu/g)	≤10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10

*These parameters were not assessed for every time point in the study.
-: not specified/not collected
ND: not detected
Powder stored under ambient warehouse conditions, between 50°F and 85°F and a relative humidity not exceeding 75%.

III. DIETARY EXPOSURE

A. INTENDED EFFECT

The intended effect of adding AlphaGOS® to powdered, ready-to-feed, and concentrated liquid versions of non-exempt term infant formulas and selected conventional foods is to increase oligosaccharide intake in formula-fed infants and the general population.

B. HISTORY OF USE

AlphaGOS® is currently sold in the European Union. This product is not considered a novel food, as it is derived from the field pea (*Pisum sativum*), a food with a long history of consumption in Europe. An application from Olygose was submitted for authorization of a health claim pursuant to Article 13(5) of Regulation (EC) No 1924/2006 via the Competent Authority of France, the EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA). EFSA was asked to deliver an opinion on the scientific substantiation of a health claim related to AlphaGOS® and a reduction of post-prandial glycemic responses [EFSA NDA Panel (EFSA Panel on Dietetic Products, Nutrition and Allergies), 2014. Scientific opinion on the substantiation of a health claim related to AlphaGOS® and a reduction of post-prandial glycaemic responses pursuant to Article 13(5) of Regulation (EC) No 1924/2006. EFSA Journal 2014;12(10):3838, 10 pp. doi:10.2903/j.efsa.2014.3838]. A claim on non-digestible carbohydrates and reduction of post-prandial glycaemic responses had already been assessed by the EFSA Panel with a favorable outcome. The previous evaluation, including the proposed wording and the conditions of use, also applies to the application for AlphaGOS®. The EFSA Panel concluded that a cause and effect relationship has been established between the consumption of foods/beverages containing non-digestible carbohydrates and a reduction of post-prandial glycemic responses as compared with sugar-containing foods/beverages.

In Canada, AlphaGOS® is regulated as a standard foodstuff by the Food and Drugs Act. The alpha-GOS sugar, raffinose, is in breads (wheat, rye and spelt) and muesli at levels of 20 - 38 mg/100g depending on the foods (Biesiekierski et al., 2011). Raffinose and stachyose (both alpha-GOS) are the main short chain carbohydrates in legumes, particularly lima and red beans; lentils and chick peas (Biesiekierski et al., 2011) and found in significant quantities in Jerusalem artichokes (Muir et al., 2009).

C. INTENDED USE

AlphaGOS® is suitable for use in selected general foods and non-exempt infant formulas. Table 15 provides the food and product categories and proposed levels of incorporation used to calculate the Estimated Daily Intake (EDI).

Table 15. Foods Proposed To Contain AlphaGOS®

Food Category	Proposed Level (g)/serving	Reference Amounts Customarily Consumed (21 CFR Part 101) (2016)	Corresponding Product Categories	Proposed Level (% incorporation)
Beverages: carbonated/non-carbonated, juice, flavored water	6	360 mL (considering 1 mL = 1 g)	Carbonated and non-carbonated beverages, wine coolers, water	1.67
Ready to drink iced coffee and teas	6			
Sports drinks	6			
Energy drinks	2.5			
Soups	2.5	245 g	Soups – all varieties	1.02
Meal-replacement drinks	6	240 mL (considering 1 mL = 1 g)	Milk, milk- based drinks, e.g., instant breakfast, meal replacement, cocoa	2.50
Processed fruits and vegetable juices	2.5	240 mL (considering 1 mL = 1 g)	Juices, nectars, fruit drinks	1.04
Dairy products analogs	2.5	170 g	Yogurt	1.47
Dairy and analogs	2.5	240 mL (considering 1 mL = 1 g)	Milk, milk substitute, and fruit juice concentrates	1.04
Sugars and sweets	2	15 g	Hard candies, others; powdered candies, liquid candies	13.33
Coffee and tea	1.5	360 mL (considering 1 mL = 1 g)	Coffee or tea, flavored and sweetened	0.42
Infant formulas				0.8 % (8 g/L)
Growing-up milks				0.8 % (8 g/L)
Baby cereals	0.5	110 g	Cereals, prepared, ready-to-serve	0.45
Baby foods	0.5	110 g	Dinners, desserts, fruits, vegetables or soups, ready -to-serve, junior type. Dinners, desserts, fruits, vegetables or soups, ready -to-serve, strained type	0.45
Toddler foods	0.5	170 g	Dinners, stews or soups for toddlers, ready-to-serve	0.29

D. ESTIMATED DAILY INTAKE

The assessment of the consumption of AlphaGOS® by the U.S. population resulting from the selected uses of AlphaGOS® (Table 16) was conducted using SAS and Matlab®. Estimates for the intake of AlphaGOS® were based on the food uses and maximum use level in Table 16 in conjunction with food consumption data included in the National Center for Health Statistics' (NCHS) 2013-2014 National Health and Nutrition Examination Surveys (NHANES) (CDC, 2016; USDA, 2016; USDA, Agricultural Research Service. 2016.). Calculations for the mean and 90th percentile intakes were performed for representative food uses of AlphaGOS® combined. The intakes were reported for the following population groups:

- infants, age 0 to 1 year
- toddlers, age 1 to 2 years,
- children, ages 2 to 5 years,
- children, ages 6 to 12 years,
- teenagers, ages 13 to 19 years,
- adults, ages 20 years and up,
- total population (all age groups combined, excluding ages 0-2 years),

1. Food Consumption Survey Data

a. Survey Description

The National Health and Nutrition Examination Surveys (NHANES) for the years 2013-2014 are available for public use. NHANES are conducted as a continuous, annual survey, and are released in 2-year cycles. In each cycle, approximately 10,000 people across the U.S. completed the health examination component of the survey. Any combination of consecutive years of data collection is a nationally representative sample of the U.S. population. It is well established that the length of a dietary survey affects the estimated consumption of individual users and that short-term surveys, such as the typical 1-day dietary survey, overestimate consumption over longer time periods (Hayes and Kruger, 2014). Because two 24-hour dietary recalls administered on 2 non-consecutive days (Day 1 and Day 2) are available from the NHANES 2013-2014 survey, these data were used to generate estimates for the current intake analysis.

The NHANES provide the most appropriate data for evaluating food-use and food-consumption patterns in the United States, containing 2 years of data on individuals selected via stratified multistage probability sample of civilian non-institutionalized population of the U.S. NHANES survey data were collected from individuals and households via 24-hour dietary

recalls administered on 2 non-consecutive days (Day 1 and Day 2) throughout all 4 seasons of the year. Day 1 data were collected in-person in the Mobile Examination Center (MEC), and Day 2 data were collected by telephone in the following 3 to 10 days, on different days of the week, to achieve the desired degree of statistical independence. The data were collected by first selecting Primary Sampling Units (PSUs), which were counties throughout the U.S. Small counties were combined to attain a minimum population size. These PSUs were segmented and households were chosen within each segment. One or more participants within a household were interviewed. Fifteen PSUs are visited each year. For example, in the 2009-2010 NHANES, there were 13,272 persons selected; of these 10,253 were considered respondents to the MEC examination and data collection. 9754 of the MEC respondents provided complete dietary intakes for Day 1 and of those providing the Day 1 data, 8,405 provided complete dietary intakes for Day 2. The release data do not necessarily include all the questions asked in a section. Data items may have been removed due to confidentiality, quality, or other considerations. For this reason, it is possible that a dataset does not completely match all the questions asked in a questionnaire section. Each data file has been edited to include only those sample persons eligible for that particular section or component, so the numbers vary.

In addition to collecting information on the types and quantities of foods being consumed, the NHANES surveys collect socioeconomic, physiological, and demographic information from individual participants in the survey, such as sex, age, height and weight, and other variables useful in characterizing consumption. The inclusion of this information allows for further assessment of food intake based on consumption by specific population groups of interest within the total population.

Sample weights are incorporated with NHANES survey data to compensate for the potential under-representation of intakes from specific population groups as a result of sample variability due to survey design, differential non-response rates, or other factors, such as deficiencies in the sampling frame (CDC, 2013).

b. Statistical Methods

Consumption data from individual dietary records, detailing food items ingested by each survey participant, were collated by computer in Matlab and used to generate estimates for the intake of AlphaGOS® by the U.S. population. Estimates for the daily intake of AlphaGOS® represent projected 2-day averages for each individual from Day 1 and Day 2 of NHANES data; these average amounts comprised the distribution from which mean and percentile intake estimates were produced. Mean and percentile estimates were generated incorporating sample weights in order to provide representative intakes for the entire U.S. population. “All-user” intake refers to the estimated intake of AlphaGOS® by those individuals consuming food

products containing AlphaGOS®. Individuals were considered users if they consumed 1 or more food products containing AlphaGOS® on either Day 1 or Day 2 of the survey.

2. Food Usage

a. Food Data

Food codes representative of each approved use were chosen from the Food and Nutrition Database for Dietary Studies (FNDDS) for the corresponding biennial NHANES survey. In FNDDS, the primary (usually generic) description of a given food is assigned a unique 8-digit food code (USDA, 2018).

b. Food Survey Results

The estimated "all-user" total intakes of AlphaGOS® from all proposed food uses of AlphaGOS® in the U.S. by population group are summarized in Table 16. In summary, 83.1% of the total U.S. population of all users 2+ years was identified as consumers of AlphaGOS® from the selected food uses in the 2013-2014 survey. The mean intakes of AlphaGOS® by all AlphaGOS® consumers age 2+ ("all-user") from all selected food uses were estimated to be 4.3 g/person/day or 0.065 g/kg body weight/day. The heavy consumer (90th percentile all-user ages 2 and up) intakes of AlphaGOS® from all selected food-uses were estimated to be 8.5 g/person/day or 0.1 g/kg body weight/day for ages 2 and up.

Table 16. Estimated "All-user" Daily Intake (EDI) of AlphaGOS® in Targeted Foods by Population Group (2013-2014 NHANES Data)

Population Group	N users	N population	% users	Mean mass (kg)	Mean EDI (g)	90th Percentile EDI (g)	Mean EDI (g/kg)	90th Percentile EDI (g/kg)
ages 0-1	349	379	92.1	8.0	0.7	1.7	0.09	0.2
ages 1-2	225	290	77.6	13.7	1.9	3.5	0.1	0.3
ages 2-5	886	1196	74.1	15.9	2.0	3.5	0.1	0.2
ages 6-12	1207	1564	77.2	37.2	3.1	5.8	0.08	0.2
ages 13-19	1078	1239	87.0	68.8	4.6	8.7	0.07	0.1
ages 20 and up	4944	5769	85.7	81.5	4.8	9.6	0.06	0.1
ages 2 and up	8115	9768	83.1	66.5	4.3	8.5	0.07	0.1

IV. SELF-LIMITING LEVELS OF USE

There are no known self-limiting levels of use.

V. COMMON USE IN FOOD BEFORE 1958

Galacto-oligosaccharides (GOSs) are a group of nondigestible oligosaccharides consisting of galactose units linked via α - or β- glycosidic bonds to galactose, a terminal glucose, or a sucrose. The linkages are not digested by human and animal pancreatic or intestinal enzymes (Ohtsuka et al., 1990; Wisker et al., 1985; Chonan et al., 2004). The raffinose family of oligosaccharides (RFOs), including raffinose, stachyose, and verbascose, are a group of alpha-GOS consisting of galactose or galactoses and a sucrose linked via α-1,6-glycosic bonds that are naturally present in grains and legumes, ranging from 5% to 8% of dry matter (DM) (Asp, 1995; Hou et al., 2009; Choct et al., 2010; Biesiekierski et al., 2011). Melibiose (Nagura et al., 1995-1996) the nonfructosylated raffinose disaccharide, formed by an α-1,6 linkage between galactose and glucose, is present in legumes (Rackis, 1974). In addition to melibiose, the nonfructosylated raffinose family of α-1,6 linked GOS includes manninotriose and verbascotetraose which have been found naturally in foods, such as cocoa beans and raw and processed soybeans and other plant sources (Cerbulis, 1954, Molnar-Perl et al., 1984; Takano et al., 1991; Rehms and Barz, 1995; Hou et al., 2009; Zhang et al., 2009; dos Santos et al., 2013). Raffinose has been used in infant diets in Japan for many years including regular, peptide (http://www.hagukumi.ne.jp/eng/products/ ebaby/ebaby.shtml, accessed 6 February 2017), and hypoallergenic lactose-free infant formulas (http://www.hagukumi.ne.jp/eng/ products/hagukumi/hagukumi.shtml, accessed 6 February 2017; http://www.hagukumi.ne.jp/eng/products/ ebaby/ebaby.shtml, accessed 6 February 2017; http://www.hagukumi.ne.jp/ products/specialmilk/newma1.shtml, accessed 6 February 2017).

VI. NARRATIVE ON THE CONCLUSION OF GRAS STATUS

Published pivotal genotoxicity, subchronic rat toxicology and neonatal piglet studies of AlphaGOS® document the safety and GRAS status of the product for use in general foods and non-exempt infant formula (Kruger et al., 2017a; Kruger et al., 2017b). Published clinical studies of AlphaGOS® also corroborate the safety of intake at the proposed levels of use. Thus, the publicly available information establishes that there is reasonable certainty of no harm to consumers from ingesting AlphaGOS® for the intended uses and use levels. AlphaGOS® is therefore GRAS as an ingredient in conventional food and beverages, and in non-exempt infant formula at the intended use levels.

AlphaGOS® was not genotoxic in an Ames test or an *in vitro* chromosome aberration study (Kruger et al., 2017a). Additionally, in an Organization for Economic Cooperation and Development (OECD)-compliant 90-day rodent toxicology study the no observed adverse effect level (NOAEL) for AlphaGOS® was determined to be at least 2000 mg/kg/day (Kruger et al., 2017a), which is consistent with the NOAELs established in rodent toxicology studies of other indigestible oligosaccharides, specifically beta-linked GOS (reviewed in GRN 729). Together these results provide an adequate margin of safety for the proposed EDI of AlphaGOS® of 65 and 128 mg/kg/day or 4.3 g/day and 8.5 g/day for the mean and 90th percentile consumers (age 2 and up) consistent with the margin of safety between an EDI of up to 25.3 g/day used in the GRAS assessments of beta-linked GOS with NOAELs in published studies ranging from 825 to 2250 mg GOS/kg/day.

Although the OECD-compliant 90-day rodent toxicology study of AlphaGOS® provides pivotal information about the safety of AlphaGOS® (Kruger et al., 2017a), the neonatal piglet is generally accepted to be the best preclinical model of for understanding the effects of infant formula ingredients on infant development (Thorsrud et al., 2010; Guilloteau et al., 2010; Alizadeh et al., 2016). Therefore, a study of AlphaGOS® in neonatal piglets was conducted (Kruger et al., 2017b). Female and male 3 day-old piglets were fed swine milk replacer in the absence and presence of AlphaGOS®, at a level of 8 mg/mL (8 g/L), for three consecutive weeks. All animals underwent evaluations, including clinical observations, body weight, feed consumption, clinical pathology, and gross necropsy with histopathology of selected tissues. AlphaGOS® was determined to be safe and well tolerated at this level of ingestion and supports the proposed use in non-exempt infant formulas at 7.8 g/L.

The safe use of other non-digestible beta-linked GOS products in non-exempt infant formulas and selected conventional foods at levels up to 7.8 g/L and 11 g/serving, respectively, has been widely accepted as GRAS (GRN 334, 484, 495, 518, 569, 620, 721, 729) and corroborates the safety of non-digestible alpha-linked GOS for the proposed uses.

A. ABSORPTION, DISTRIBUTION, METABOLISM AND EXCRETION

GOS are non-digestible oligosaccharides consisting of 1 to 7 galactose units linked via glycosidic bonds to either a terminal glucose or galactose. AlphaGOS® is a mixture of three non-digestible oligosaccharides, melibose, manninotriose, and verbascotetraose. These sugars contain 1 to 3 galactose units linked via 1-6 α-glycosidic bonds to a terminal glucose.

The α-1,6 glycosidic bonds are hydrolyzed by α-galactosidase. Because humans do not produce this enzyme, alpha-GOS passes through the upper gastrointestinal tract without being hydrolyzed and reaches the lower intestinal tract intact where it may then be fermented by gut microflora (Stipanuk 2006). AlphaGOS® is not expected to be absorbed. Following fermentation by the microflora in the colon, short chain fatty acids, lactate, methane, CO_2, and H_2 gas are produced. Short-chain fatty acids, CO_2, and H_2 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). EFSA agreed that AlphaGOS® is a non-digestible carbohydrate resistant to hydrolysis and absorption in the small intestine (EFSA, 2014).

The absorption of the alpha-GOS containing sugar raffinose (Gal pα1 → 6Glc pα1 → 2 Fru) has been shown to be between 0.16 – 0.26% in healthy individuals via urine analysis (Hessels et al., 2003; Lobley et al., 1990; Menzies, 1974). More recently, a study of seven healthy volunteers found that 97.1% ± 2.4% of the raffinose consumed orally could be recovered from the terminal ileum (Shimaya et al., 2009). These data support the accepted definition that AlphaGOS® is a non-digestible carbohydrate (EFSA 2014).

B. GENOTOXICITY STUDIES

Genotoxicity of AlphaGOS® was assessed in an Ames test and in vitro chromosomal aberration test. Neither test found evidence of genotoxicity of AlphaGOS® with or without metabolic activation (S9) treatment (Kruger et al., 2017a). A micronucleus test was not performed, as AlphaGOS® is not expected to be absorbed by the gut, but rather fermented by the gut microflora.

Both studies were performed at CiToxLAB France, BP 563, 27005 Evreux, France, in compliance with CiToxLAB France's standard operating procedures and the following principles of Good Laboratory Practice: OECD Principles of Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM (98) 17 and all subsequent OECD consensus documents, Directive 2004/10/EC of the European Parliament and of the Council of 11 February 2004 on the harmonization of laws, regulations and administrative provisions relating to the application of the Principles of Good

Laboratory Practice and the verification of their applications for tests on chemical substances (OJ No. L50 of 20.2.2004), and Article Annexe 2 à l'article D523-8 du code de l'environnement concernant les principes de l'OCDE des Bonnes Pratiques de Laboratoire (BPL).

1. Bacterial Reverse Mutation (Ames) Test

The study design was based on the following guidelines: OECD guideline No. 471, adopted on 21st July 1997, and Council Regulation (EC) No. 440/2008 of 30 May 2008, laying down test methods pursuant to Regulation (EC) No. 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), Annex, Part B.13/14 p. 248.

a. Methods

AlphaGOS® was dissolved in water and tested in two independent experiments, both with and without metabolic activation. Metabolic activation of AlphaGOS® was achieved through treatment with S9, prepared from a liver post-mitochondrial fraction (S9 fraction) of rats induced with Aroclor 1254. The S9 fraction was purchased from Moltox (Molecular Toxicology, Inc., Boone, North Carolina, USA). Treatments were performed according to the direct plate incorporation method except for the second experiment with S9 mix, which was performed according to the pre-incubation method (60 minutes, 37°C). Five strains of bacteria *Salmonella typhimurium* were used: TA1535, TA1537, TA98, TA100, and TA102. Each strain was exposed to five doses of the test item (three plates/dose-level): 312.5, 625, 1250, 2500, and 5000 µg/plate. Each strain was also treated with the indicated positive controls, dissolved in dimethylsulfoxide (DMSO), except for mitomycin C (MMC), which was dissolved in water. After 48 to 72 hours of incubation at 37°C, the revertant colonies were scored. The evaluation of the toxicity was performed on the basis of the observation of the decrease in the number of revertant colonies and/or a thinning of the bacterial lawn. Each main experiment was considered valid if the following acceptance criteria were fully met: the mean number of revertants in the vehicle controls is consistent with historical data, in each strain and test condition; at least five analyzable dose-levels (i.e. including at least three non-cytotoxic dose-levels) are obtained for each strain and test condition; the mean number of revertants in the positive controls is higher than that of the vehicle controls (at least 2-fold increase (for the TA98, TA100, and TA102 strains) or at least 3-fold increase (for the TA1535 and TA1537 strains)).

b. Results

The mean number of revertants for the vehicle and positive controls met the acceptance criteria (Table 17). No precipitate or toxicity was noted in the five *S. typhimurium* strains used, either with or without S9 mix (data not shown). The test item did not induce any noteworthy increase in the number of revertants, in any of the five strains, either with or without S9 mix. These results met the criteria of a negative response.

Under the experimental conditions of this study, AlphaGOS® did not show any mutagenic activity in the bacterial reverse mutation test with *Salmonella typhimurium* strains, either in the presence or absence of a rat liver metabolizing system.

2. *In Vitro* Chromosomal Aberration Test in Cultured Human Lymphocytes

The study design was based on the following guidelines: OECD guideline No. 473, adopted on 26 September 2014, and Council Regulation (EC) No. 440/2008 of 30 May 2008, laying down test methods pursuant to Regulation (EC) No. 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH), Annex, Part B.10, p. 225.

a. Methods

i. Cells

Cultures of human lymphocytes were prepared from whole blood samples obtained from young (*i.e.* 18 to 35 years old), healthy, non-smoking donors and collected into heparinized sterile tubes.

ii. Metabolic Activation

The S9 fraction for metabolic activation was purchased from Moltox (Molecular Toxicology, Inc., Boone, North Carolina, USA). The S9 metabolic activation mix was prepared at +4°C immediately before use and maintained at this temperature until added to the culture medium. The composition of S9 mix is as follows: glucose-6-phosphate 5 mM, NADP 4 mM, KCl 33 mM, MgCl2 8 mM, sodium phosphate buffer pH 7.4 100 mM, and S9 fraction 10% (v/v). The final concentration of S9 fraction in the culture medium was 1.5% (*i.e.* 15% S9 mix).

Table 17. Results of Bacterial Reverse Mutation (Ames) Test When Exposed to AlphaGOS®, Direct Plate Incorporation Method

Strain *S. typhimurium*		Dose AlphaGOS® µg/plate						Positive Control	
		0	312.5	625	1250	2500	5000	Treatment	Colonies
TA100	-S9	132.7 ± 5.9	138.3 ± 16.1	149.0 ± 15.4	144.7 ± 4.5	152.7 ± 11.5	138.3 ± 5.8	NaN_3 1 µg/plate	1168.7 ± 4.6
	+S9	158.7 ± 14.8	153.3 ± 12.1	179.7 ± 16.2	168.0 ± 13.1	182.7 ± 7.4	173.0 ± 18.0	BaP 20 µg/plate	784.0 ± 10.4
TA1535	-S9	11.7 ± 7.5	10.0 ± 0.0	8.7 ± 3.1	13.3 ± 4.9	11.0 ± 1.0	14.7 ± 2.1	NaN_3 1 µg/plate	647.3 ± 18.2
	+S9	8.7 ± 3.1	9.3 ± 5.0	10.0 ± 4.0	13.3 ± 6.5	9.0 ± 3.5	14.7 ± 4.7	2AM 2 µg/plate	239.3 ± 16.7
TA102	-S9	417.0 ± 14.9	388.3 ± 23.9	393.7 ± 21.2	354.3 ± 29.6	395.7 ± 16.0	401.3 ± 27.9	MMC 0.5 µg/plate	2471.0 ± 40.6
	+S9	570.0 ± 27.6	518.0 ± 69.4	520.7 ± 72.0	527.0 ± 70.4	558.0 ± 84.8	553.7 ± 79.9	2AM 2 µg/plate	2341.7 ± 170.3
TA98	-S9	23.7 ± 0.6	28.3 ± 5.9	28.7 ± 9.6	31.7 ± 1.5	26.0 ± 3.0	24.3 ± 5.5	2NF 0.5 µg/plate	108.3 ± 25.6
	+S9	24.7 ± 5.1	20.7 ± 4.6	33.3 ± 7.1	27.0 ± 4.4	29.3 ± 3.8	25.7 ± 6.7	2AM 2 µg/plate	1249.0 ± 121.6
TA1537	-S9	8.3 ± 1.5	11.0 ± 3.0	11.3 ± 1.5	7.3 ± 2.5	6.7 ± 2.9	9.0 ± 6.1	9-AA 50 µg/plate	129.0 ± 68.0
	+S9	7.3 ± 3.2	7.0 ± 3.5	13.0 ± 3.0	6.7 ± 1.5	12.0 ± 1.0	10.3 ± 4.6	2AM 2 µg/plate	147.7 ± 5.0

Abbreviations: NaN_3, Sodium Azide; 9AA, 9-Aminoacridine; 2NF, 2-Nitrofluorene; MMC, Mitomycin C
Data shown as average number of colonies ± standard deviation, three plates/dose

iii. Preliminary Toxicity Test

To assess the cytotoxicity of AlphaGOS®, a preliminary test was conducted, using one culture/dose, both with and without S9 mix. The doses used for treatment of the preliminary test were 15.6, 31.3, 62.5, 125, 250, 500, 1000, and 2000 µg/mL. At the highest tested dose, 2000 µg/mL, the pH of the culture medium was approximately 7.1 (the same pH as the vehicle control) and the osmolality equal to 291 mOsm/kg H_2O (286 mOsm/kg for the vehicle control). Therefore, none of the tested doses was considered to produce extreme culture conditions. No precipitate was observed in the culture medium at the end of the treatment periods, at any of the tested dose-levels.

iv. Main Cytogenetic Experiment

Using two cultures/dose, each culture was tested in the presence and absence of S9 mix, with 125, 250, 500, 1000, or 2000 µg/mL of AlphaGOS®, the vehicle control, and the appropriate positive control. Two known clastogens, dissolved in "water for injections," were used to check the sensitivity of the test system. For tests without S9 mix, mitomycin C was used at final concentrations of 2 and 3 µg/mL for the 3-hour of treatment and at 0.2 and 0.3 µg/mL for the continuous treatment. For tests with S9 mix, cyclophosphamide (CPA, used at final concentrations of 12.5 and 25 µg/mL). For each condition, both cultures were prepared from the blood of one donor.

To prepare each culture, 0.5 mL of heparinized whole blood was added to 5 mL of RPMI 1640 medium containing 20% fetal calf serum, L-glutamine (2 mM), antibiotic, antimycotic, and phytohemagglutinin (PHA: a mitogen to stimulate the lymphocytes to divide). The cultures were then incubated at 37°C for about 48 hours.

Lymphocyte cultures with or without S9 mix were exposed to the test or control items for 3 hours and then washed. Then, the cultures were incubated in fresh medium at 37°C until harvest. Harvest time was 20 hours after the beginning of treatment, corresponding to approximately 1.5 normal cell cycles. Three hours before harvest, each culture was treated with a Colcemid® solution (10 µg/mL) to block cells at the metaphase-stage of mitosis.

v. Preparation of Microscope Slides

At harvest, the cells were collected by centrifugation and submitted to a hypotonic treatment (KCl 0.075 M). The cells were then fixed in a methanol/acetic acid mixture (3/1; v/v), spread on glass slides and stained with Giemsa. All slides were coded, so that the scorer was unaware of the treatment group of the slide under evaluation ("blind" scoring).

vi. Evaluation of the Test Item Cytotoxicity

The cytotoxicity of the test item was assessed by the observation of mitotic inhibition in treated cultures in comparison to vehicle control cultures. The Mitotic Index (MI) was evaluated in each culture, in each experiment, by scoring the number of cells in mitosis on a total of 1000 cells per culture. Scoring of MI was undertaken without blinding.

Mitotic Index (%) = (number of cells in mitosis/number of cells scored) x 100.

vii. Metaphase Analysis

For each condition tested in the main cytogenetic experiments, metaphase analysis was performed for three dose-levels of the test item, the vehicle control and one concentration of the positive control.

Analysis was undertaken on 300 metaphases/dose-level (on metaphases which contained 44 to 46 chromosomes). Whenever possible, 150 metaphases were scored for each culture. Only 50 metaphases/culture were analyzed when at least 10% cells with structural chromosome aberration were observed.

The following structural aberrations were recorded for each metaphase: gaps, chromatid and chromosome breaks and exchanges, and others (multiple aberrations and pulverizations). In addition, the following numerical aberrations were recorded when encountered: polyploidy, endoreduplication and hyperdiploidy.

The experiment was considered valid if the following acceptance criteria were met: the frequency of cells with structural chromosome aberration in the vehicle and positive controls is consistent with (but not necessary within) historical data, and the frequency of aberration is statistically significantly higher in the positive controls than in the vehicle controls ($p \leq 0.05$ by χ^2 test). A result was considered positive for inducing chromosomal aberrations if, in any of the experimental conditions examined, a statistically significant increase in the frequency of cells with structural chromosome aberration is observed at one or more dose levels, a dose-response relationship is demonstrated by a statistically significant trend test, or if any of the results are above the historical data range of the corresponding vehicle control. A result was considered negative for inducing chromosomal aberrations if, in all experimental conditions, none of the criteria for a positive response are met.

b. Results

Since the test item was found freely soluble and non-cytotoxic in the preliminary test (data not shown), the highest dose-level selected for the main experiments was 2000 μg/mL,

according to the criteria specified in the international guidelines as the limit dose. The nominal dose-levels selected for the main experiments ranged from 125 to 2000 µg/mL.

i. Preliminary Toxicity Test

Using a test item concentration of 110 mg/mL and a treatment volume of 100 µL/5.5 mL culture medium, the highest recommended dose-level of 2000 µg/mL was achievable. No cytotoxicity was observed at any of the tested dose-levels, either with or without S9 mix, as shown by the absence of any noteworthy decrease in the mitotic index.

ii. Main Cytogenic Experiments

The frequencies of cells with structural chromosome aberrations for the vehicle and positive controls were as specified in the acceptance criteria. The study was therefore considered to be valid. Following the 3- and 20-hour treatments, no noteworthy decrease in the mitotic index was observed at any of the tested doses with or without S9 treatment. For the 3- and 20-hour treatments, the dose levels selected for metaphase analysis were 500, 1000, and 2000 µg/mL. No statistically significant increase in the frequency of cells with structural chromosomal aberrations was noted at any of the analyzed dose levels in comparison to the vehicle controls, either after the 3- or 20-hour treatment, with or without S9 treatment (Table 18). No dose-response relationship was demonstrated by the linear regression. Moreover, the frequencies of cells with structural chromosomal aberrations remained within the vehicle control historical ranges. These results met the criteria of a negative response.

Table 18. Results of AlphaGOS® in the *In Vitro* Chromosome Aberration Test (Main Test)

Dose AlphaGOS® µg/mL	S9 mix	Trt-Rec time (h)	No. cells analyzed	Cells with structural chromosome aberrations	
				% + Gaps	%-Gaps
0	-	3-20	150	1.0	1.0
500	-	3-20	150	1.3	1.3
1000	-	3-20	150	0.3	0.3
2000	-	3-20	150	1.7	1.7
MMC 2 µg/mL	-	3-20	50	48.0	47.0*
0	+	3-20	150	1.0	1.0
500	+	3-20	150	0.7	0.7
1000	+	3-20	150	1.0	1.0
2000	+	3-20	150	0.7	0.7
CPA 12.5 µg/mL	+	3-20	50	17.0	17.0*
0	-	20-20	150	0.7	0.7
500	-	20-20	150	1.0	1.0
1000	-	20-20	150	0.7	0.7
2000	-	20-20	150	1.7	1.7
MMC 0.2 µg/mL	-	20-20	50	26.0	26.0*

MMC, Mitomycin C. CPA, Cyclophosphamide
*p<0.001 (performed only for cells with structural aberrations excluding gaps), chi-squared test

Under the experimental conditions of this study, AlphaGOS® did not induce chromosome aberrations in cultured human lymphocytes, either in the presence or absence of a rat liver metabolizing system.

C. TOXICOLOGY STUDIES

1. Summary

The toxicity of AlphaGOS® was assessed in an OECD-compliant, GLP 90 day subchronic toxicity study in rats (Kruger et al, 2017a) and in a three-week neonatal piglet study (Kruger et al., 2017b). No AlphaGOS®-related toxicity was reported in rats administered 2000 mg/kg/day AlphaGOS® or in piglets treated with 8 mg/mL AlphaGOS®.

2. Subchronic Toxicity of AlphaGOS® in Rats

a. Summary

A 90-day rat toxicology study was completed by CiTox-LAB France under good laboratory practices (GLP) conditions and complied with OECD guideline 408 with the exception of the use of only one dose level (Kruger et al., 2017a). One dose level was considered appropriate, due to the known safety profile for other galactooligosaccharides

b. Methods

i. Study Design and Study Animals

Sprague Dawley rats were obtained from Charles River Laboratories, Calco, Italy. Upon receipt, two same-sex animals from the same group were housed per cage. Prior to starting the treatment, animals were acclimated to the study environment for 9 days (temperature: 22 ± 2ºC; humidity: 50 ± 20%; light/dark cycle: 12 h/12 h). Rats were 5–6 weeks old and had a mean body weight of 171 g and 139 g for male and female rats, respectively. Ad libitum feed and water were provided to the animals. Ten male and ten female rats were given either 2000 mg/kg/day AlphaGOS® or vehicle (reverse osmosis-treated drinking water) in the morning via gavage for 90 days. The single dose of 2000 mg/kg/day was chosen because there was no reasonable expectation of toxicity for an oligosaccharide like AlphaGOS®, and previous clinical studies in humans found no serious adverse events at doses up to 18 g/day (Morel et al., 2015).

Animals were monitored twice daily for mortality and once daily for aberrant clinical signs. Detailed clinical examinations (skin, fur, eye, mucous membrane changes; occurrence of secretions and/or excretion; autonomic activity, changes in gait, posture and response to handling; clonic or tonic movements), individual body weight and feed consumptions (per cage) were recorded weekly. Animals underwent a functional observation battery (assessment of reactivity to manipulation and different stimuli and motor activity) during week 12 of treatment. Animals had an ophthalmological exam before dosing and during week 13 of treatment.

Fasting (at least 14 hours) urine and blood samples were collected following the completion of the treatment period, and animals were killed for full macroscopic and microscopic post-mortem examination. Organs were weighed at the completion of the study included adrenals, brain (medulla/pons, cerebellar and cerebral cortex), epididymides, heart, kidneys, liver, ovaries (including oviducts), spleen, testes, thymus and uterus (with horns and cervix). The following tissues were collected and preserved: adrenals, aorta, brain (medulla/pons, cerebellar and cerebral cortex), cecum, colon, duodenum, epididymides, esophagus, eyes (with Harderian glands), femoral bone (with articulation), gut associated lymphoid tissue, heart, ileum, jejunum, kidneys, liver, lungs (with bronchi), lymph nodes (mandibular and mesenteric), mammary glands, ovaries (including oviducts) pancreas, pituitary gland, prostate (dorsolateral and ventral), rectum, salivary glands (sublingual and submandibular), sciatic nerve, seminal vesicles (including coagulation gland), skeletal muscle, skin, spinal cord (cervical, thoracic and lumbar), spleen, sternum (with bone marrow), stomach (with forestomach), testes, thymus, thyroids (with parathyroids), tongue, trachea, urinary bladder, uterus (horns and cervix) and vagina. All organs, except for the eyes, femoral bone, skeletal muscle and tongue, were examined microscopically.

ii. Test Article

AlphaGOS® was dissolved in water. Test article composition was analytically verified to comply with product specifications and supplied to the testing laboratory with a certificate of analysis. Test article and vehicle only were delivered via oral gavage at 10 mL/kg/day. The Test article was stable for 2 weeks in solution under refrigerated conditions protected from light.

iii. Statistical Analysis

Statistical analysis was conducted using either Citox (D.7) software (body weight, feed consumption, hematology, blood biochemistry and urinalysis) or PathData (6.2d2) (organ weight). Specific statistical tests are specified where indicated in the text.

c. Results

AlphaGOS® was well-tolerated throughout the study. There were no mortalities during the trial. AlphaGOS® did not affect feed consumption in either male or female rats when compared to control rats. No test article-related, toxicologically significant differences were observed in the clinical examinations, body weights, hematology, clinical chemistry, urinalysis, organ weights, gross or histopathology.

i. Clinical and Functional Observation Battery

Animals were assessed for a variety of clinical signs of distress over the course of the study. Chromodacryorrhea was observed in one male and one female each receiving

AlphaGOS®. While the chromodacryorrhea was only seen in animals receiving AlphaGOS®, chromodacryorrhea is observed in laboratory-housed Sprague Dawley rats and was not considered to be related to AlphaGOS® consumption.

In the control group, five male rats displayed no abnormalities in fur appearance throughout the study. Of the five control male rats that did exhibit some fur abnormalities, there were five cases of scabbing (lasting 3–4 days) and one case of thinning hair (lasting from day 43 until sacrifice). Seven of the 10 male rats receiving AlphaGOS® had no signs of abnormal fur; there were 2 cases of scabbing (lasting 14 or 26 days) and 1 case of thinning hair (lasting 14 days). Six of the 10 female control rats showed no signs of abnormal fur. Scabs presented on two control female rats (lasting 1–9 days) and there was one case of alopecia (from day 59 to sacrifice). Eight female rats receiving AlphaGOS® presented with no fur abnormalities. The two female rats that did present with fur abnormalities exhibited thinning hair and alopecia from 71 days to 72 days, respectively, until terminated.

At the 12-week functional observational battery (FOB), both sexes in the treatment and control groups were normal for touch escape, reactivity to handling, touch response, auditory startle reflex, tail pinch response, forelimb grip strength, gait, posture and breathing. No animal in the study showed signs of salivation, lacrimation, piloerection, tremors, convulsions, ataxia, hypotonia, stereotypy or any excessive or abnormal defecation/urination. Stress-induced grooming was noticed in one male rat from both the control and AlphaGOS® groups and two control female rats and one female given AlphaGOS®. As this behavior was seen in both control and AlphaGOS® groups and is also part of normal behavior, it was not attributed to the test item.

In both male rats and female rats, the calculated mean landing foot splay was slightly higher in the control group. Landing foot splay measurements were taken twice, and a mean was calculated for each rat. In male rats, the higher mean landing foot splay in the control group was mainly due to an isolated animal for which both assays gave high values, leading to a higher mean when compared to other animals in the same group. In female rats, this was attributed to highly variable individual measurements in some control group animals, which increased the mean.

ii. Body Weight

AlphaGOS® did not affect the body weights of the rats compared to control, as shown by weight change over time (Tables 19 and 20). However, the body weight change was statistically lower in the female AlphaGOS® treated rats compared to the control female rats (7 g vs. 12 g, respectively) between days 64 and 71. There were no differences in feed consumed between groups or sexes at any point in the study (Table 21). As this finding was only observed during 1 week of feeding and did not significantly affect total body weights, it was considered incidental and not toxicologically relevant.

Table 19. Mean Body Weight Change (g) in Rats Fed AlphaGOS® in the Subchronic Toxicity Study

Dose AlphaGOS® (mg/kg/day)	Males		Females	
	0	2000	0	2000
Days 1 - 8	64 ± 4	67 ± 6.6	24 ± 4.6	28 ± 10.2
Days 8 - 15	60 ± 7.3	60 ± 6.7	24 ± 6.1	22 ± 3.7
Days 15 - 22	50 ± 8.2	51 ± 10.2	16 ± 5.8	14 ± 6.8
Days 22 - 29	35 ± 6.8	36 ± 6.3	13 ± 6.9	14 ± 8.8
Days 29 - 36	34 ± 9.1	35 ± 6.1	13 ± 6.5	13 ± 5.1
Days 36 - 43	35 ± 5.8	34 ± 6.8	14 ± 5.2	11 ± 4.1
Days 43 - 50	21 ± 5.6	25 ± 4.9	9 ± 6.7	5 ± 7.3
Days 50 - 57	21 ± 5	21 ± 5.4	6 ± 8.5	6 ± 9.8
Days 57 - 64	11 ± 6.4	16 ± 5.8	4 ± 6.7	8 ± 5.8
Days 64 - 71	19 ± 7.9	19 ± 5.8	12 ± 5.3	7 ± 5.3*
Days 71 - 78	14 ± 6.3	14 ± 4.5	6 ± 5.5	5 ± 5.5
Days 78 - 85	-5 ± 6.7	-6 ± 3.3	-7 ± 4.1	-3 ± 6.1
Days 85 - 91	9 ± 6.4	8 ± 8.6	13 ± 6.9	13 ± 5.7
Days 1 - 91	365 ± 47.7	380 ± 33.4	149 ± 20.3	141 ± 20.1
*p < 0.05, n =10 rats/group, statistical test – Dunnett Test				

Table 20. Body Weight (g) of Rats Fed AlphaGOS® in the Subchronic Toxicity Study

Dose (day)	Male		Female	
	Control	2000 mg/kg/day	Control	2000 mg/kg/day
-7	106 ± 6.6	107 ± 6.1	96 ± 5.9	96 ± 5.8
1	170 ± 10.8	172 ± 7.8	139 ± 8.9	138 ± 9.5
8	234 ± 12.9	238 ± 10.6	163 ± 7.6	166 ± 16.8
15	294 ± 17.8	299 ± 13.5	187 ± 9.8	187 ± 19.2
22	343 ± 19.9	350 ± 20.5	203 ± 13.4	202 ± 19.5
29	378 ± 23.2	385 ± 20.4	216 ± 16.8	216 ± 24
36	412 ± 26.5	420 ± 22.8	230 ± 16	229 ± 22.9
43	446 ± 31.2	454 ± 23.1	244 ± 17.4	240 ± 24.1
50	467 ± 34.2	479 ± 24.8	253 ± 21.8	245 ± 25.0
57	488 ± 35.2	500 ± 26.6	259 ± 23.6	251 ± 28.1
64	499 ± 34.7	516 ± 27.4	263 ± 19.8	257 ± 26.7
71	518 ± 40.5	535 ± 27.7	275 ± 22.0	265 ± 26.3
78	532 ± 43.6	549 ± 27.2	281 v 24.4	270 ± 29.3
85	527 ± 47.0	543 ± 28.7	274 ± 24.8	266 ± 27.5
91	536 ± 48.4	551 ± 28.5	288 ± 25.3	279 ± 24.2
N = 10 for all groups at all time points				

Table 21: Average Feed Consumed/day (g)in the Rat Subchronic Toxicity Study														
Days of Study		**1 - 8**	**8 - 15**	**15 - 22**	**22 - 29**	**29 - 36**	**36 - 43**	**43 - 50**	**50 - 57**	**57 - 64**	**64 - 71**	**71 - 78**	**78 - 85**	**85 - 91**
Male	0 mg/kg AlphaGOS®	25.9 ± 1.1	29.3 ± 1.8	30.5 ± 2.2	31.1 ± 1.9	31.0 ± 2.2	30.9 ± 1.4	30.4 ± 1.5	29.6 ± 1.4	29.7 ± 1.6	29.8 ± 2.1	28.1 ± 5.9	26.2 ± 1.9	29.1 ± 2.0
	2000 mg/kg AlphaGOS®	26.8 ± 1.9	30.3 ± 2.0	32.1 ± 2.5	32.7 ± 2.0	32.2 ± 2.1	32.5 ± 1.6	32.3 ± 2.6	31.1 ± 1.8	31.5 ± 0.9	31.2 ± 1.4	31.1 ± 1.9	26.4 ± 1.6	29.2 ± 1.3
Female	0 mg/kg AlphaGOS®	17.7 ± 1.3	18.6 ± 1.4	19.0 ± 2.4	19.5 ± 1.8	20.7 ± 2.1	19.4 ± 1.6	20.3 ± 2.6	19.7 ± 2.3	20.1 ± 2.4	19.9 ± 2.7	19.7 ± 2.9	17.6 ± 2.5	20.4 ± 2.6
	2000 mg/kg AlphaGOS®	18.4 ± 1.4	18.4 ± 1.4	18.6 ± 1.7	20.2 ± 1.9	19.4 ± 1.9	19.2 ± 1.9	19.0 ± 1.5	19.5 ± 2.6	19.9 ± 2.1	19.3 ± 2.5	19.9 ± 2.0	17.1 ± 1.5	20.5 ± 1.7
Data shown as mean feed consumed/day ± standard deviation. N = 5 for each group.														

iii. Hematology

Hematology parameters were not adversely impacted by test article administration (Table 22). The female rats receiving AlphaGOS® had a statistically significant increase in mean corpuscular hemoglobin concentration (MCHC) compared to the control group (34.2 g/dL vs. 33.8 g/dL, $p < 0.05$). In view of the low magnitude of this change and given that both MCHC values are within historical limits for this breed of rat at CiToxLAB France, and not observed in male rats, this isolated finding was not considered biologically relevant.

Table 22. Hematology Results in the Subchronic Toxicity Study

Parameter	Male		Historical Data Range (Male)†	Female		Historical Data Range (Female)†
	0 mg/kg/day	2000 mg/kg/day		0 mg/kg/day	2000 mg/kg/day	
WBC (g/L)	10.5 ± 2.2	10.9 ± 2.3	5.5 – 45	6.1 ± 1.7	6.0 ± 2.2	1.92 – 17.9
Neutrophils (g/L)	1.6 ± 0.4	1.9 ± 0.6	0.1 - 35.4	0.9 ± 0.3	0.7 ± 0.5	0.4 – 2.6
Eosinophils (g/L)	0.2 ± 0.07	0.2 ± 0.06	0.04 – 0.4	0.1 ± 0.02	0.1 ± 0.05	0 – 0.5
Basophils (g/L)	0.02 ± 0.01	0.02 ± 0.01	0 – 0.2	0.01 ± 0.006	0.01 ± 0.006	0 – 0.07
Lymphocytes (g/L)	8.3 ± 2.1	8.4 ± 2.0	4.8 – 15.6	5.0 ± 1.6	4.9 ± 1.8	1.4 – 13.1
Monocytes (g/L)	0.3 ± 0.06	0.3 ± 0.08	0.08 – 1.9	0.1 ± 0.03	0.2 ± 0.07	0.02 – 0.5
RBC (T/L)	9.2 ± 0.3	9.1 ± 0.3	7.9 – 11.3	8.7 ± 0.4	8.6 ± 0.4	7.0 – 9.5
Hemoglobin (g/dL)	16.1 ± 0.4	15.9 ± 0.3	13.3 – 17.7	16.0 ± 0.6	16.0 ± 0.6	12 – 17
Hematocrit (L/L)	0.5 ± 0.01	0.5 ± 0.01	0.41 – 0.56	0.5 ± 0.02	0.5 ± 0.02	0.4 – 0.5
MCHC (g/dL)	33.4 ± 0.5	33.0 ± 0.3	29.9 – 34.4	33.8 ± 0.4	34.2 ± 0.2*	30.9 – 35.5
MCV (fL)	52.6 ± 1.6	52.9 ± 1.2	47.4 – 55.8	54.5 ± 1.3	54.1 ± 1.8	50.5 – 60
MCH (pg)	17.6 ± 0.6	17.5 ± 0.5	14.9 – 18.4	18.4 ± 0.5	18.5 ± 0.5	16.7 – 19.3
Platelet (g/L)	956 ± 121.4	954 ± 122.8	375 - 1626	775 ± 158.5	880 ± 151.3	68 – 1407

*p < 0.05 Dunnett Test
† Historical data based on 121 male rats and 150 female Sprague Dawley rats from CiToxLab France
N = 10 for all groups at all time points
Abbreviations: WBC: white blood cell count, RBC: red blood cell count, MCHC: mean corpuscular hemoglobin concentration, MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin

iv. Clinical Chemistry

In male rats from in the AlphaGOS®-treated group, fasting blood glucose level (Table 23) was higher than in the control group (6.9 mmol/L vs. 6.0 mmol/L, $p < 0.05$) mainly due to two animals with concentrations at 9.4 and 8.1 mmol/L, which increased the mean glucose level for the AlphaGOS® treated group. Female rats from the AlphaGOS® treated group had no change in blood glucose level compared to the controls.

The level of potassium (Table 23) was slightly lower in blood of female rats from AlphaGOS®-treated group compared to the control group (3.5 mmol/L vs. 3.8 mmol/L, $p < 0.01$). However, male rats showed no significant changes in potassium levels. For both potassium and blood glucose, all values were within the limits of historical data from CiToxLab France and not considered clinically adverse. The lack of significant changes in clinical chemistries across both sex groups corroborates the conclusion that any changes were not likely to have been test article related.

Table 23. Clinical Chemistry Results in Subchronic Toxicity Study

Parameter	Male		Female	
	0 mg/kg/day	2000 mg/kg/day	0 mg/kg/day	2000 mg/kg/day
Sodium (mmol/L)	145.5 ± 0.8	145.0 ± 0.8	143.9 ± 0.9	143.8 ± 0.9
Potassium (mmol/L)	3.9 ± 0.2	3.9 ± 0.2	3.8 ± 0.2	3.5 ± 0.1**§
Chloride (mmol/L)	104.2 ± 1.1	104.0 ± 0.5	104.5 ± 1.1	104.4 ± 1.6
Calcium (mmol/L)	2.8 ± 0.04	2.8 ± 0.06	2.8 ± 0.09	2.8 ± 0.1
Phosphorus (mmol/L)	2.2 ± 0.1	2.3 ± 0.2	1.7 ± 0.2	1.6 ± 0.1
Glucose (mmol/L)	6.0 ± 0.4	6.9 ± 1.0*†	6.8 ± 0.6	6.6 ± 0.3
Urea (mmol/L)	3.6 ± 0.4	3.6 ± 0.4	4.8 ± 0.7	4.5 ± 0.8
Creatinine (µmol/L)	27.5 ± 1.0	28.3 ± 1.2	32.0 ± 2.8	30.2 ± 3.6
Bilirubin (µmol/L)	0.1 ± 0.3	0.5 ± 0.6	1.1 ± 0.8	0.9 ± 0.7
Protein (g/L)	64.4 ± 1.4	63.8 ± 2.1	67.7 ± 3.7	68.5 ± 4.7
Albumin (g/L)	40 ± 0.9	39 ± 1.2	43 ± 2.2	44 ± 3.4
A/G ratio	1.7 ± 0.08	1.6 ± 0.09	1.7 ± 0.07	1.8 ± 0.08
Cholesterol (mmol/L)	1.6 ± 0.4	1.7 ± 0.4	1.8 ± 0.3	1.9 ± 0.4
Triglycerides (mmol/L)	0.7 ± 0.2	0.9 ± 0.4	0.4 ± 0.07	0.4 ± 0.2
ALP (U/L)	232 ± 40.0	214 ± 23.7	146 ± 36.6	124 ± 36.0
AST (U/L)	89 ± 24.8	77 ± 7.8	94 ± 19.6	78 ± 17.4
ALT (U/L)	37 ± 5.6	33 ± 5.2	35 ± 9.8	35 ± 9.8

*p < 0.05
**p < 0.01
†Historical data (135 Sprague Dawley female rats) = 2.85 - 4.93 mmol/L (CiToxLab, France)
§ Historical data (104 Sprague Dawley male rats) = 5.11 - 9.65 mmol/L (CiToxLab, France)
Statistical test – Dunnett Test
N = 10 for all groups at all time points
ALP = Alkaline Phosphatase; ASAT = Aspartate aminotransferase; ALT = Alanine Aminotransferase

v. Urine Analysis

In male rats of the treated group, the urine of 3 of 10 animals tested positive for nitrites. A few white blood cells were also observed in the urine of one male rat receiving AlphaGOS®. However, these animals lacked kidney pathology at histopathologic exam and did not display any clinical signs or kidney weight changes. Urine from female rats was not affected by ingestion of 2000 mg/kg/day of AlphaGOS®.

vi. Organ Weights

The absolute liver weight in female rats was not changed between test and control groups; however, the liver weight relative to body weight was significantly increased in the female rats receiving AlphaGOS® compared to controls (Table 24). This was not a consistent finding across genders, as there was no significant finding in the liver weights (absolute or as a percent of total body weight) in the male rats receiving AlphaGOS® compared to the control group. There were no adverse effects seen in clinical chemistries and no corroborating findings in histopathology that would indicate an adverse effect on the liver. Therefore, the effect noted in the relative liver weights in female rats was not considered toxicologically significant.

The absolute and relative weight of the testes in male rats fed AlphaGOS® were significantly decreased compared to control rats. However, the absence of a similar trend in the epididymis, a lack of adverse findings in histopathology, and corroboration that the weight of the testes was within historical limits (0.74 – 4.416 g) indicated that this finding was not an adverse effect related to test article intake.

The slight lymphoid hyperplasia observed in the spleen of one AlphaGOS® treated rat is occasionally seen in animals of this age. As this was an isolated observation, it was considered not to be test article related. No other differences in absolute or relative organ weights were noted between treated and control groups (Table 24).

Table 24. Absolute and Relative Organ Weights Results in the Subchronic Toxicity Study

Organ	Male		Female	
	0 mg/kg/day	2000 mg/kg/day	0 mg/kg/day	2000 mg/kg/day
Adrenal Gland (g) %Adrenal gland/body	0.062 ± 0.012 (0.012 ± 0.002)	0.069 ± 0.01 (0.013 ± 0.002)	0.068 ± 0.007 (0.026 ± 0.003)	0.069 ± 0.007 (0.027 ± 0.004)
Brain (g) %Brain/body	2.15 ± 0.1 (0.42 ± 0.04)	2.13 ± 0.11 (0.41 ± 0.024)	1.97 ± 0.067 (0.74 ± 0.06)	1.92 ± 0.065 (0.75 ± 0.07)
Heart (g) %Heart/body	1.42 ± 0.14 (0.28 ± 0.016)	1.5 ± 0.12 (0.29 ± 0.02)	0.92 ± 0.13 (0.34 ± 0.032)	0.89 ± 0.08 (0.35 ± 0.032)
Kidneys (g) %Kidneys/body	3.1 ± 0.29 (0.61 ± 0.022)	3.11 ± 0.24 (0.60 ± 0.034)	1.77 ± 0.085 (0.67 ± 0.063)	1.8 ± 0.17 (0.70 ± 0.065)
Liver (g) %Liver/body	12.32 ± 1.82 (2.4 ± 0.18)	13.19 ± 0.99 (2.52 ± 0.17)	6.74 ± 0.50 (2.52 ± 0.13)	6.93 ± 0.653 (2.68 ± 0.14)*
Spleen (g) %Spleen/body	0.90 ± 0.16 (0.18 ± 0.019)	0.96 ± 0.13 (0.183 ± 0.024)	0.6 ± 0.051 (0.23 ± 0.022)	0.55 ± 0.074 (0.21 ± 0.026)
Thymus (g) %Thymus/body	0.31 ± 0.078 (0.6 ±0.013)	0.33 ± 0.094 (0.063 ± 0.017)	0.28 ± 0.045 (0.10 ± 0.019)	0.24 ± 0.049 (0.92 ± 0.015)
Testes (g) %Testes/body	3.8 ± 0.40 (0.75 ± 0.091)	3.45 ± 0.19# (0.66 ± 0.059)#	NA	NA
Epididymides (g) %Epididymides/body	1.52 ± 0.15 (0.3 ± 0.037)	1.48 ± 0.15 (0.28 ± 0.028)	NA	NA
Uterus (g) %Uterus/body	NA	NA	0.88 ± 0.33 (0.33 ± 0.13)	0.81 ± 0.28 (0.32 ± 0.12)
Ovaries (g) %Ovaries/body	NA	NA	0.16 ± 0.03 (0.059 ± 0.009)	0.15 ± 0.027 (0.057 ± 0.012)

5% level significance with Wilcoxon's Test
*5% level significance with Student's Test
N = 10 for all groups at all time points
NA = Not Applicable

vii. Histopathology

No adverse findings in gross pathology or histopathology were attributed to the test article. Slight lymphoid hyperplasia was observed in the spleen of a single male treated with AlphaGOS®. As this observation may be occasionally seen in animals of this age, this isolated observation was considered not to be treatment related. Other changes were observed at a similar incidence and/or severity between both control and treated groups and were considered to be part of the normal background commonly seen in animals of this age.

3. Developmental Toxicity of AlphaGOS® in Piglets

a. Summary

A study of AlphaGOS® in neonatal piglets corroborates the safety and tolerance of this product for ingestion by human infants (Kruger et al., 2017b). In the current study, both female

and male 3-day-old piglets were fed swine milk replacer (Solustart II, Land O'Lakes Animal Milk Products Co., Shoreview, Minnesota, USA) in the absence, and presence of AlphaGOS®, at a level of 8 mg/mL, for three consecutive weeks. All animals underwent evaluations, including clinical observations, body weight, feed consumption, clinical pathology, and gross necropsy with histopathology of selected tissues. The study performed was approved by the Experimur Institutional Animal Care and Use Committee (IACUC), following an approved Animal Care Use Protocol.

There were some statistically significant reductions in a few clinical chemistry parameters between control and treated males. However, since toxicity is associated with elevation in these values (Gowda et al., 2009; Martin 2016), the decreases noted are not considered toxicologically or clinically meaningful. A significant increase in TRIGs was noted in treated males; however, because the TRIG value is within the historical control range for piglets of this age and strain (11.0–108.0 gm/dL), it is not considered clinically adverse. No statistically significant differences were reported in the female piglets in the study. In summary, the statistically significant changes in clinical chemistry and hematology parameters noted in the treated males were not considered to be the result of any adverse AlphaGOS®-treatment-related effects and were most likely attributed to normal biological variation.

The current study demonstrated that formula supplemented with 8 mg/mL of AlphaGOS® is safe and well tolerated in neonatal piglets and supports the safe use of AlphaGOS® in non-exempt infant formulas.

b. Methods

The study complied with the Guide for the Care and Use of Laboratory Animals (https://grants.nih.gov/grants/olaw/guide-for-the-care-and-use-of-laboratory-animals.pdf, accessed 23 May 2017) and the Guide for the Care and Use of Agricultural Animals in Research and Teaching (https://www.aaalac.org/about/Ag_Guide_3rd_ed.pdf, accessed 23 May 2017). The study design was approved by the Experimur Institutional Animal Care and Use Committee, following an approved Animal Care Use Protocol. In addition, the animals were euthanized in accordance with the recommendations of the American Veterinary Medical Association Panel on Euthanasia (https://www.avma.org/KB/Policies/Documents/euthanasia.pdf, accessed 23 May 2017).

i. Animals and Husbandry

Farm piglets (Yorkshire crossbred) were used in the current study for AlphaGOS® since this species has been identified to be an appropriate neonatal model for safety evaluation of ingredients in infant formula. The animals were obtained from Oak Hill Genetics, Ewing, Illinois, USA. At the supplier, the neonatal piglets were allowed to nurse from the sow for at least 2 days after birth to provide them with maternal colostrum. In order to minimize the risk of failure to thrive due to low body weights, 12 male and 12 female animals of at least 1.5 kg were selected and shipped in an environmentally controlled vehicle by the supplier. At receipt, each piglet was given a physical examination (day 1) and body weights were recorded. Each animal was acclimated to the feeding containers. Animals were randomly assigned to two groups, each having six piglets/sex, based on body weight using an in-house computer-based randomization process. Each piglet was individually identified at the supplier with a plastic ear tag number. Identification numbers were assigned to the animals that were unique to this study within the animal room used. The animals were individually housed in tandem stainless steel suspended cages equipped with rubberized flooring. Each cage had an electric heating pad designed for piglets that allowed the animals to control and maintain their body temperature. Individual cage cards containing at minimum the study and animal numbers were provided. Environmental controls were set to maintain a temperature of at least 25ºC (77ºF) and a relative humidity range of approximately 30–70%. Lighting controls were set to maintain a 12-h light/12-h dark cycle.

ii. Diets

All animals were offered swine milk replacer alone or supplemented with AlphaGOS® for three consecutive weeks at a dose volume of 500 mL/kg/day (Table 25). The constant dose volume of 500 mL/kg/day contained sufficient water to keep the animals hydrated during the study. On the day of animal receipt (day 1), the piglets were introduced to bowl feeding approximately every 3 h until they became acclimated. All of the animals successfully learned to eat from the feeders and were fed six times a day the first day to allow for a total daily dose volume of 500 mL/kg. The control group received base formula alone, while the test group received the same formula supplemented with 8 mg/mL of AlphaGOS®. The level of AlphaGOS® used in the current study is based on the levels used in infant clinical studies (Moro et al., 2006; van Hoffen et al., 2009) and proposed levels of GOS products in several GRAS determinations that received “no questions” letters from the FDA (GRN 286, 334, 569) and in the Commission Delegated Regulation on Infant Formula of European Union (https://www.avma.org/KB/Policies/Documents/euthanasia.pdf, accessed 23 May 2017).

Table 25. Diets Design for All Animals				
No. Males	**No. Females**	**Treatment**	**Dose Concentration (mg/mL)**	**Dose Volume (mL/kg/day)**
6	6	Control	0	500
6	6	AlphaGOS®	8	500

iii. Experiment Design

During the entire study, all animals were observed for moribundity, mortality, and any abnormal clinical signs at least twice daily on weekdays and at least once on weekends and holidays. A detailed clinical examination was performed on all animals prior to feeding on days 1, 4, 8, 11, 15, 18, and 21. Specific emphasis was placed on the fecal color and consistency. The body weights of all animals were recorded at receipt (day 1) and daily for the first week and every other day thereafter as well as on the day of necropsy. Feed consumption for all animals was documented daily. Feed efficiency and compound consumption were calculated based on formulas shown in equations (1) and (2), respectively.

Equation (1): Feed Efficiency (FE)

$$\text{Percent FE} = \frac{\text{Mean Body Weight Gain (g)}}{\text{Total mean Feed Consumed (g)}} \text{ X } 100$$

FE is the feed efficiency expressed in percentage (amount of body weight gained per gram of feed consumed); body weight gain is the total amount gained during the 21-day study period, and the feed consumed is the total amount consumed during the 21-day study period.

Equation (2): Compound Consumption (CC)

$$\text{CC (mg/kg/day)} = \frac{\text{Total mean Feed Consumed (g) x Conc. (mg/mL)}}{\text{Interval x Density (g/mL) x mean Body Weight (kg)}}$$

CC is the compound consumption, or dose level, conc. Is the concentration of the test article in the milk replacer, interval is the duration of administration (21 days), density is the density of the test article formula and milk replacer with test article measured at preparation, mean body weight is the mean body weight for the 21-day study period.

iv. Blood Collection

On day 22, blood for clinical chemistry and hematology was collected via the brachiocephalic trunk just prior to the scheduled necropsy. The animals were not fasted, since the pigs were neonates. The following hematology parameters were measured: red blood cell count (RBC), white blood cell count (WBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), platelet count (PLAT), mean platelet volume (MPV), red blood cell

distribution width (RDW), reticulocyte count (absolute and relative) (Retic), automated differential leukocyte count (absolute and relative) including neutrophil (NEUT), lymphocyte (LYMPH), monocytes (MONO), eosinophils (EOS), large unstained cell (LUC), and basophils (BASO), prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrinogen (F). The following clinical chemistry parameters were also measured: albumin (A), A/G ratio, alanine aminotransferase (ALT), alkaline phosphatase (ALKP), aspartate aminotransferase (AST), total bilirubin (T. BIL), blood urea nitrogen (BUN), calcium (Ca), chloride (Cl), cholesterol (CHOL), creatinine (CREA), creatine phosphokinase (CPK), gamma glutamyl transferase (GGT), globulin (G), glucose (GLU), lactate dehydrogenase (LDH), phosphate (PO_4), potassium (K), total protein (T. PRO), sodium (Na), triglycerides (TRG).

v. Postmortem

All animals were sedated via intramuscular injection of a mixture of ketamine (VET One, Boise, Idaho, USA), xylazine (VET One), and acepromazine (Phoenix Pharmaceutical, Saint Joseph, Missouri, USA) at a mixing ratio of 1:1:1 with concentrations of 100, 100, and 10 mg/mL, respectively, followed by killing with an injection of 100 mg/kg Fatal Plus® (Vortech Pharmaceuticals, Dearborn, Michigan, USA) on day 22. All animals received a complete necropsy including examination of the external surface of the body, all orifices, the cranial, thoracic, and peritoneal cavities, and their contents. Testes were fixed in Davidson's solution; all other tissues were fixed in 10% neutral-buffered formalin (EKI Chemical, Jolliet, Illinois, USA). The following tissues were weighed at necropsy: adrenals, brain (forebrain, midbrain, cerebellum), kidneys, liver, parathyroid, spleen, testes, thymus and thyroid. Organ to body and organ to brain weight ratios were calculated. Paired organs were weighed together. The lymph nodes, any gross lesions, and small and large intestines were also collected. The full gastrointestinal tract from each animal was macroscopically examined (from mouth to rectum). The small intestine (from stomach to cecum) was sectioned anteriorly at the junction of the stomach with the duodenum and posteriorly at the junction of the ileum with the cecum. The large intestine (cecum and colon) was sectioned anteriorly at the junction of the ileum with the cecum and posteriorly to the end of the colon. These two sections were weighed for each animal after a saline rinse. The intestinal contents were collected from cecum and proximal colon and measured for pH in triplicate.

vi. Histopathology

All tissues were processed by routine histological methods, stained with hematoxylin and eosin (StatLab Medical Products, McKinney, Texas, USA) using a CV5030 Autostainer XL (Leica Biosystems; Buffalo Grove, IL, USA) and evaluated microscopically by a blinded board-certified veterinary pathologist.

vii. Statistical Analysis

Continuous data were analyzed for homogeneity of variance using Levene's test. If the variances are homogeneous ($p > 0.001$), the data were further analyzed by analysis of variance. If a significant F value is observed ($p \leq 0.05$), the treatment group was compared to the vehicle control group using Dunnett's two-tailed t test. Statistical significance was declared at $p \leq 0.05$ for Dunnett's test. If Levene's test was significant ($p \leq 0.001$), an appropriate transformation was applied to the data (e.g. log transformation or rank transformation) and the analyses were performed on the transformed data. Experimental results are expressed as means with their standard deviations. Analyses were performed using Systat® (Systat, San Jose, California, USA).

c. Results

i. Clinical Observations and Viability

All animals appeared normal at study initiation, with the exception of diarrhea in some animals, and remained alive until scheduled necropsy on day 22. No diarrhea was recorded in the control males but was recorded in five-treated males (one on day 2, one on days 2–4, one on days 3–4 and 11, one on day 4, and one on day 15). In females, it was noted in three controls (one on day 5, one on day 15, one on days 3–4, 8–15, and 17–18) and six-treated animals (one on days 5 and 7, two on day 7, one on day 4, and two, each, on days 3–4). Most cases of diarrhea were limited to a short duration (1–2 days) or single occurrences during the first week of treatment; however, in the control females, it was noted up to 2 weeks into the dosing period and ranged from 1 day to 8 days in duration. One control female was noted to be slightly emaciated on day 18, which correlated to an extended period of diarrhea and some body weight loss during the second week of the study. One male from the treated group was noted with scaly skin on the ventral neck on days 15 and 18 but returned to normal afterward. These observations were considered incidental. The majority of animals had no further abnormal clinical observations after the first week of the study.

ii. Body Weight, Feed Consumption, and Feed Efficiency

The mean body weights of females and males in the AlphaGOS® treated groups were slightly higher and lower, respectively, than in the corresponding control groups (Figure 3); however, the differences between groups were not statistically significant. A similar pattern, without statistical significance, between the control and treated groups was noted in feed consumption (Figure 4).

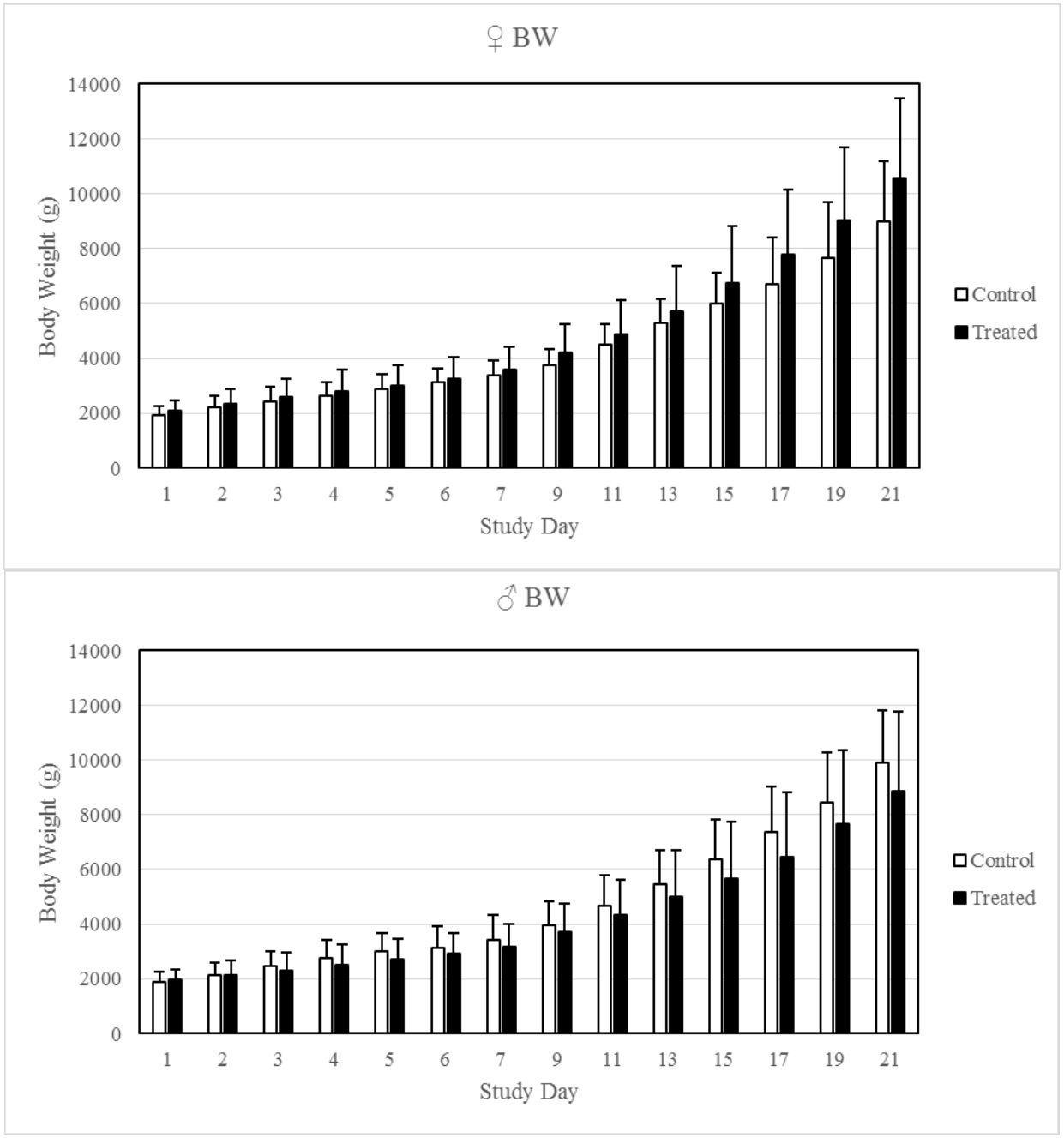
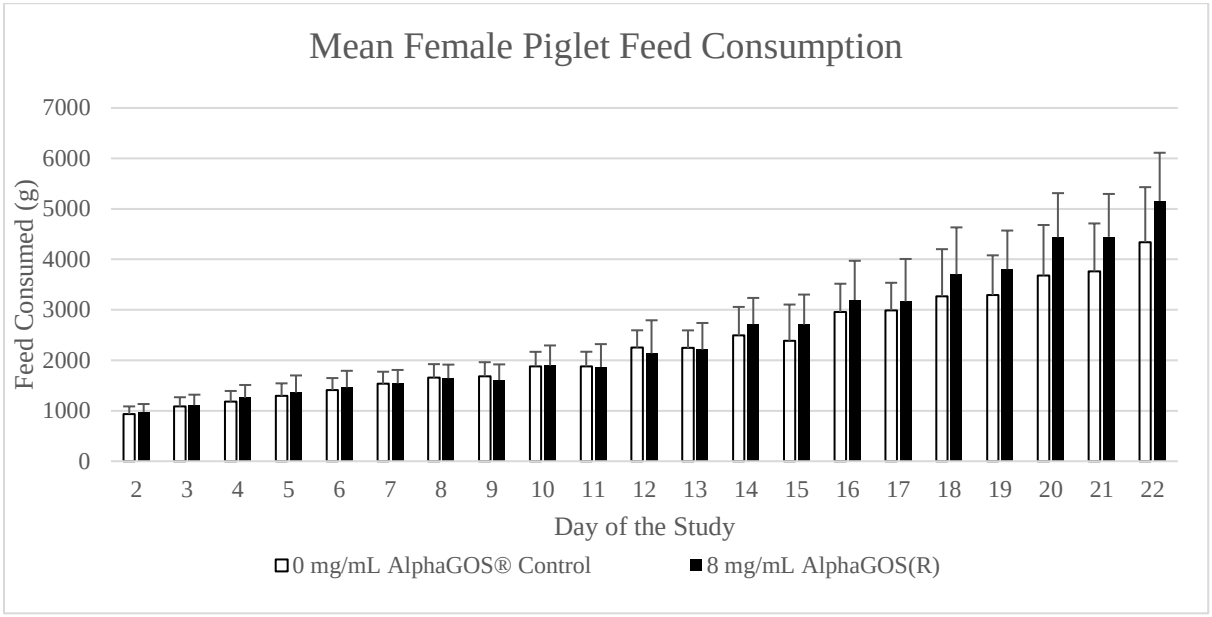


Figure 3. Female (Above) and Male (Below) Mean Body Weight (BW) During Study, Error Bars Represent Standard Deviation.



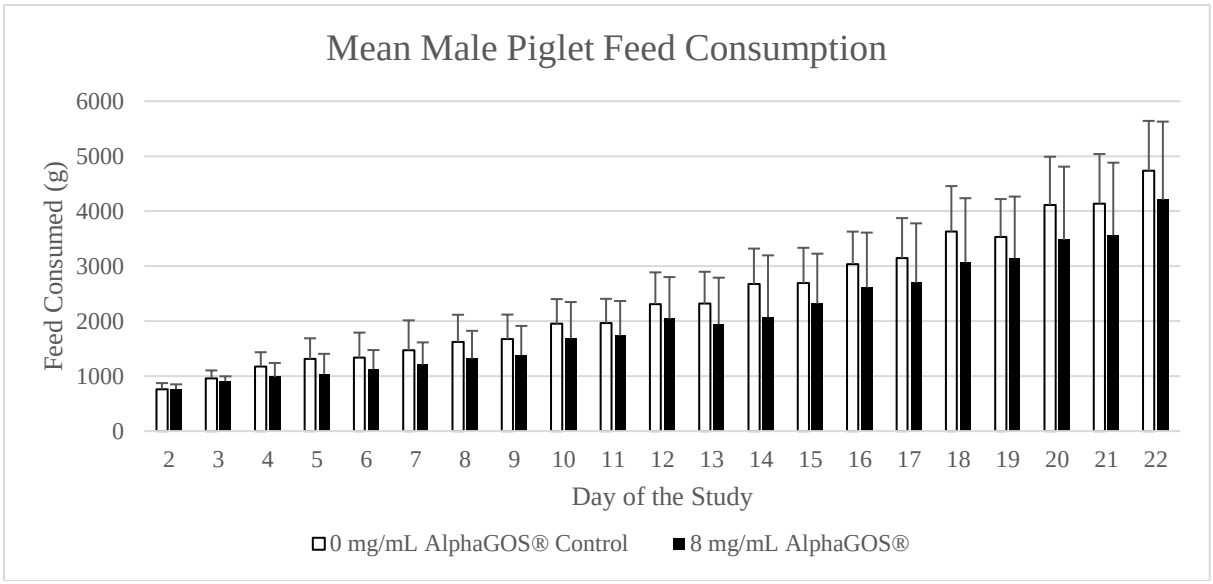


Figure 4. Female (Above) and Male (Below) Mean Feed Consumption During Study, Error Bars Represent Standard Deviation.

In addition, feed efficiency was calculated (equation (1)) to evaluate the growth of these piglets over the entire treatment period (days 1–21). The mean feed efficiency was statistically significantly increased in treated females (16.2%) compared to control females (14.4%), which may be caused by the remarkably decreased body weight gain of one control female during the study (noted in the clinical observation results). There was no statistically significant difference in males in terms of mean feed efficiency between control (15.9%) and AlphaGOS® -treated piglets (15.8%).

iii. AlphaGOS® Consumption

The mean AlphaGOS® consumption for male and female piglets was 3697 and 3900 mg/kg/day, respectively, meaning the consumption of alpha-linked galactooligosaccharides (which is 97% of the AlphaGOS® product) was 3336 and 3520 mg/kg/day, respectively.

iv. Hematology and Clinical Chemistry

Clinical chemistry parameters that were statistically significantly different between control and treated males included alkaline phosphatase (ALKP: 491 ± 47.9 vs. 420 ± 37.9 U/L in control), alanine aminotransferase (ALT: 24 ± 2.1 vs. 20 ± 1.9 U/L in control), aspartate aminotransferase (AST: 32 ± 5.0 vs. 22 ± 2.9 U/L in control), gamma glutamyl transferase (GGT: 30.8 ± 6.1 vs. 22.5 ± 6.1 U/L in control), lactate dehydrogenase (LDH: 669 ± 86.1 vs. 531 ± 40.1 U/L in control), triglycerides (TRIG: 32 ± 5.2 vs. 51 ± 13.0 mg/dL in control), and blood urea nitrogen (BUN: 17 ± 2.2 vs. 14 ± 2.3 mg/dL in control) (Table 26). No toxicity was associated with elevation in ALKP, ALT, AST, GGT, and LDH (Gowda et al., 2009; Martin 2016); therefore, the decreases noted were not considered toxicologically or clinically meaningful. The reduction in blood urea nitrogen (BUN) may be due to the increased nitrogen utilization by a larger microbial population induced by oligosaccharide intake (Kawasaki et al., 2015). The significant increase in TRIGs is within the historical control range for piglets of this age and strain (11.0–108.0 gm/dL) and is therefore not considered clinically adverse. No statistically significant changes were noted between females in the control and treated groups.

Table 26. Summary of Clinical Chemistry in Neonatal Piglets Fed AlphaGOS® for Three Weeks

Clinical Chemistry Parameters	Male		Female	
	0 mg/mL (n = 6)	8 mg/mL (n = 6)	0 mg/mL (n = 6)	8 mg/mL (n = 6)
ALB (g/dL)	3.4 ± 0.3	3.1 ± 0.4	3.1 ± 0.4	3.3 ± 0.4
ALKP (U/L)	491 ± 47.9	420 ± 37.9*	426 ± 150.1	437 ± 72.6
ALT (U/L)	24 ± 2.1	20 ± 1.9*	20 ± 1.7	23 ± 3.9
AST (U/L)	32 ± 5	22 ± 2.9*	21 ± 4.7	25 ± 10.8
T. BIL (mg/dL)	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.1
Ca (mg/dL)	11.8 ± 0.2	11.7 ± 0.3	11.5 ± 0.6	11.9 ± 0.5
CHOL (mg/dL)	81 ± 16.9	76 ± 17.1	81 ± 23.4	85 ± 14.3
CPK (U/L)	503 ± 234.5	285 ± 63.1	287 ± 67	331 ± 207.3
CREA (mg/dL)	0.69 ± 0.1	0.65 ± 0.1	0.65 ± 0.1	0.68 ± 0.0
GGT (U/L)	30.8 ± 6.1	22.5 ± 6.1*	25.6 ± 7.3	25 ± 8.0
GLU (mg/dL)	158 ± 9.7	158 ± 11.9	141 ± 16.8	144 ± 9.4
LDH (U/L)	669 ± 86.1	531 ± 40.1*	544 ± 31.7	583 ± 80.1
PO_4 (mg/dL)	10.9 ± 0.8	10.6 ± 0.7	10.7 ± 0.3	10.7 ± 0.3
T. PRO (g/dL)	5 ± 0.3	4.5 ± 0.4	4.7 ± 0.3	4.9 ± 0.4
TRIG (mg/dL)	32 ± 5.2	51 ± 13.0*	31 ± 19.6	48 ± 15.9
BUN (mg/dL)	17 ± 2.2	14 ± 2.3*	15 ± 2.5	15 ± 2.3
GLB (g/dL)	1.5 ± 0.1	1.5 ± 0.2	1.6 ± 0.1	1.6 ± 0.1
A/G	2.3 ± 0.3	2.2 ± 0.4	2.1 ± 0.3	2.1 ± 0.2
Na (mmol/L)	143 ± 1.8	143 ± 2.4	145 ± 2.2	144 ± 1.7
K (mmol/L)	6.9 ± 1.1	6.4 ± 1	5.7 ± 0.8	6.5 ± 0.6
Cl (mmol/L)	105 ± 1.5	104 ± 1.9	105 ± 1.5	105 ± 1.9

ALB: Serum Albumin; ALKP: alkaline phosphatase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; T. BIL: total bilirubin; Ca: calcium; CHOL: cholesterol; CPK: creatine phosphokinase; CREA: creatinine; GGT: gamma glutamyl transferase; GLB: Globulin; GLU: glucose; LDH: lactate dehydrogenase; PO_4: phosphate; T. PRO: total protein; TRIG: triglycerides; BUN: blood urea nitrogen; A/G: albumin/globulin; Na: sodium; K: potassium; Cl: chloride.
*Significantly different from the control ($p < 0.05$).

In the AlphaGOS®-treated males, a significant difference in the hematocrit (HCT: 34 ± 4.5% vs. 38.6 ± 1.9% in control), relative monocytes (MONO: 3.4 ± 0.3% vs. 5.2 ± 1.2% in control), and relative eosinophils (EOS: 0.8 ± 0.2% vs. 0.5 ± 0.1% in control) was noted compared to controls (Table 27). However, these values are within historical control range for piglets of this age and strain (27.5 – 43.8%, 0 – 8.0%, and 0 – 7.1%, respectively) and piglets used in other studies (Weiner et al., 2015; Hanlon and Thorsrud 2014; Fedorova-Dahms et al., 2014). Therefore, the findings are most likely associated with normal biological variation rather than an adverse treatment-related effect. No statistically significant changes in hematology parameters were noted in females between the control and treated groups.

Table 27. Summary of Hematology in Neonatal Piglets Fed AlphaGOS® for Three Weeks

Hematology Parameters	Males		Females	
	0 mg/mL (n = 6)	8 mg/mL (n = 6)	0 mg/mL (n = 6)	8 mg/mL (n = 6)
WBC (10^3 cells/µL)	10.64 ± 4.50	9.72 ± 2.12	9.12 ± 2.32	10.92 ± 3.40
RBC (10^6 cells/µL)	6.03 ± 0.31	5.45 ± 0.97	5.71 ± 0.67	5.57 ± 0.24
HGB (g/dL)	10.7 ± 0.6	9.4 ± 1.3	10.1 ± 1.1	10.3 ± 0.7
HCT (%)	38.6 ± 1.9	34.0 ± 4.5*	36.3 ± 3.8	36.1 ± 2.9
MCV (fL)	64.0 ± 1.2	63.2 ± 6.4	63.8 ± 3.2	64.8 ± 3.8
MCH (pg)	17.8 ± 0.7	17.5 ± 2.2	17.8 ± 1.4	18.5 ± 1.1
MCHC (g/dl)	27.8 ± 1.0	27.7 ± 0.7	27.9 ± 0.9	28.6 ± 0.5
PLAT (10^3 cells/µL)	800 ± 145	625 ± 136	817 ± 208	674 ± 108
MPV (fL)	8.5 ± 0.4	9.2 ± 0.6	8.7 ± 0.4	8.7 ± 0.2
RDW (%)	19.3 ± 1.2	20.1 ± 2.2	19.9 ± 2.0	19.0 ± 1.6
NEUT				
(10^3 cells/µL)	3.05 ± 0.70	4.14 ± 2.12	3.77 ± 1.62	3.67 ± 1.28
(%)	31.1 ± 8.1	40.8 ± 11.7	40.2 ± 10.2	34.9 ± 11.8
LYMPH				
(10^3 cells/µL)	6.78 ± 3.89	4.99 ± 0.84	4.80 ± 0.99	6.34 ± 2.89
(%)	61.6 ± 7.2	52.9 ± 11.6	53.7 ± 10.2	56.8 ± 10.3
MONO				
(10^3 cells/µL)	0.58 ± 0.34	0.33 ± 0.06	0.36 ± 0.16	0.59 ± 0.26
(%)	5.2 ± 1.2	3.4 ± 0.3*	3.9 ± 1.7	5.6 ± 2.2
EOS				
(10^3 cells/µL)	0.05 ± 0.02	0.07 ± 0.03	0.07 ± 0.04	0.08 ± 0.02
(%)	0.5 ± 0.1	0.8 ± 0.2*	0.8 ± 0.2	0.8 ± 0.2
LUC				
(10^3 cells/µL)	0.12 ± 0.06	0.19 ± 0.05	0.13 ± 0.07	0.13 ± 0.11
(%)	1.3 ± 0.8	2.0 ± 0.6	1.3 ± 0.4	1.2 ± 0.8
BASO				
(10^3 cells/µL)	0.06 ± 0.13	0.01 ± 0.00	0.02 ± 0.02	0.10 ± 0.14
(%)	0.4 ± 0.6	0.1 ± 0.0	0.1 ± 0.1	0.8 ± 1.0
Retic				
(10^9 cells/L)	680.3 ± 152.9	826.4 ± 206.0	673.2 ± 240.3	672.4 ± 137.6
(%)	11.36 ± 2.71	15.73 ± 5.16	11.82 ± 3.79	12.07 ± 2.35
PT (sec)	13.3 ± 0.5	13.1 ± 0.6	13.3 ± 0.9	13.0 ± 0.8
aPTT (sec)	13.3 ± 1.5	14.1 ± 2.2	14.7 ± 1.0	14.2 ± 1.7
FIB (sec)	214.5 ± 46.2	187.3 ± 19.8	209.0 ± 35.8	187.5 ± 35.0

WBC: white blood cell count, RBC: Red blood cell count, HGB: hemoglobin, HCT: hematocrit, MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration, PLAT: platelet count, MPV: mean platelet volume, RDW: red blood cell distribution width, Retic: reticulocyte count, NEUT: neutrophil, LYMPH: lymphocyte, MONO: monocytes, EOS: eosinophils, LUC: large unstained cell, BASO: basophils, PT: prothrombin time, aPTT: activated partial thromboplastin time, FIB: fibrinogen.
*Significantly different from the control ($p < 0.05$).

v. Organ Weights

Administration of AlphaGOS® resulted in an increase in intestinal weight, particularly large intestines (Table 28). This finding is commonly seen with substances that are incompletely digested and poorly absorbed in the small intestine and thus are subjected to microbial metabolism in both the cecum and colon (England et al.; 2015; Weiner et al., 2015; Houdijk et al., 1998; Chonan et al., 1995; Haschek et al., 2010). Although it is generally agreed that the cecum and colon are the main sites of fermentation in pigs, there is already substantial microbial activity in the ileum (Leser et al., 2002). This can explain why the small intestine (ileum) weight also tended to increase in the treated animals yet to a lesser extent as compared to the increase seen in the weight of the large intestine (colon and cecum). The pH of intestinal content also corroborated the effect of AlphaGOS® on the increase of microbial metabolic products, such as short chain fatty acids (Table 28).

Table 28. Summary of Intestinal Contents pH in Neonatal Piglets Fed AlphaGOS® for Three Weeks

Sex	Description	0 mg/mL AlphaGOS®	8 mg/mL AlphaGOS®
Male	Cecum pH	6.23 ± 0.19	5.87 ± 0.25
	Colon pH	6.69 ± 0.29	6.11 ± 0.25
Female	Cecum pH	6.39 ± 0.18	6.21 ± 0.51
	Colon pH	6.79 ± 0.28	6.54 ± 0.57

The absolute adrenal weight for the control males was slightly lower than that for the control females (0.89 vs. 1.07 g, respectively) and lower than historical control data (1.275 ± 0.186 and 1.342 ± 0.184 for males and females, respectively). This may account for the increase in the absolute adrenal weights in the treated males compared to control. There were no histopathologic findings noted during the microscopic examination of the adrenal cortex and the adrenal medulla. Due to the lack of any microscopic changes in the adrenals of the treated males, it is unlikely that the apparent increase in adrenal weight is toxicologically meaningful. Additionally, the adrenal weight increase was limited to only one gender; there was no statistically significant effect on the adrenal weight noted in treated females compared to controls (Table 29).

Table 29. Summary of Absolute and Relative Organ Weights of Intestines and Adrenals in Neonatal Piglets Fed AlphaGOS® for Three Weeks

Sex	Tissue	Organ Weight	Control (0 mg/mL)	AlphaGOS® (8 mg/mL)	Percent Change from Control
Male	Small Intestine (between stomach and cecum)	Abs Wt, g	456 ± 163	456 ± 90	0%
		% body wt	4 ± 2	5 ± 1	25%
		% brain wt	879 ± 299	859 ± 141	-2%
Female	Small Intestine (between stomach and cecum)	Abs Wt, g	436 ± 77	524 ± 68	20%
		% body wt	5 ± 0	5 ± 1	0%
		% brain wt	839 ± 109	986 ± 86	18%
Male	Large Intestine (cecum and colon)	Abs Wt, g	122.8 ± 27.9	135.5 ± 38.3	10%
		% body wt	1.2 ± 0.1	1.4 ± 0.1	17%*
		% brain wt	238.0 ± 39.9	254.3 ± 60.4	7%
Female	Large Intestine (cecum and colon)	Abs Wt, g	115.8 ± 29.3	151.8 ± 41.6	31%
		% body wt	1.2 ± 0.1	1.3 ± 0.2	8%
		% brain wt	221.3 ± 40.8	283.3 ± 60.3	28%
Male	Adrenal	Abs Wt, g	0.89 ± 0.14	1.04 ± 0.08	17%*
		% body wt	0.008 ± 0.001	0.012 ± 0.004	50%
		% brain wt	1.73 ± 0.23	1.98 ± 0.13	14%*
Female	Adrenal	Abs Wt, g	1.07 ± 0.17	1.21 ± 0.17	13%
		% body wt	0.011 ± 0.002	0.011 ± 0.002	0%
		% brain wt	2.06 ± 0.29	2.29 ± 0.29	11%

Mean values ± SD; Wt = Weight; Abs = absolute
*Significantly different from control ($p<0.05$), Dunnett's two-tailed test

vi. Histopathology Findings

Microscopic evaluation was conducted for tissues collected including adrenal glands, brain (cerebellum, forebrain, and midbrain), intestine (ileum), kidneys, liver, lymph nodes (mesenteric), spleen, testes (males), thymus, and thyroids/parathyroid glands. The testes from all the male animals were found to be immature, which is expected since they were neonatal animals. There were a few findings in all groups that were considered incidental and not related to the test articles. One control male showed pigmentation in the left kidney. One treated male had minimal diffuse hepatocellular vacuolation. Another treated male was noted with minimal multifocal accumulation of erythrocytes in the mesenteric lymph node. One treated female had a finding of a mild focal infarct of one kidney. Histopathological evaluation did not reveal any adverse changes that could be attributed to the AlphaGOS® treatment.

4. Corroborative Animal Studies with alpha-GOS

There are studies in the literature in which alpha-linked oligosaccharides, such as raffinose or stachyose from soybean or other sources, were fed to animals; however, it is important to note that the focus of these studies was not safety. Zhang et al. (2004) investigated the effects of 100, 200 and 400 mg/kg bw/day of oral stachyose (from *Rehmannia glutinosa*)

administered for 6 days in induced hyperglycemic and alloxan-induced diabetic rats. Stachyose ingestion lowered blood glucose levels, and no toxic effects were reported.

Other studies that evaluated raffinose or stachyose provided by the oral route to animals are summarized in Table 30 and described briefly below. No safety data were reported for these studies (see Table 30). Mitamura et al. (2004) examined the effects of oral raffinose on intestinal calcium absorption in ovariectomized rats, and its ingestion resulted in increased calcium absorption levels, similar to non-ovariectomized, control-fed rats. Nakamura et al. (2012) evaluated the effects of germanium and raffinose in the intestinal microbiota of rats. A diet containing raffinose (5%) was given alone or in combination with Ge-132. Raffinose increased Bifidobacterium and Lactobacillus counts, and statistically increased ceacal content and cecal wall weight.

Nagura et al. (2002) focused on the immunosuppressive potential of a diet supplemented with 50 mg raffinose/kg for 2 weeks in a transgenic mouse model of allergy responsive to ovalbumin injection (OVA). Raffinose increased interleukin 12 secretion from Peyer's patch (PP) cells in vitro compared to control diet in wild type mice. Raffinose ingestion suppressed the levels of immunoglobulin E induced by ovalbumin in the transgenic OVA mouse model of allergy. Watanabe et al. (2004) also focused on immune response modulation by raffinose. This group found that a dietary intake of raffinose at 50 g/kg of diet for 20 days in rats led to a suppression of the allergic eosinophil airway response. In summary, no adverse effect on immune response is expected from ingestion of alpha-GOS.

Table 30. Corroborative Studies of Supplementation with Raffinose and Stachyose in Animals				
Reference	**Model**	**Test Article**	**Route of Administration, Dose, Duration**	**Relevant Findings and Safety**
Nagura et al., 2002	BalbC mice Transgenic mouse model of allergy	Raffinose	Oral 50 mg /kg diet 2 wks	• Raffinose increased interleukin 12 secretion from Peyer's patch (PP) cells in WT mice. • Raffinose ingestion suppressed the levels of immunoglobulin E induced by ovalbumin in transgenic mice. • No safety data were reported in this study.
Mitamura et al., 2004	Ovariectomized rats	Raffinose	Oral 30 g/kg diet 4 wks	• Raffinose restored calcium absorption in the large intestine of ovariectomized rates. • No safety data were reported in this study.
Watanabe et al., 2004	Brown Norway rats	Raffinose	Oral 50 g/kg diet 20 days	• Suppression of the allergic eosinophil airway response in rats. • No safety data were reported in this study.
Zhang et al., 2004	Rat model of induced diabetes	Stachyose (f. *Rehmannia glutinosa*)	Oral 100, 200 and 400 mg/kg bw 6 days	• Stachyose ingestion lowered blood glucose levels • No safety data were reported in this study.
Nakamura et al., 2012	Male Wistar Rats	Raffinose	Oral 5% raffinose diet 2 wks	• Raffinose increased *Bifidobacterium* spp. and *Lactobacillus* spp. counts. • Increased ceacal content and ceacal wall weight. • No safety data was reported in this study.

D. CLINICAL STUDIES

1. AlphaGOS®

AlphaGOS® has been studied in two published clinical trials, both described in Morel et al., (2015). Both of these trials reported that AlphaGOS® was well tolerated in human subjects with no test-article related adverse events recorded. AlphaGOS® has also been investigated in several unpublished clinical trials sponsored by Olygose. In these trials, AlphaGOS® was well tolerated and no serious adverse events were reported.

a. Randomized, Placebo Controlled, Double-Blind, Parallel, Four-Arm Clinical Trial in Overweight Adults (Morel et al., 2015)

i. Summary

In a published, randomized, placebo controlled, double-blind, parallel, four-arm clinical trial the effects of the degree of polymerization of AlphaGOS® on appetite and energy intake in overweight subjects were evaluated (Morel et al., 2015). To address this objective, both a dose effect study and a formulation effect study were performed. In each study, 88 subjects were randomly assigned to 1 of 4 intervention groups (n = 22/group) and given tea supplemented with AlphaGOS® for 14 days. For the dose effect study, subjects consumed tea twice a day supplemented with either 3, 6, or 9 g of AlphaGOS® or a glucose control. In the formulation effect study, subjects consumed tea twice a day supplemented with 6 g/day of dried glucose syrup (placebo, Group 1), 12 g/day of AlphaGOS® with either high content of melibiose (degree of polymerization 2, DP2), manninotriose (DP3), or verbascotetraose (DP4). Appetite ratings and 24-hour dietary recall were conducted on days 4, 8, and 11 of the study. Height, weight, BMI, waist and hip circumferences, and body fat were evaluated at the beginning and end of the study. The three intervention groups in the dose effect study reported increased feelings of satiety and fullness, as well as exhibited increased numbers of Bifidobacteria in the stool and decreased plasma lipopolysaccharide versus the control group. Overall, AlphaGOS® produced positive effects on appetite ratings, food and nutrient consumption, and the gut microbiota, although no impact of the degree of polymerization was observed. No serious adverse effects were reported, but some individuals in the AlphaGOS®-treated groups reported increased flatulence and bloating from days 8-14, but the bloating resolved by day 14. Consumption of AlphaGOS® for 14 days dose-dependently reduced appetite, food intake, and inflammation in overweight adults with no impact of the AlphaGOS® composition.

ii. Methods

Study designs. At the Institute of Nutrition and Health Food (Tongji University, Shanghai, China), two single-center, double-blind, randomized, placebo-controlled, parallel 4-arm studies were conducted separately. Both studies were performed in accordance with the Declaration of Helsinki as revised in 1983, the guidelines of Good Clinical Practice (guidelines

E6 from The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Care), and local regulations. These studies were granted approval by the Shanghai Nutrition Society Ethics Committee. All participants provided written informed consent before any research activities were initiated.

Participants. The two studies were conducted separately, with distinct patient cohorts. In both studies, 88 healthy, free-living men and women, aged 18–45 y, with a BMI (in kg/m^2) between 25 and 28, and with a stable weight (63 kg) for at least 3 months were included. Exclusion criteria were as follows: having participated in another trial within the 2 previous months, having attempted to lose weight in the past 3 months, having taken medication or dietary supplements that could influence study outcomes within the 3 months before the study, having a contraindication to dietary fiber supplementation, having a previous known allergic reaction to wheat products (gluten intolerance or celiac disease), having endocrine or gastrointestinal disease(s), being pregnant or lactating, exhibiting alcohol or drug dependence, consuming >3 drinks of alcohol/day, and smoking. Subjects had to report any medication/treatment that they were taking to the investigator during the study. Each medication/treatment, except for paracetamol up to a maximum dose of 1 g/day, was discussed between the investigator and study monitor to decide whether or not to keep the subject in the study. All medication was recorded.

Intervention. In each study, qualified subjects were randomly assigned to 1 of 4 groups. In the dose-effect study, subjects drank 250 mL bottled oolong tea (water, oolong tea leaves, vitamin C, sodium hydrogen carbonate; 0 kcal) twice a day to which either 3, 6, or 9 g AlphaGOS® [with >98% polymers of D-galactose residues linked by α(1→6) bonds with a terminal α (1→6)-linked D-glucose] or a control (dried glucose syrup C*DRY 01934; Cargill) were added. Study groups were as follows: 6 g AlphaGOS®/d (3 g AlphaGOS®twice per day), 12 g AlphaGOS® /d (6 g AlphaGOS® twice per day), and 18 g AlphaGOS® /d (9 g AlphaGOS® twice per day). In the formulation-effect study, subjects drank 250 mL bottled oolong tea twice per day to which one of the following was added: 6 g AlphaGOS® (with >98% AlphaGOS® dry matter) with a high content of DP2 (high DP2; including >98% melibiose alpha-GOS content), DP3 (high DP3; including >90% manninotriose alpha-GOS content), or DP4 (high DP4; including >80% verbascotetraose alpha-GOS content) or a control (dried glucose syrup, C*DRY 01934). In each study, participants consumed 1 bottle in the morning during breakfast (08:00 h) and 1 during the afternoon (between 15:00 and 16:00 h). All subjects consumed the control drink during the first day of the study (day 0) and consumed the tested products or control daily from days 1 to 15 (i.e., an intervention period of 14 d). No follow-up was conducted after the end of the intervention.

Outcomes. The primary endpoint of both studies was an appetite rating evaluated on 5 appetite dimensions (hunger, fullness, satiety, desire to eat, and prospective consumption) according to a preload and test meal design. The preload meal (day 0) was a standardized

breakfast consumed in 15 min. All participants consumed the entire breakfast. The test meal was an ad libitum lunch during which participants were informed that they could eat as much or as little food as they desired. Appetite ratings during the preload test meal were assessed on day 0 (before the intervention) and day 15 (after the intervention) with the use of visual analog scales (VASs) just before the start of a preload meal (consumed at 08:00 h); 30, 60, 120, 180, and 240 min after the preload meal; and 30, 60, 120, 180, and 240 min after the test meal (consumed at 12:00 h). A 9-point numerical scale ranging from 0 (not at all) to 9 (extremely) was used for evaluating subject feelings on the 5 appetite dimensions: hunger, fullness, satiety, desire to eat, and prospective consumption. The area under the curve (AUC) from the start of the preload meal to 240 min after the test meal was calculated for each of the appetite categories.

Secondary endpoints in both studies included food intake and anthropometric measurements. Food and beverage intakes during the test meal were determined by weighing food and drinks before and after the meal. In addition, a 24-h dietary recall (after a training session) was used to calculate the daily intakes of calories, protein, fat, carbohydrate, and fiber at days 0 and 15. Anthropometric variables, including height, weight, BMI, waist and hip circumferences (measured in centimeters as the minimum value between iliac crest and the lateral costal margin and the maximum value over the buttocks, respectively, with each variable measured twice), and body fat percentage (measured by impedancemetry by using an Omron Karada Scan Body Composition Monitor HBF-306), were measured on days 0 and 15.

Overnight fasting blood samples were drawn on days 0 and 15 and collected into depyrogenated tubes (Becton Dickinson) containing EDTA. Plasma liposaccharide (LPS) was measured in samples stored at –70^{o}C with the Limulus Amebocyte Lysate chromogenic endpoint assay HIT302 kit from Hycult, Uden, The Netherlands. C-reactive protein (CRP) was measured for samples stored at –20^{o}C with ELISA kits from R&D Systems, Minneapolis, Minnesota, USA. Fecal bifidobacteria and total bacteria were quantified in the formulation effect study by extracting DNA from fecal samples and quantifying *Bifidobacterium spp*. via 16S gene expression. Gastrointestinal tolerance was self-monitored at days 0 and 15 with a VAS rating ranging from 0 to 9 for flatulence, borborygmi, bloating, abdominal pain, stool frequency, and stool consistency.

Sample size and power consideration. Sample size calculations were based on the primary endpoint criterion of these studies, appetite scores. These calculations revealed that requiring the detection of a minimum difference of 10% in the AUC for each VAS rating (27) with a patient cohort of 22 individuals (allocation ratio of 1:1:1:1, 10% attrition rate) would show 80% power.

Randomization. For both studies, 88 subjects were randomly assigned to 1 of the 3 intervention groups (n = 22) or to the control group (n = 22). Subjects were stratified by age (18–30 or 31–45 y) and sex and assigned by using a permuted block design.

Statistical analyses. Continuous variables were statistically assessed for verisimilitude of the normal distribution assumption by using the Shapiro-Wilk test. Continuous outcome variables are reported as means ± 6 standard deviations. One-factor ANOVA was used to evaluate between-group differences for baseline and postintervention subject characteristics. When a significant difference was detected with ANOVA, multiple tests with the use of Fisher's least significant difference technique were performed without correction of the significance level. Comparisons across all groups were performed in both studies. Repeated-measures ANOVA was used to evaluate between-group changes in appetite scores. Spearman rank correlation coefficients (r) were calculated to assess correlations between changes in the AUC for appetite VAS scores in response to the test meal or plasma LPS and either doses or formulation. Data related to anthropometric variables and energy intake are presented as changes rather than as endpoints to limit interindividual variability. All analyses were conducted according to the intent-to-treat principle. The last observation carried forward method was used to impute missing outcome data. A significance threshold for statistical tests was set at $P < 0.05$ (2-sided). All analyses were performed by using SAS 9.3 for Windows (SAS Institute).

iii. Results

Baseline patient characteristics. A total of 88 subjects (22/group) participated in both studies. No patients dropped out of the dose-effect study, whereas 2 individuals (1 in the control group and 1 in the high-DP3-formula group) withdrew in the middle of the formulation-effect study for personal reasons. Patient baseline characteristics were well matched across study groups (Table 31). No significant differences were detected between groups within each study.

Appetite. There were no significant differences between study groups at baseline for all appetite dimensions. In the dose-effect study, after 14 d of intervention, a significant effect of 3 doses of AlphaGOS® was observed on mean appetite scores over the 240 min after preload meal consumption (except for the fullness score in the group who received 6 g AlphaGOS®/d) and over the 240 min after the test meal. The mean scores for hunger, desire to eat, and prospective consumption were lower in the 3 AlphaGOS® groups than in the control group, whereas mean fullness and satiety scores were higher in the 3 AlphaGOS® groups than in the control group for both periods. All appetite dimension profiles are shown in Figure 5. The AUCs calculated for all appetite dimensions during the preload test meal experiment were significantly different between groups (Figure 6). Significant positive correlations were detected between AlphaGOS® doses and changes in the AUC for fullness ($r = +0.55$, $P < 0.001$) and satiety ($r = +0.68$, $P < 0.001$), whereas negative correlations were found between changes in AUC for hunger ($r = –0.69$, $P < 0.001$), desire to eat ($r = –0.65$, $P < 0.001$), and prospective consumption ($r = –0.63$, $P < 0.001$) in response to the preload test meal period. This finding indicated a dose-response effect of AlphaGOS® on appetite scores.

Table 31. Baseline Characteristics of the Overweight Adults Who Participated in the Studies Described in Morel et al., 2015

Characteristics	Dose-Effect Study				Formulation-Effect Study			
	AlphaGOS®				AlphaGOS® with High DP, 12 g/d			
	Control	6 g/d	12 g/d	18 g/d	Control	High DP2	High DP3	High DP4
Subjects (M/F), n	11/11	11/11	11/11	11/11	11/11	11/11	11/11	11/11
Age, y	30 ± 9	32 ± 8	32 ± 9	31 ± 7	35 ± 10	39 ± 9	38 ± 11	34 ± 10
Weight, kg	71.6 ± 8.3	71.6 ± 6.6	71.7 ± 9	71.8 ± 7.4	73 ± 8	73.6 ± 8.4	72.4 ± 8.7	74.8 ± 6.8
Height, cm	164 ± 9	165 ± 8	165 ± 9	164 ± 7	166 ± 7	166 ± 8	164 ± 8	167 ± 7
BMI, kg/m^2	26.4 ± 0.7	26.4 ± 0.7	26.3 ± 0.9	26.6 ± 0.7	26.3 ± 0.9	26.5 ± 1.1	26.7 ± 1.3	26.8 ± 1.2
Waist circumference, cm	90.4 ± 5.2	90.5 ± 5.3	90.4 ± 6.4	90.6 ± 5.4	86.7 ± 6.2	89.1 ± 8	87.6 ± 7.4	88 ± 7.8
Hip circumference, cm	96.7 ± 4.9	96.6 ± 5.8	96.8 ± 4.8	96.8 ± 5.4	92.2 ± 8.8	93.3 ± 8.8	92.1 ± 10.3	93.9 ± 11.2
Waist-to-hip ratio	0.94 ± 0.05	0.94 ± 0.05	0.93 ± 0.04	0.94 ± 0.05	0.94 ± 0.06	0.96 ± 0.06	0.96 ± 0.06	0.94 ± 0.05
Body fat, %	27.2 ± 2.2	27.2 ± 2.4	27.3 ± 2.4	27.1 ± 2	26.4 ± 4.3	26.8 ± 3.9	27.8 ± 4.1	28.8 ± 3.4
Body fat mass, kg	19.4 ± 2.1	19.4 ± 1.5	19.5 ± 2.3	19.4 ± 2	19.3 ± 4.2	19.6 ± 3	20.2 ± 4.2	21.5 ± 3
Fat-free mass, kg	52.2 ± 6.8	52.2 ± 6.1	52.1 ± 7.4	52.4 ± 6.1	53.7 ± 6	53.9 ± 7.6	52.2 ± 6.4	53.3 ± 5.7
Daily energy intake, kcal	3062 ± 457	3036 ± 429	3014 ± 511	3009 ± 418	2887 ± 351	2682 ± 350	2639 ± 416	2683 ± 451
Daily fiber intake, g	31.6 ± 3.1	31.6 ± 3.7	31.2 ± 3.4	31.6 ± 2.3	30.4 ± 2.7	29.1 ± 2.6	29 ± 3	29.4 ± 2.8

Values are means ± SDs, n = 22. DP, degree of polymerization; High DP2, alpha-GOS s with >98% melbiose in alpha-GOS content; High DP3, alpha-GOS s with >90% manninotriose in alpha-GOS content; High DP4, alpha-GOS with >80% verbascotetraose in alpha-GOS content;

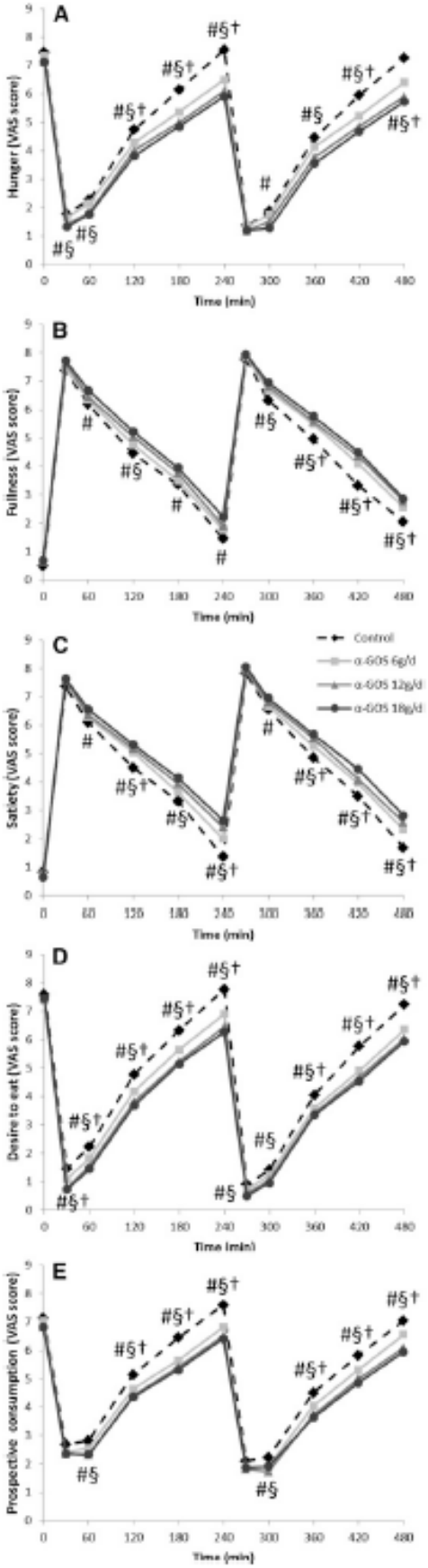


Figure 5. Appetite Scores Reported in Morel et al., 2015

Hunger (A), fullness (B), satiety (C), desire to eat (D), and prospective consumption (E) after meals in overweight adults who consumed a mix of 6, 12, or 18g α-GOSs/d for 14d. Values are means±SEM, n=22. *$p<0.05$ between the 18g/d α-GOS and control groups; §$p<0.05$ between 12 g/d α-GOS and control groups; †$p<0.0$ between 6 g/day α-GOS and control groups. Subjects rated 5 appetite dimensions on a 9-point numerical scale, ranging from 0 (not at all) to 9 (extremely). VAS, visual analogy scale.

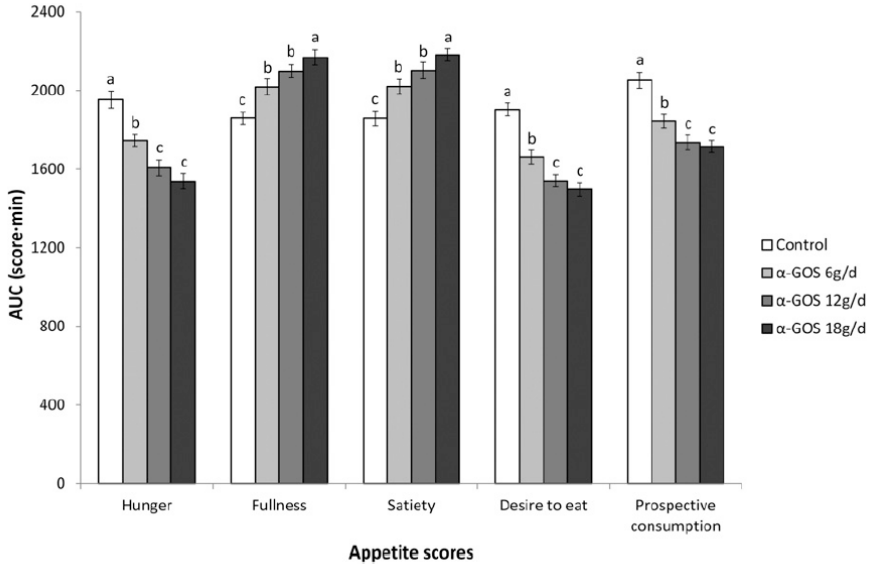


Figure 6. Change from Day 0 to Day 15 in AUCs for Appetite Scores in Morel et al., 2015

Assessed with visual analog scales in overweight adults who consumed 6, 12, or 18 g AlphaGOS®/day. Values are means ± SEMs, n = 22. Labeled means without a common letter differ, $P < 0.05$.

In the formulation-effect study, the mean scores for hunger, desire to eat, and prospective consumption were lower in the three groups with different AlphaGOS® formulas than in the control group and the mean scores for fullness and satiety were higher in the three groups with different AlphaGOS® formulas than in the control group over the 240 min after the preload meal and over the 240 min after the test meal. Moreover, the AUC for appetite scores was significantly affected in those groups who received formula with a high content of DP2, DP3, or DP4 compared with the control group. Specifically, hunger, desire to eat, and prospective consumption were significantly lower in the three groups who received different AlphaGOS® formulas than in the control group, whereas fullness and satiety were significantly higher. However, there was no significant difference among the three formulas.

Food intake. In the dose-effect study, the change from day 0 to 15 in food and nutrient (except for fat) intake during the test meal was significantly increased by AlphaGOS® intake, with greater differences in groups who received higher doses of AlphaGOS® (Table 32). The change from day 0 to day 15 in 24-h recall of energy intake during lunch and dinner was significantly increased by AlphaGOS® intake, with greater differences in the groups who received higher doses of AlphaGOS® (Table 33). In the formulation-effect study, changes from day 0 to day 15 in food weight and carbohydrate and dietary fiber intake during the test meal were greater for all three AlphaGOS® formula groups than for the control group. The reduction in energy and protein intake from day 0 to day 15 was significantly higher in the group who received the high-DP4 formula than in the control group. However, no significant difference was observed for changes in energy intake among the three AlphaGOS® formula groups (high DP2,

DP3, or DP4 content) during the test meal. The 24-h recall of energy intake indicated that the reduction in energy intake during lunch was higher in the high-DP3 and -DP4 groups (–25 ± 54 and –29 ± 46 kcal, respectively) than in the control group (+20 ± 71 kcal; $P < 0.05$). There were no significant differences detected among the three AlphaGOS® formula groups. In both studies, change in daily energy intake measured for the 24-h recall did not differ among groups, with the exception of the high-DP3 group in the formulation-effect study (–233 ± 559 kcal), which was significantly lower than that in the controls (–85 ± 647 kcal).

Table 32. Changes in Food Intake During a Test Meal (consumed 4 h after a preload meal) of Overweight Adults After 14d of Consumption of 6, 12, or 18 g AlphaGOS®/d from Morel et al., 2015

	Food intake during test meal, Δ day 15–day0			
	Control	AlphaGOS®		
		6 g/d	12 g/d	18 g/d
Weight, g	4.7 ± 19.2[a]	-11.4 ± 16.9[b]	-22.5 ± 19.1[b]	-28.8 ± 18.6[c]
Energy, kcal	6.1 ± 20.8[a]	-12.6 ± 18.6[b]	-25.7 ± 21.6[c]	-32.1 ± 22.0[c]
Protein, g	0.2 ± 0.6[a]	-0.3 ± 0.5[b]	-0.7 ± 0.6[c]	-0.9 ± 0.6[c]
Fat, g	-0.02 ± 0.29	-0.06 ± 0.18	-0.07 ± 0.1	-0.14 ± 0.3
Carbohydrates, g	1.5 ± 4.9[a]	-2.9 ± 4.3[b]	-6 ± 5.0[c]	-7.4 ± 5.3[c]
Dietary fiber, g	0.1 ± 0.4[a]	-0.2 ± 0.3[b]	-0.4 ± 0.4[b]	-0.6 ± 0.4[c]
Values are means ± SDs, n = 22. Labeled means without a common letter differ, $P < 0.05$. Δ, change				

Table 33. Changes in Energy Intake (assessed by a 24-h recall) of Overweight Adults After Consumption of 6, 12, or 18 g AlphaGOS®/d for 14 d from Morel et al., 2015

	Energy intake, Δ Day 15–Day 0			
	Control	AlphaGOS®		
		6 g/d	12 g/d	18 g/d
Lunch, kcal	4.2 ± 30.9[a]	-23.2 ± 29.5[b]	-36.8 ± 33.0[b]	-40.8 ± 29.7[b]
Dinner, kcal	2.2 ± 24.3[a]	-25 ± 45.6[b]	-32.3 ± 46.2[b]	-38 ± 57.1[b]
Daily total, kcal	-117 ± 436	-92 ± 432	-208 ± 372	-196 ± 401
Values Are means ± SDs, n=22. Labeled means without a common letter differ, $P < 0.05$. Δ, change.				

Inflammatory markers. In both studies, there were no significant between-group differences in the concentration of plasma LPS at baseline. Plasma LPS at day 15 was significantly lower after AlphaGOS® intake (Figure 7). A significant negative correlation was observed between dose and changes in plasma LPS ($r = –0.57$, $P < 0.001$). Plasma LPS did not differ among the 3 AlphaGOS® formula groups (high content in DP2, DP3, or DP4). In the formulation-effect study, there were no significant between-group differences in the concentration of plasma CRP at baseline. The plasma CRP concentration was significantly lower in all AlphaGOS® formula groups than in the control group after the study period (Figure 8); however, there was no significant difference among the groups who consumed high-DP2, -DP3, or -DP4 formulas.

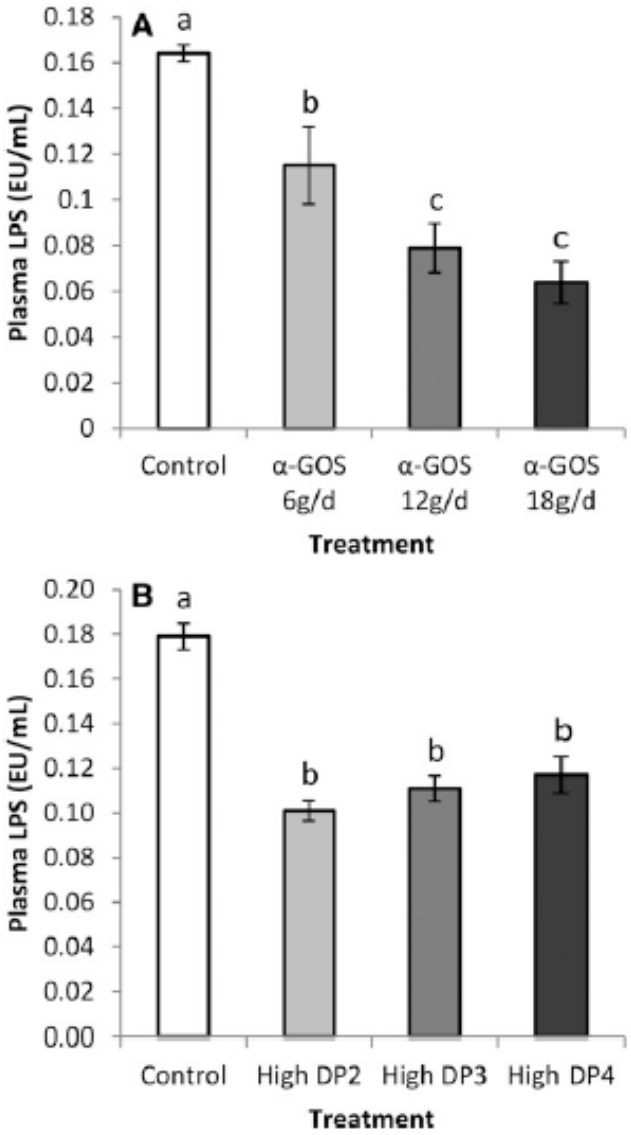


Figure 7. Plasma LPS Concentrations in Overweight Adults After 14 d of AlphaGOS® Consumption in Morel et al., 2015

(A) Dose effect. (B) Formulation effect. Groups consumed 0, 6, 12, or 18 g/d of a mix of alpha-GOS (n = 22/group) and 0 (control, n = 21) or 12 g AlphaGOS®/d with >98% melbiose (High DP2, n = 22), >90% manninotriose (High DP3, n = 21), or >80% of verbascotetraose (High DP4, n = 22) in alpha-GOS content. Values are means ± SEMs. Labeled means without a common letter differ, $P < 0.05$. DP, degree of polymerization.

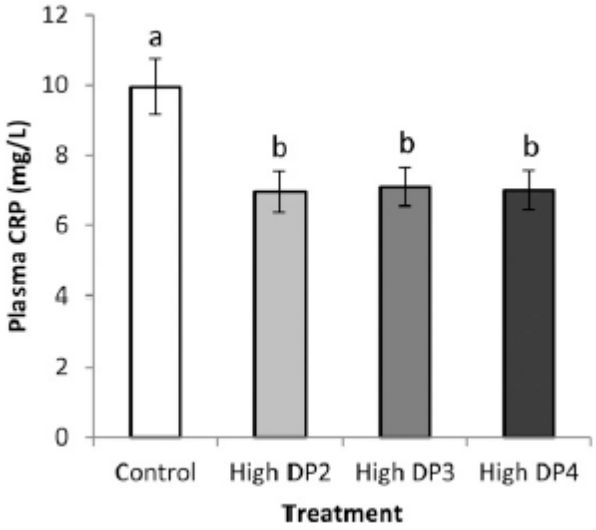


Figure 8. Plasma CRP Concentrations in Overweight Adults After 14 d of Consumption of 12 g/d of Different Formulations of AlphaGOS® in Morel et al., 2015

Groups consumed 0 (control, n = 21) or 12 g AlphaGOS® /d with >98% melbiose (High DP2, n = 22), >90% manninotriose (High DP3, n = 21), or >80% of verbascotetraose (High DP4, n = 22) in alpha-GOS content. Values are means 6 SEMs. Labeled means without a common letter differ, P > 0.05. CRP, C-reactive protein; DP, degree of polymerization.

Anthropometric variables. In both studies, there was no significant difference among groups in anthropometric measurements at baseline and post intervention. However, in the dose-effect study, changes in body weight, BMI, and body fat mass from baseline showed significant pairwise differences between AlphaGOS® supplementation in the 12 and 18 g/day groups vs. the control group (Table 34). Significant pairwise differences were also observed for changes in the waist-to-hip ratio between the group who consumed 18 g AlphaGOS®/day and the control group. A significant negative correlation was detected between changes in body weight, BMI, waist-to-hip ratio, body fat percentage, and body fat mass from baseline and AlphaGOS® dose amounts (all $P < 0.05$). In the formulation-effect study, the AlphaGOS® formulas with high DP2, DP3, or DP4 did not significantly affect anthropometric variables (body weight change: 0.02 ± 1.15, 0.07 ± 1.22, and –0.06 ± 1.17 kg for the high-DP2, -DP3, and -DP4 groups, respectively, vs. 0.35 ± 1.14 kg for the control group; $P > 0.05$).

Table 34. Changes in Anthropometric Variables in Overweight Adults after Consumption of 6, 12, or 18 g AlphaGOS® /day for 14 days in Morel et al., 2015

	Anthropometric variables, Δ day 15 – day 0			
		AlphaGOS®		
	Control	6 g/day	12 g/day	18 g/day
Weight, kg	0.04 ± 0.16[a]	-0.06 ± 0.15 [a,b]	-0.11 ± 0.17 [b,c]	-0.21 ± 0.24[c]
BMI, kg/m^2	0.01 ± 0.38[a]	-0.01 ± 0.06 [a,b]	-0.1 ± 0.27[b]	-0.08 ± 0.09[b]
Waist circumference, cm	0.05 ± 0.38	0 ± 0.44	-0.09 ± 0.29	-0.18 ± 0.5
Hip circumference, cm	-0.05 ± 0.2	0 ± 0.31	-0.05 ± 0.21	0.05 ± 0.21
Waist-to-hip ratio	0.0009 ± 0.004[a]	-0.00001 ± 0.005 [a,b]	-0.0005 ± 0.002 [a,b]	-0.0023 ± 0.005[b]
Body fat, %	0.1 ± 0.56	-0.06 ± 0.12	-0.07 ± 0.13	-0.08 ± 0.19
Body fat mass, kg	0.09 ± 0.43[a]	-0.06 ± 0.10 [a,b]	-0.08 ± 0.11[b]	-0.11 ± 0.16[b]
Fat-free mass, kg	-0.05 ± 0.46	-0.01 ± 0.11	-0.04 ± 0.13	-0.1 ± 0.17
Values are means ± SDs, n=22. Labeled means without a common letter differ, $P < 0.05$. Δ, change.				

Gut microbiota. There was no significant difference among groups in the amount of fecal bifidobacteria at day 0. After intervention, fecal bifidobacteria amounts, as assessed by bifidobacterial-specific DNA detection, were significantly higher for all AlphaGOS® formulas vs. the control group, whereas there were no differences detected among the AlphaGOS® formula groups (Figure 9A). Moreover, no significant difference was observed for total fecal bacteria among the three AlphaGOS® formulation groups and controls at baseline and postintervention (Figure 9B).

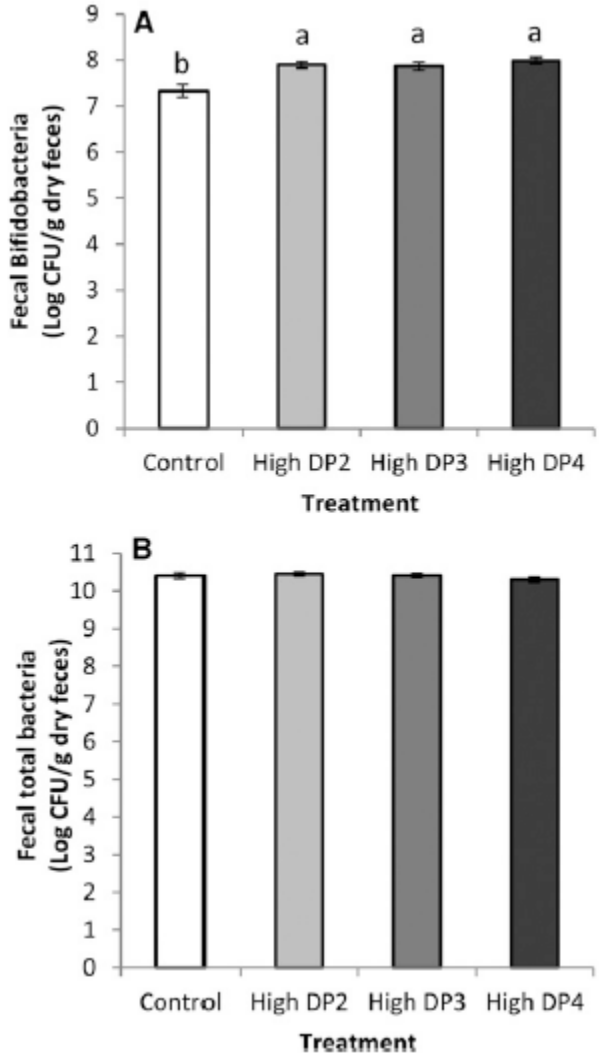


Figure 9. Change in Fecal Bifidobacteria (A) and Total Bacteria (B) from Baseline After 15 d of Consumption of 12 g/d of Different Formulations of AlphaGOS® in Overweight Adults in Morel et al., 2015

Groups consumed 12 g AlphaGOS®/d with >98% melibiose (High DP2), >90% manninotriose (High DP3), of >80% verbascotetraose (High DP4). Values are means ± SEMs, n = 22. Labeled means without a common letter differ, $P < 0.05$. DP, degree of polymerization.

Tolerance. None of the subjects experienced abdominal pain throughout the studies. Borborygmi, bloating scores, and stool consistency did not significantly differ between days 0 and 15 in either of the studies. In the high-DP3 and -DP4 groups, the flatulence score was slightly increased compared with that in the control group. In the formulation-effect study, stool frequency was increased in all AlphaGOS® groups compared with that in the control group.

a) Unpublished Studies

Three unpublished clinical trials of AlphaGOS® in healthy adults corroborate the safety of AlphaGOS® ingestion. The doses of AlphaGOS® used in these studies are well outside the proposed EDI of AlphaGOS® (65 mg/kg/day for the mean and 128 mg/kg/day for the 90th percentile consumers). Two of the three unpublished clinical trials had no adverse events following a single dose of AlphaGOS® (45 g or 33 g liquid).

The third clinical study had multiple doses of 20 or 30 g AlphaGOS® over the course of 46 days with a seven day washout period. These subjects reported adverse abdominal events following consumption of 30 g of AlphaGOS®, including constipation. Although abdominal events occurred in the third unpublished study, these events are unlikely to occur in mean or 90th percentile consumers of AlphaGOS®. The 90th percentile consumer of AlphaGOS® will consume approximately 25% of the highest dose used in the study. No abdominal or gastrointestinal adverse events were observed in the 20 g group. No serious adverse events occurred (see Table 35 for more details).

1) Randomized, Double-blind, Placebo-controlled Clinical Study (2009)

An unpublished study (2009) evaluated the acute tolerance associated with the intake of several prebiotic formulations through a randomized, double-blind, placebo-controlled clinical trial. One hundred twenty healthy men and women 20-40 years old were randomly distributed into six groups (n = 20/group). Following baseline assessment, subjects were administered a single dose of placebo (34 g glucose control; Group 1), Synergy 1 (22 g inulin-oligofructose; Group 2), GOS 550 (45 g galacto-oligosaccharide; Group 3), soybean oligosaccharide (23 g, Group 4), hydrolyzed soybean oligosaccharide (35 g, Group 5), or raffinose (20 g, Group 6) and monitored for 24 hours. Acute tolerance was determined according to the Acute Tolerance Questionnaire and the Gastro-intestinal Tolerability Questionnaire. These questionnaires were completed at baseline and at 24 hours. Baseline age, % female subjects, and frequency of gastrointestinal symptoms did not differ among the groups. No subjects withdrew from the study, and no adverse events were reported in any group. Groups 2-6 reported increased gastrointestinal symptoms versus placebo (Group 1), but these remained “very infrequent” overall. These findings support the safety and tolerability of the test articles under the conditions employed (Table 35).

2) Prospective, Controlled, Crossover, Open-Label, Randomized Clinical Study (2011)

In a prospective, controlled, crossover, open-label, randomized clinical study (2011), the glycemic and insulinemic responses to AlphaGOS® were examined in 10 healthy male and female volunteers. The test article consisted of 33 g of AlphaGOS® syrup (containing 25 g

carbohydrate), and the control solution consisted of 25 g glucose. Each subject was seen 5 times: 1) baseline measurements and 2-hour, 75 g oral glucose tolerance test; 2) control solution; 3) control or test solution; 4) test or control solution (whichever was not administered during session 3); and 5) control solution. Subjects fasted for 12 h before each session. Blood was taken 15, 30 60, 90, and 120 minutes after administration of the appropriate solution. AlphaGOS® ingestion had no impact on blood pressure or heart rate. Subjects reported one case of diarrhea and one case of stomach ache, but no serious adverse events were reported. The control solution produced a characteristic kinetic response for blood glucose and insulin, while AlphaGOS® produced no change in either parameter. The glycemic index for AlphaGOS® was 7.1 ± 4.21. The insulinemic index was 1.0 ± 0.51. Overall, AlphaGOS® administration did not result in a glycemic or insulinemic response (Table 35). These data are consistent with the fact that AlphaGOS® is a non-digestible carbohydrate.

3) Randomized, Double-blind, Placebo-controlled, Cross-over Study (2013)

An unpublished randomized, double-blind, placebo-controlled, cross-over study (2013) compared the gastrointestinal tolerance of AlphaGOS® to isomalt or placebo. Thirty-four healthy male and females 18-45 years old were randomized into five groups; each group consumed the 5 treatments in a random order. The treatments were placebo (30 g), AlphaGOS® low dose (20 g + 10 g placebo), AlphaGOS® high dose (30 g), isomalt low dose (20 g + 10 g placebo), and isomalt high dose (30 g) dissolved in sugar-free fresh tea. Subjects were screened on day 0, then test articles were administered on days 14, 22, 30, 38, and 46. Subjects received a single dose of the test article diluted in 200 mL of a flavored, non-carbonated beverage 2 hours after a normal breakfast. Gastrointestinal symptoms were monitored for 24 hours after product administration.

A total of six subjects withdrew from the trial: two following adverse events (acute nasopharyngitis, menstrual pain, headache, stomach acidity) and four due to non-compliance. Both AlphaGOS® and isomalt significantly increased the frequency and intensity of abdominal bloating, borborygmi, flatulence, and abdominal pain versus the placebo. For both products, the incidence of abdominal symptoms was greater at the high dose. Twenty-eight subjects reported a total of 51 adverse events. These included respiratory events (cold, fever, asthma attack, sore throat, nasal congestion), GI events outside of those included in the protocol (diarrhea, stomach acidity, constipation), headache/migraine, and others (menstrual pain, fungal infection, back pain, knee pain, toothache, and ferropenic anemia). Constipation was considered an effect of the study protocol (limited dietary fiber intake); the other adverse events were not considered related to the test articles. No serious adverse events occurred (Table 35).

Table 35. Unpublished Corroborative AlphaGOS® Clinical Trials

Year Study Performed	Study Type	Primary Objective	AlphaGOS® exposure	Subjects	Outcomes	Adverse Events (Number reported)
2009	Randomized, double-blind, placebo-controlled	Study intestinal acute tolerance of several food ingredients, including AlphaGOS®	Single dose liquid AlphaGOS®, 45 g, monitored for 24 hours	120 healthy men and women aged 20-40, n=20/group	No statistically significant differences vs. placebo on bloating, borboryghmi, abdominal pain Higher flatulence/return to placebo levels after 8 days	No adverse events reported
2011	Prospective, Controlled, Crossover, Open-Label, Randomized	Determine the glycemic and insulinaemic response of AlphaGOS®	33 g liquid AlphaGOS®, monitored for 2 hours	10 healthy men and women, aged 18-65	AlphaGOS® produced no change blood glucose or insulin. The glycemic index for AlphaGOS® was 7.1 ± 4.21. The insulinemic index was 1.0 ± 0.51.	No adverse event was linked to AlphaGOS® intake.
2013	Phase IV, monocentric, cross-over, double-blind, randomized, placebo-controlled	Study gastrointestinal tolerance of AlphaGOS® compared to another non-digestible fiber, Isomalt.	Single dose 20g or 30g dried AlphaGOS® on days 14, 22, 30, 38 and 46, followed by a 7 days washout period	34 healthy men and women aged 18-45, 25 subjects completed the trial	No difference in gastrointestinal distress when comparing either dose of AlphaGOS® to another nondigestible fiber, Isomalt. Gastrointestinal distress was higher in both AlphaGOS® and Isomalt compared to placebo control.	Respiratory events, including cold, fever, asthma attack, sore throat, and nasal congestion. (n = 12) Gastrointestinal events not described in the protocol including diarrhea, stomach acidity, and constipation. (n = 8) Headache/migraine (n = 16) Other adverse events including menstrual pain, fungal infection, back pain, knee pain, toothache and ferropenic anemia (n = 15)

2. Alpha-Linked Galacto-Oligosaccharides

a. Summary

Four clinical studies have examined the effects of raffinose and stachyose, alpha-linked oligosaccharides (Benno et al., 1987; Hayakawa et al., 1990; Kapadia et al., 1995; Fujisaki et al., 1994; Table 36). The study published by Fujisaki et al. (1994) was in Japanese with only the abstract was available in English. Although these studies focused mostly on the effect of dietary soy-derived stachyose and raffinose on gut microbiota and other gastrointestinal parameters in humans, there were no reports of serious adverse events.

Kapadia et al., (1995) studied the effect of soy oligosaccharides on transit time, stool weight, and bowel movement frequency in seven healthy male and female subjects who consumed a diet supplemented with 150 g soy oligosaccharides for 4 to 7 days. There was no effect transit time, stool weight, or bowel movement frequency and no adverse events were reported. A soybean oligosaccharide extract providing 3.0 g of alpha-GOS (2.3 g stachyose and 0.7 g raffinose) per day was consumed by six healthy male adults for 3 weeks with no adverse events reported (Hayakawa et al., 1990). Supplementation with 15 g raffinose/day for 4 weeks induced diarrhea in 3 of 7 male and female volunteers in another study (Benno et al., 1987). Fujisaki and colleagues studied the effects of 3, 4 or 5 g raffinose/day on bifidobacteria. Ingestion of raffinose increased bifidobacterial but did not affect the concentration of organic acids (Fujisaki et al., 1994).

Table 36. Clinical Studies of Alpha-linked Galacto-Oligosaccharides in Humans

Reference	Study Design	Subjects	Treatment and Numbers of Subjects	Intake and Duration	Results
Benno et al., 1987 Aim: To assess the effects of raffinose intake on human fecal microflora	Dietary intervention, not blinded or randomized	7 healthy adults 5 males, 2 females 26-37 y	Group 1: 15 g raffinose/d after usual diet	4 wks	• Three subjects had diarrhea after onset of treatment • Increased Bifidobacteria counts and percentage of *Bifidobacteria spp.* in stool • Lower Clostridium counts and percentage of *Clostridium* spp. in stool
Hayakawa et al., 1990 Aim: To assess the effects of soybean oligosaccharides on human feacal microflora	Cross-over Study, no information on randomization or blinding	6 healthy males 28-48 y	Group 1: control diet Group 2: 10 g soybean oligosaccharide extract 2.3 g stachyose, 0.7 g raffinose per day Group 2: 10 g soybean oligosaccharide extract 2.3 g stachyose, 0.7 g raffinose per day + *B. longum* ($6x10^9$ cfu)	3 wks	• No adverse events reported • Increased Bifidobacterium counts
Fujisaki et al., 1994 (Abstract only available in English, publication in Japanese)	No information	10 healthy subjects	3, 4 or 5 g raffinose/day	Not reported	• Organic acids and putrefactive products were not changed • Increased Bifidobacteria • Decreased branched chain fatty acids • No adverse events reported
Kapadia et al., 1995 Aim: To assess the influence of fiber supplemented diets on bowel function and short chain fatty acid production	Randomized, cross-over study	11 healthy subjects (no age specified)	Group 1 (Control Group): self-selected diet Group 2: fiber free diet Group 3: 15g total dietary fiber/L from oat fiber Group 4: 15 g total dietary fiber/L from soy oligosaccharide fiber Group 5: 15 g total dietary fiber/L from soy polysaccharide fiber	4-7 d	• No adverse events reported • No effect on bowel movement and transit time • Increased butyrate and total short chain fatty acids

b. Other Studies with Alpha-Linked Galactoligosaccharides

An in vitro study was performed using the microbiome from two healthy human volunteers to understand the digestion and fermentation of alpha-GOS in the adult human gut (Poeker et al., 2017).

A three continuous *in vitro* fermentation PolyFermS model was used to study the modulating effect of dietary fibers, including alpha-GOS, on two distinct human adult proximal colon microbiota. Supplementation of dietary fibers, equivalent to a 9 g daily intake, induced a consistent metabolic response depending on the donor microbiota. Irrespective to the dietary fiber supplemented, the *Bacteroidaceae-Ruminococcaceae* dominated microbiota produced more butyrate (up to 96%), while the *Prevotellaceae-Ruminococcaceae* dominated microbiota produced more propionate (up to 40%). These results further demonstrate the alpha-GOS, including the saccharides found in AlphaGOS® are fermented by the human microbiome.

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).

1. Summary

Recent reports in the literature describe an allergy to red meat triggered by exposure to galactose-α1,3-galactose (an alpha linked oligosacharride, referred to as alpha-gal) through tick bites (Commins et al., 2013; Steinke et al., 2015; Hilger et al., 2019). AlphaGOS® is unlikely to elicit a red meat allergy due to the differences in linkages in the oligosaccharides. Furthermore, there are no known reports of allergies to galactose-α-1,6 linkages.

AlphaGOS® is unlikely to cause an IgE-mediated immune response and is free of known allergens. To rule out the possibility that AlphaGOS® is allergenic, the allergenic potential was assessed through immunoblotting and ELISA inhibition. The ELISA inhibition experiments were performed with pea protein extract, isolated from dun pea (*Pisum sativum* arvense). The results of these two assays demonstrated the absence of proteins and dun pea allergens in AlphaGOS®.

2. Residual Allergenicity of AlphaGOS® Compared to Dun Pea Protein

The allergenic potential of AlphaGOS® was assessed through immunoblotting for proteins (potential allergens) and ELISA inhibition. In the immunoblotting assay, three batches of AlphaGOS® were submitted to gel electrophoresis under reducing and denaturing conditions and stained for protein with Coomassie blue. No protein was detected in any of the three batches of AlphaGOS® (Figure 10).

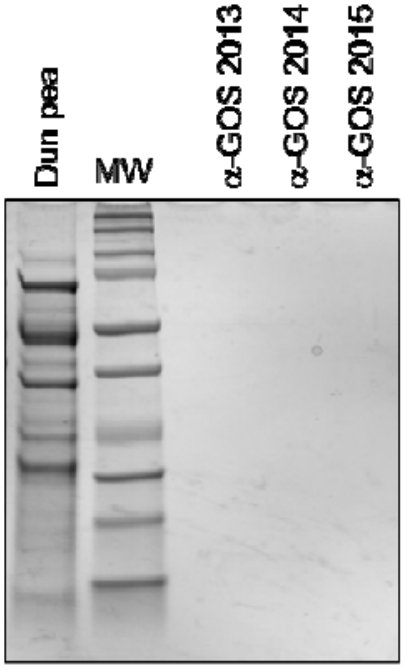


Figure 10. Electrophoresis and Coomassie Blue Staining of AlphaGOS® Solutions

Lane 1: dun pea protein, positive control, Lane 2: MW, molecular weight marker, Lanes 3-5: three different batches of AlphaGOS® . Each lane is loaded with 1 mg/mL weight/volume AlphaGOS®.

To search for allergens in AlphaGOS® solutions, an immunoblot using serum from a dun pea allergic patient was performed. The trace of proteins present in AlphaGOS® solutions were first separated by electrophoresis and then subjected to immunoblotting using serum containing specific IgE to dun pea. A protein extract prepared from dun pea was used as a positive control. Although the serum was able to detect allergens with different level of reactivity in the dun pea extract, there were no identified allergens in the AlphaGOS® solutions (Figure 11).

Figure 11. Immunoblot of AlphaGOS® Solutions Using Serum from an Allergic Patient

Since the results of the immunoblotting assays did not detect allergens, a more sensitive ELISA inhibition assay was performed (Richard et al., 2015). In the ELISA experiments, dun pea proteins were coated in wells and used as baits to detect specific IgE to pea in serum. In the ELISA inhibition, serum was pre-incubated with dun pea extract as a positive control or AlphaGOS® . If dun pea allergens were present in the sera, specific IgE detected would be lower than those measured in the absence of inhibitors. The detection decrease is proportional to the amount of inhibitor present in preincubation solutions (Figure 12).

Fifty-three percent inhibition was observed with 0.3 ppm of dun pea proteins and 8 ppm increased to approximately 81% inhibition, indicating that the dub pea-specific IgE was present in the serum of dun pea allergic individuals (Figure 12).

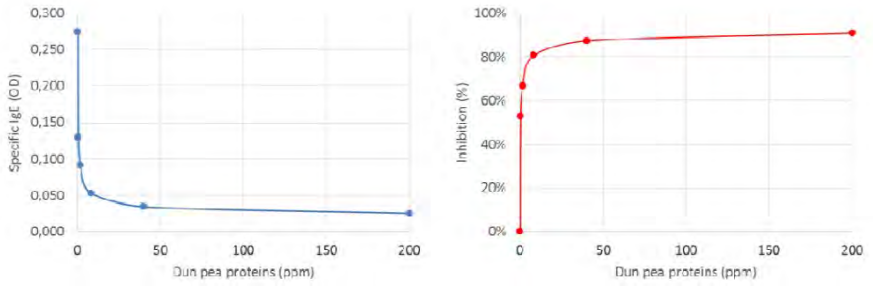


Figure 12. ELISA Inhibition with Dun Pea Extract

Left, ELISA results when incubated with increasing amounts of dun pea proteins. Right, % inhibition calculated from ELISA results.

Dun pea proteins, either 0.3 ppm or 40 ppm were added to AlphaGOS® solution and the level of inhibition was measured using allergic serum (Figure 13). If dun pea allergens are present in AlphaGOS®, there would be an increase in inhibition. The levels of inhibition were similar between the three AlphaGOS® solutions as well as compared to standard conditions. The results suggested that dun pea allergens were not detectable in AlphaGOS® solutions. Given the amounts of proteins tested here, all three AlphaGOS® solutions contain less than 0.3 ppm of pea allergens, the limit of detection for the assay.

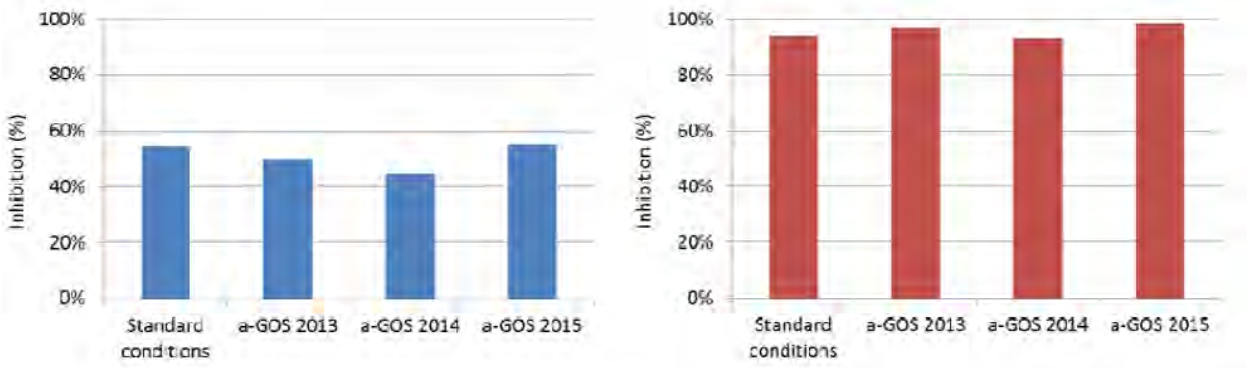


Figure 13. ELISA Inhibitions with AlphaGOS® Solutions Spiked with 0.3 ppm (left) or 40 ppm (right) Dun Pea Proteins

F. REGULATORY APPROVALS ACROSS THE WORLD

Currently AlphaGOS® is considered a non-digestible carbohydrate in the European Union. In Canada, it is regulated as a standard foodstuff by the Food and Drugs Act. Alpha-linked oligosaccharides like raffinose are found in breads (wheat, rye and spelt) and muesli from about 20-38 mg/100g depending on the foods (Biesiekierski et al., 2011). Raffinose and stachyose (both alpha-GOS) are the main short chain carbohydrates in legumes, particularly lima and red beans and lentils and chick peas (Biesiekierski et al., 2011) and are found in significant quantities in Jerusalem artichokes (Muir et al., 2009).

VII. SUPPORTING DATA AND INFORMATION

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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 AlphaGOS®. AlphaGOS® 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 AlphaGOS® 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 uses of AlphaGOS® as an ingredient for the intended uses in foods and beverages, as listed above, has been determined to be safe through scientific procedures set forth under 21 CFR §170.30(b) based on the following:

1. AlphaGOS® is an alpha-linked galacto-oligosaccharide (alpha-GOS) manufactured using pea solubles derived from *Pisum sativum*.

2. All raw materials and processing aids used to produce AlphaGOS® comply with appropriate US federal regulations. The production process takes place in a facility operating under Good Manufacturing Practice (GMP) and is controlled to ensure a consistently reproducible product, free of known contaminants.

3. AlphaGOS® consists of 1 to 3 glucose units linked via α(1→6) glycosidic bonds to a terminal galactose and is a non-digestible oligosaccharide.

    a. Alpha-GOS pass 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, similar to other fermentation products following the ingestion of other non-digestible materials.

    b. Alpha-GOS is naturally present in certain foods, such as legumes, from which AlphaGOS® is derived. Raffinose and stachyose (both alpha-GOS) are the main short chain carbohydrates in legumes, particularly lima beans, red beans, lentils and chick peas, and found in other foods like Jerusalem artichokes.

    c. AlphaGOS® has a history of consumption in many countries including France, Germany, United Kingdom, Italy, Netherlands, Canada, Spain, Czech Republic, South Korea, and Australia.

4. The pivotal toxicology studies supporting the GRAS status of AlphaGOS® are a 90-day subchronic toxicity study in rats, an Ames test and an in vitro chromosome

aberration study and a 3-week neonatal piglet study (Kruger et al., 2017a; Kruger et al., 2017b).

a. AlphaGOS® was not genotoxic in an Ames test or an in vitro chromosome aberration study

b. The subchronic toxicity study established a no observed adverse effect level (NOAEL) of at least 2000 mg AlphaGOS®/kg body weight/day, the highest dose tested.

5. Safety and tolerance in an infant population was addressed in a neonatal piglet study using piglet formula supplemented with 8 mg/mL (8 g/L) of AlphaGOS®.

6. Two published clinical trials of AlphaGOS® (Morel et al. 2015) reported that AlphaGOS® is well tolerated in human subjects with no test-article related adverse events recorded at levels up to 12 g/day.

Therefore, AlphaGOS® is safe and GRAS at the proposed levels of addition to the intended foods and beverages and infant formulas listed in this GRAS document. AlphaGOS® is 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. Together, these results confirm safety of the proposed EDI of AlphaGOS® in foods and beverages of 65 and 128 mg/kg/day or 4.3 g/day and 8.525 g/day for the mean and 90th percentile consumers (age 2 and up) and the safety of intake from infant formulas at a level of addition of 8 g/L.

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

Signature: [redacted]
Date: November 13, 2019

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

Signature: [redacted]
Date: November 13, 2019

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

Signature: [redacted]
Date: November 13, 2019

Claire Kruger, PhD, DABT
Scientific Advisor to the Panel
Spherix Consulting Group, Inc.

Signature: [redacted]
Date: November 13, 2019

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

GENERALLY RECOGNIZED AS SAFE *(GRAS)* NOTICE (Subpart E of Part 170)

Form Approved: OMB No. 0910-0342; Expiration Date: 09/30/2019
(See last page for OMB Statement)

FDA USE ONLY

GRN NUMBER	DATE OF RECEIPT
896	Nov 15, 2019
ESTIMATED DAILY INTAKE	INTENDED USE FOR INTERNET
NAME FOR INTERNET	
KEYWORDS	

Transmit completed form and attachments electronically via the Electronic Submission Gateway *(see Instructions)*; OR Transmit completed form and attachments in paper format or on physical media to: Office of Food Additive Safety *(HFS-200)*, Center for Food Safety and Applied Nutrition, Food and Drug Administration,5001 Campus Drive, College Park, MD 20740-3835.

SECTION A – INTRODUCTORY INFORMATION ABOUT THE SUBMISSION

1. Type of Submission *(Check one)*

☒ New ☐ Amendment to GRN No. _____ ☐ Supplement to GRN No. _____

2. ☒ All electronic files included in this submission have been checked and found to be virus free. *(Check box to verify)*

3 Most recent presubmission meeting *(if any)* with FDA on the subject substance *(yyyy/mm/dd):* _____

4 For Amendments or Supplements: Is your amendment or supplement submitted in response to a communication from FDA? *(Check one)* ☐ Yes ☐ No If yes, enter the date of communication *(yyyy/mm/dd):* _____

SECTION B – INFORMATION ABOUT THE NOTIFIER

1a. Notifier

Name of Contact Person	Position or Title
Francois Delbaere	CEO
Organization *(if applicable)*	
Olygose	
Mailing Address *(number and street)*	
Parc Technologique des Rives de l'Oise	

City	State or Province	Zip Code/Postal Code	Country
BP 50149	Compiegne Cedex	F-60201	France

Telephone Number	Fax Number	E-Mail Address
+33 (0)3 44 90 78 13		francois.delbaere@olygose.com

1b. Agent or Attorney *(if applicable)*

Name of Contact Person	Position or Title
Claire Kruger	Managing Partner
Organization *(if applicable)*	
Spherix Consulting Group, Inc.	
Mailing Address *(number and street)*	
11821 Parklawn Drive	

City	State or Province	Zip Code/Postal Code	Country
Rockville	Maryland	20852	United States of America

Telephone Number	Fax Number	E-Mail Address
301-775-9476		ckruger@spherixgroup.com

RECEIVED
NOV 18 2019
OFFICE OF
FOOD ADDITIVE SAFETY

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

GENERALLY RECOGNIZED AS SAFE *(GRAS)* NOTICE (Subpart E of Part 170)

Form Approved: OMB No. 0910-0342; Expiration Date: 09/30/2019
(See last page for OMB Statement)

FDA USE ONLY

GRN NUMBER 000896	DATE OF RECEIPT
ESTIMATED DAILY INTAKE	INTENDED USE FOR INTERNET
NAME FOR INTERNET	
KEYWORDS	

Transmit completed form and attachments electronically via the Electronic Submission Gateway *(see Instructions)*; OR Transmit completed form and attachments in paper format or on physical media to: Office of Food Additive Safety *(HFS-200)*, Center for Food Safety and Applied Nutrition, Food and Drug Administration,5001 Campus Drive, College Park, MD 20740-3835.

SECTION A – INTRODUCTORY INFORMATION ABOUT THE SUBMISSION

1. Type of Submission *(Check one)*
☒ New ☐ Amendment to GRN No._____ ☐ Supplement to GRN No. _____

2. ☒ All electronic files included in this submission have been checked and found to be virus free. *(Check box to verify)*

3 Most recent presubmission meeting *(if any)* with FDA on the subject substance *(yyyy/mm/dd)*: _____

4 For Amendments or Supplements: Is your amendment or supplement submitted in response to a communication from FDA? *(Check one)* ☐ Yes ☐ No If yes, enter the date of communication *(yyyy/mm/dd)*: _____

SECTION B – INFORMATION ABOUT THE NOTIFIER

1a. Notifier

Name of Contact Person	Position or Title
Francois Delbaere	CEO
Organization *(if applicable)*	
Olygose	
Mailing Address *(number and street)*	
Parc Technologique des Rives de l'Oise	

City	State or Province	Zip Code/Postal Code	Country
BP 50149	Compiegne Cedex	F-60201	France

Telephone Number	Fax Number	E-Mail Address
+33 (0)3 44 90 78 13		francois.delbaere@olygose.com

1b. Agent or Attorney *(if applicable)*

Name of Contact Person	Position or Title
Claire Kruger	Managing Partner
Organization *(if applicable)*	
Spherix Consulting Group, Inc.	
Mailing Address *(number and street)*	
11821 Parklawn Drive	

City	State or Province	Zip Code/Postal Code	Country
Rockville	Maryland	20852	United States of America

Telephone Number	Fax Number	E-Mail Address
301-775-9476		ckruger@spherixgroup.com

SECTION C – GENERAL ADMINISTRATIVE INFORMATION

1. Name of notified substance, using an appropriately descriptive term

Alpha-Galacto-Oligosaccharides (AlphaGos®)

2. Submission Format: *(Check appropriate box(es))*

☐ Electronic Submission Gateway

☒ Electronic files on physical media

☒ Paper

If applicable give number and type of physical media

1 CD

3. For paper submissions only:

Number of volumes _____

Total number of pages _____

4. Does this submission incorporate any information in CFSAN's files? *(Check one)*

☐ Yes *(Proceed to Item 5)* ☒ No *(Proceed to Item 6)*

5. The submission incorporates information from a previous submission to FDA as indicated below *(Check all that apply)*

☐ a) GRAS Notice No. GRN _____

☐ b) GRAS Affirmation Petition No. GRP _____

☐ c) Food Additive Petition No. FAP _____

☐ d) Food Master File No. FMF _____

☐ e) Other or Additional *(describe or enter information as above)* _____

6. Statutory basis for conclusions of GRAS status *(Check one)*

☒ Scientific procedures *(21 CFR 170.30(a) and (b))* ☐ Experience based on common use in food *(21 CFR 170.30(a) and (c))*

7. Does the submission (including information that you are incorporating) contain information that you view as trade secret or as confidential commercial or financial information? (see 21 CFR 170.225(c)(8))

☐ Yes *(Proceed to Item 8*

☒ No *(Proceed to Section D)*

8. Have you designated information in your submission that you view as trade secret or as confidential commercial or financial information *(Check all that apply)*

☐ Yes, information is designated at the place where it occurs in the submission

☐ No

9. Have you attached a redacted copy of some or all of the submission? *(Check one)*

☐ Yes, a redacted copy of the complete submission

☐ Yes, a redacted copy of part(s) of the submission

☐ No

SECTION D – INTENDED USE

1. Describe the intended conditions of use of the notified substance, including the foods in which the substance will be used, the levels of use in such foods, and the purposes for which the substance will be used, including, when appropriate, a description of a subpopulation expected to consume the notified substance.

AlphaGOS® will be added to beverages (carbonated/non-carbonated, juice, flavored water), ready to drink iced coffee and teas, sports drinks, energy drinks, soups, meal-replacement drinks, processed fruits and vegetable juices, dairy products analogs, dairy and analogs, sugars and sweets, coffee and tea, non-exempt infant formulas, baby cereals, baby foods, and toddler foods.

2. Does the intended use of the notified substance include any use in product(s) subject to regulation by the Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture?

(Check one)

☐ Yes ☒ No

3. If your submission contains trade secrets, do you authorize FDA to provide this information to the Food Safety and Inspection Service of the U.S. Department of Agriculture?

(Check one)

☐ Yes ☐ No , you ask us to exclude trade secrets from the information FDA will send to FSIS.

SECTION E – PARTS 2 -7 OF YOUR GRAS NOTICE

(check list to help ensure your submission is complete – PART 1 is addressed in other sections of this form)

☒ PART 2 of a GRAS notice: Identity, method of manufacture, specifications, and physical or technical effect (170.230).

☒ PART 3 of a GRAS notice: Dietary exposure (170.235).

☒ PART 4 of a GRAS notice: Self-limiting levels of use (170.240).

☒ PART 5 of a GRAS notice: Experience based on common use in foods before 1958 (170.245).

☒ PART 6 of a GRAS notice: Narrative (170.250).

☒ PART 7 of a GRAS notice: List of supporting data and information in your GRAS notice (170.255)

Other Information

Did you include any other information that you want FDA to consider in evaluating your GRAS notice?

☐ Yes ☒ No

Did you include this other information in the list of attachments?

☐ Yes ☐ No

SECTION F – SIGNATURE AND CERTIFICATION STATEMENTS

1. The undersigned is informing FDA that Francois Delbaere
(name of notifier)

has concluded that the intended use*(s)* of Alpha-Galacto-Oligosaccharides (AlphaGos®)
(name of notified substance)

described on this form, as discussed in the attached notice, is (are) not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on your conclusion that the substance is generally recognized as safe recognized as safe under the conditions of its intended use in accordance with § 170.30.

2. Olygose agrees to make the data and information that are the basis for the conclusion of GRAS status available to FDA if FDA asks to see them; agrees to allow FDA to review and copy these data and information during customary business hours at the following location if FDA asks to do so; agrees to send these data and information to FDA if FDA asks to do so.
(name of notifier)

Parc Technologique des Rives de l'Oise, BP 50149, F-60201 Compiegne Cedex, France
(address of notifier or other location)

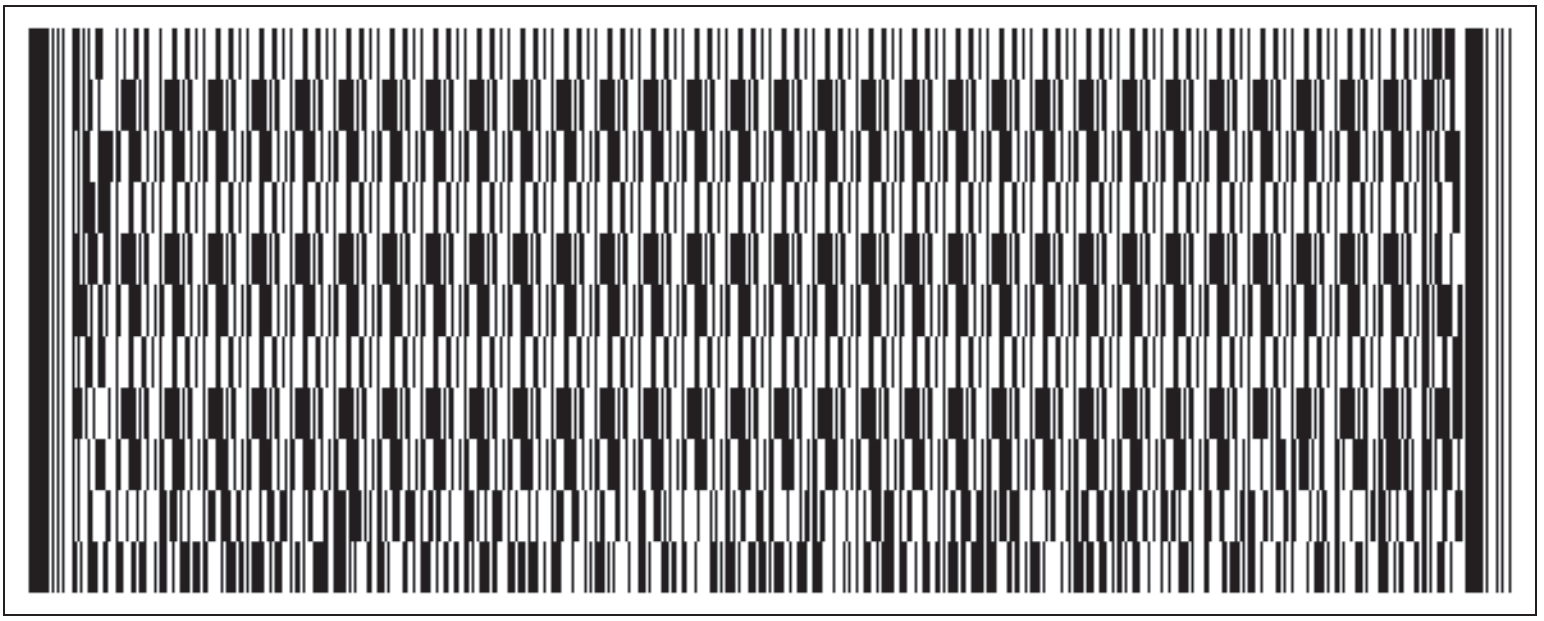
The notifying party certifies that this GRAS notice is a complete, representative, and balanced submission that includes unfavorable, as well as favorable information, pertinent to the evaluation of the safety and GRAS status of the use of the substance.The notifying party certifies that the information provided herein is accurate and complete to the best or his/her knowledge. Any knowing and willful misinterpretation is subject to criminal penalty pursuant to 18 U.S.C. 1001.

3. Signature of Responsible Official, Agent, or Attorney	**Printed Name and Title**	**Date (mm/dd/yyyy)**
Claire L. Kruger, PhD Digitally signed by Claire L. Kruger, PhD Date: 2019.11.15 09:19:59 -05'00'	Claire Kruger, Managing Partner, Spherix Consulting	11/15/2019

SECTION G – LIST OF ATTACHMENTS

List your attached files or documents containing your submission, forms, amendments or supplements, and other pertinent information. Clearly identify the attachment with appropriate descriptive file names (or titles for paper documents), preferably as suggested in the guidance associated with this form. Number your attachments consecutively. When submitting paper documents, enter the inclusive page numbers of each portion of the document below.

Attachment Number	Attachment Name	Folder Location (select from menu) (Page Number(s) for paper Copy Only)
	Olygose AlphaGOS GRAS Dossier Final 11-13-19.pdf	Submission
	All References	Submission



Viebrock, Lauren

From: kbrailer@spherixgroup.com
Sent: Wednesday, April 29, 2020 5:18 PM
To: Viebrock, Lauren
Cc: ckruger@spherixgroup.com; 'Dietrich Conze'; 'Jennifer Symonds'
Subject: FW: Question regarding GRN 000896
Attachments: Chen 2013.pdf; Response to FDA Questions on GRN896 4-29-20.pdf; Wu 2007.pdf

Dear Dr. VieBrock,

Attached please find our response to your questions regarding GRN 000896. Should you need any additional information, please don't hesitate to ask.

Best regards,

Kathy Brailer
Director of Administrative Services
Spherix Consulting Group, Inc.
11821 Parklawn Drive, Suite 310
Rockville, MD 20852
+1-301-557-0375
kbrailer@spherixgroup.com
www.spherixgroup.com

From: Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
Sent: Thursday, April 16, 2020 10:14 AM
To: ckruger@spherixgroup.com
Subject: Question regarding GRN 000896

Dear Dr. Kruger,

During our review of GRAS Notice No. 000896, we noted further questions that need to be addressed and are attached to this email.

We respectfully request a response within **10 business days**. If you are unable to complete the response within that time frame, please contact me to discuss further options.

If you have questions or need further clarification, please feel free to contact me. Thank you in advance for your attention to our comments.

Regards,
Lauren

Lauren VieBrock
Consumer Safety Officer/Microbiology Reviewer

Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration
Tel: 301-796-7454
lauren.viebrock@fda.hhs.gov

Spherix Consulting Group

April 29, 2020

Lauren VieBrock, Ph.D.
Consumer Safety Officer/Microbiology Reviewer
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
US Food and Drug Administration
5001 Campus Drive, HFS-225
College Park, MD 20740

RE: Questions Regarding GRN 000896

Dear Dr. VieBrock:

In response to your email of April 16, 2020, following are our responses to your request for additional information regarding GRN 000896. FDA's questions are italicized and our responses are in plain text.

1. *Please provide a specification for Salmonella serovars and batch analysis data to support that manufacturing can meet the specification.*

   Olygose has set a specification of "Negative in 125 g" for *Salmonella spp*, which is assessed by the compendial method, NF EN ISO 6579. *Salmonella spp*. was negative in two batches of AlphaGOS® powder and three batches of AlphaGOS® syrup (Supplementary Table). Tables 3 and 4 in the GRAS Notification, which list the product specifications and batch compliance, have been updated with this new information and are provided below. This information will be added to the GRAS dossier on pages 10-11, Section II.E.1. Product Specifications.

Supplementary Table. *Salmonella spp.* are Not Detected in Batches of AlphaGOS® Syrup and Powder

AlphaGOS® Syrup Batch Number	**2015 01 60**	**2017 01 201**	**2017 09 01**
Salmonella spp.	Negative/125 g[b]	Negative/125 g[b]	Negative/125 g[a]
AlphaGOS® Powder Batch Number	**2018 01 01**		**2018 01 02**
Salmonella spp.	Negative/125 g[b]		Negative/125 g[b]

[a]Method: NF EN ISO 6579(A), performed by Wessling Paris, France.
[b]Method: NF EN ISO 6579-1, performed by Eurofins, Laboratoire Nord Douai, France.

11821 Parklawn Drive, Suite 310
Rockville, MD 20852
301-557-0375; info@spherixgroup.com
http://spherixgroup.html

Table 3. Specifications and Batch Analysis for AlphaGOS® Powder

Parameter	Method	Specification	LOQ	AlphaGOS® Batch		
				2014 009 12	2015 01 01	2017 01 01
Appearance	Visual	White	-	White	White	White
Dry matter (%)	Karl Fisher – Based on ISO 8534	> 95	-	97.0	96.5	96.5
Protein (%)*	Internal method based on Reg CE No. 152/2009 Kjeldhal	< 0.5	0.2	< 0.2	0.20	< 0.2
Ash (%)*	Internal Method Based on Reg CE No. 152/2009	< 0.5	0.1	< 0.2	< 0.1	< 0.2
Alpha-GOS (DP2-DP4 with Gal(n) alpha 1-6 Glc structure) (%)*	Internal method based on AOAC 997.08	96 ± 3	-	96.09	97.76	96.51
Digestible sugars (%)*,†	Internal method based on AOAC 997.08	< 5	0.2	< 0.2	< 0.3	< 0.2
Microbials *(cfu/g unless otherwise stated)*						
Aerobic plate count	NF EN ISO 4833-1 (A)	< 1000 cfu/g	-	500	< 40	<40
Escherichia. coli	NF ISO 16649-2 modified	< 1 cfu/g	-	< 1	< 1	<1
Enterobacteria	NF ISO 21528-2	< 1 cfu/g	-	< 1	< 1	<1
Coliforms	NF ISO 4832	< 1 cfu/g	-	< 1	< 1	<1
Staphylococcus aureus	NF EN ISO 6888-3 (A)	ND in 1 g	-	ND	ND	ND
Pseudomonas aeruginosa	Ph. Eur.2.6.13	ND in 1 g	-	ND	ND	ND
Yeast and Molds	NF V08-059 (A)	≤ 10 cfu/g	-	< 10	< 10	<10
Salmonella spp.	NF EN ISO 6579	Negative in 125 g	-	Negative	Negative	Negative
Additional Microbials for Infant formula ingredients *(cfu/g unless otherwise stated)*						
Cronobacter spp.	ISO TS 22964 (A)	ND in 25 g	-	ND	ND	ND
Bacillus cereus	NF EN ISO 7932 (A)	50-500/g	-	< 10	< 10	< 10
Heavy Metals *(ppm)*						
Arsenic	ISO 11885/ISO 17294-2(A)	≤ 1 ppm	-	0.058	< 0.05	0.061
Cadmium	ISO 11885/ISO 17294-2(A)	<1 ppm	-	< 0.01	< 0.01	< 0.01
Lead	ISO 11885/ISO 17294-2(A)	<1 ppm	-	< 0.02	< 0.02	< 0.02
Mercury	DIN EN 13806; ASU L 00.00-19/4(A)	<1 ppm	-	< 0.005	< 0.005	< 0.005

*Specification as percent of dry matter.
†Digestible sugars are defined as the monosaccharides glucose, galactose and fructose, as well as the disaccharides sucrose and maltose.
Abbreviations: LOQ: limit of quantitation, ISO: International Organization for Standardization, DP: degree of polymerization; Gal: galactose; Glc: glucose, AOAC: Association of Official Analytical Chemists, NF:Norme Française, EN: European Norm, Ph. Eur.: European Pharmacopaeia, cfu: colony forming units, ND: not detected, TS: technical specifications, DIN: Deutsches Institut für Normung (German Institute for Standardization), ASU: Official Collection of Testing Methods according to §64 German Food and Feed Code(LFGB) , ppm: parts per million

Table 4. Specifications and Batch Analysis for AlphaGOS® Syrup

Parameter	Method	Specification	LOQ	AlphaGOS® Batch		
				IBC 2014 02	IBC 2014 10	IBC 2016 01
Appearance	Visual	Clear	-	Clear	Clear	Clear
Dry matter (%)	Karl Fisher – Based on ISO 8534	> 72	-	79.32	72.92	73.77
Protein (%) *	Internal method based on Reg CE No. 152/2009 Kjeldhal	< 0.5	0.2	< 0.2	< 0.2	< 0.2
Ash (%) *	Internal Method Based on Req CE No. 152/2009	< 0.5	0.1	< 0.1	< 0.1	< 0.1
Alpha-GOS (DP2-DP4 with Gal(n) alpha 1-6 Glc structure) (%) *	Internal method based on AOAC 997.08	96 ± 3	-	97.75	96.87	96.20
Digestible sugars (%)*,†	Internal method based on AOAC 997.08	< 5	0.2	0.2	0.37	0.22
Microbials *(cfu/g unless otherwise state)*						
General bacterial count	NF EN ISO 4833-1	< 1000 cfu/g	-	< 10	< 10	<10
E. coli	NF ISO 16649 2	< 1cfu/g	-	< 1	< 1	<1
Enterobacteria	NF V 08 054	< 1 cfu/g	-	< 1	< 1	<1
Coliforms	NF V 08 050	< 1 cfu/g	-	< 1	< 1	<1
Staphylococcus aureus	EN ISO 6888-1:1999ce	ND in 1 g	-	ND	ND	ND
Pseudomonas aeruginosa	Ph. Eur.2.6.13	ND in 1 g	-	ND	ND	ND
Yeast and Molds	NF V 08-059	≤ 10 cfu/g	-	< 10	< 10	<10
Salmonella spp.	NF EN ISO 6579	Negative in 125 g	-	-	Negative	Negative
Additional Microbials for Infant formula ingredients *(cfu/g unless otherwise stated)*						
Cronobacter spp.	ISO TS 22964 (A)	ND in 25 g	-	ND	ND	ND
B. cereus	NF EN ISO 7932 (A)	50-500/g	-	< 10	< 10	< 10
Heavy Metals *(ppm)*						
Arsenic	ISO 11885/ISO 17294-2(A)	≤ 1 ppm	-	< 0.05	< 0.05	< 0.05
Cadmium	ISO 11885/ISO 17294-2(A)	<1 ppm	-	< 0.01	< 0.01	< 0.01
Lead	ISO 11885/ISO 17294-2(A)	<1 ppm	-	< 0.02	< 0.02	< 0.02
Mercury	DIN EN 13806; ASU L 00.00-19/4(A)	<1 ppm	-	< 0.005	< 0.005	< 0.005

*Specification as percent of dry matter.

†Digestible sugars are defined as the monosaccharides glucose, galactose and fructose, as well as the disaccharides sucrose and maltose.

-Data not available

Abbreviations: LOQ: limit of quantitation, ISO: International Organization for Standardization, DP: degree of polymerization; Gal: galactose; Glc: glucose, AOAC: Association of Official Analytical Chemists, NF:Norme Française, EN: European Norm, Ph. Eur.: European Pharmacopaeia, cfu: colony forming units, ND: not detected, TS: technical specifications, DIN: Deutsches Institut für Normung (German Institute for Standardization), ASU: Official Collection of Testing Methods according to §64 German Food and Feed Code(LFGB) , ppm: parts per million

2. *Please provide the sample sizes used that correspond to the microbial specifications provided.*

Table 3. Specification and Batch Analysis for AlphaGOS® Powder and Table 4. Specification and Batch Analysis for AlphaGOS® Syrup have been updated to include the sample sizes used in the microbial specifications. Updated tables are provided in our response to Question 1.

3. *Olygose incorrectly states the identity of the substance: the substance was described as 1 to 3 glucose units linked via a α-1,6 glyosidic bond to a terminal galactose molecule (p. 2 and p. 90), and also as a α-GOS (DP2-DP4 with Gal (n) alpha 1-4 Glc structure) in tables 3-6. However, according to the chemical structure in the notice (Figure 1, p. 5), it should be α-1,6-linked chains of galactose attached to the 6-position of d-glucose (as described on p. 4 and p. 29). Please clarify the chemical identity of the substance.*

The text has been updated to accurately represent the chemical structure of AlphaGOS® on pages 2 and 90 of the Expert Panel Statement. It shall now read:

"AlphaGOS® consists of 1 to 3 galactose units linked via α (1→6) glycosidic bonds to a terminal glucose and is a non-digestible oligosaccharide."

Similarly, Tables 5 and 6 on page 12 have been updated to accurately reflect the linkages found in the AlphaGOS® product, see below:

Table 5. Specific Oligosaccharide Content in AlphaGOS® Powder

	AlphaGOS® Powder Batch		
Parameter	**2014 009 12**	**2015 01 01**	**2017 01 01**
alpha-GOS (DP2-DP4 with Gal(n) alpha 1-6 Glc structure) %	96.09	97.76	96.51
Melibiose (DP2) %	3.96	5.88	2.67
Manninotriose (DP3) %	48.92	41.77	39.72
Verbascotetraose (DP4) %	43.21	50.11	54.12
Method: Internal method based on AOAC 997.08			

Table 6. Specific Oligosaccharide Content in AlphaGOS® Syrup

	AlphaGOS® Syrup Batch		
Parameter	**IBC 2014 02**	**IBC 2014 10**	**IBC 2016 01**
alpha-GOS (DP2-DP4 with Gal(n) alpha 1-6 Glc structure) %*	97.75	96.87	96.20
Melibiose (DP2)*	3.80	4.66	1.60
Manninotriose (DP3)*	47.66	47.74	39.52
Verbascotetraose (DP4)*	46.29	44.47	55.08
Method: Internal method based on AOAC 997.08 *as percent of dry matter.			

4. *Olygose describes alpha-galactooligosaccharides (α-GOS) as a mixture of oligosaccharides produced from the pea raffinose family, but does not provide information on the average number of galactose moieties present in the oligosaccharides. The functionality and physiological effects of α-GOS may vary based on the molecular weight or degree of polymerization. Please provide the molecular weight distribution or average molecular weight of α-GOS to support the identity and prebiotic property of the substance.*

   AlphaGOS® consists of oligosaccharides containing 1 to 3 galactose units linked via α (1→6) glycosidic bonds to a terminal glucose. The distribution of oligosaccharides in AlphaGOS® are provided in Table 1 on page 5, as well as Tables 5 and 6. Revised Tables 5 and 6 are provided in our response to Question 3 and show that AlphaGOS® syrup and powder contain approximately 4 % melibiose (one galactose and one glucose), 43% manninotriose (two galactoses and one glucose), and 49% verbascotetraose (three galactoses and one glucose) (average of the six batches provided in Tables 5 and 6).

5. *Olygose proposes a specification for "digestible sugars" of less than 5% (pp.10-11). Olygose did not describe the identity of the digestible sugars in α-GOS. Digestible sugars are too broad a term to encompass the diverse group of digestible substances in α-GOS. Please clarify what are the residual digestible sugars in α-GOS and revise the proposed specification accordingly.*

   The digestible sugars are the monosaccharides glucose, galactose and fructose, as well as the disaccharides sucrose and maltose. The footers in Table 3. Specification and Batch Analysis for AlphaGOS® Powder and Table 4. Specification and Batch Analysis for AlphaGOS® Syrup have been updated and now specify what is measured in the "digestible sugars" specification. See above, response to question 1. It shall now read:

   "†Digestible sugars are defined as the monosaccharides glucose, galactose and fructose, as well as the disaccharides sucrose and maltose."

6. *Olygose obtains the α-GOS from acid hydrolysis (80oC, 10 hrs., pH 2.3) of pea polysaccharides (p. 6). Since acid hydrolysis is not highly selective compared to enzymatic hydrolysis, please provide a narrative describing how l the variation of the lengths and desired molecular weights of the resulting α-GOS are controlled using acid hydrolysis. Further, we expect that hot strong acid causes dehydration of sugars with the formation of various furanic compounds (i.e., furfural and 5-hydroxymethyl furfural). Olygose did not discuss the sugar degradation products formed during the manufacturing process for α-GOS. Please provide a narrative discussing the formation and purification of the sugar degradation products and provide a dietary exposure for any potential sugar degradation products in α-GOS from the proposed use.*

   The removal of fructose moieties from raffinose family oligosaccharides (RFO) by acid hydrolysis is temperature, time and pH dependent (Wu et al., 2007). Acid hydrolysis removes the terminal fructose from RFOs while leaving the glucose and galactose moieties

intact. To ensure a consistent product, Olygose's development of their proprietary production process has resulted in a standard operating procedure where the temperature, time and pH of the acid hydrolysis reaction are closely monitored. Data from three non-consecutive batches of AlphaGOS® powder and syrup show that the manufacturing process produces a finished product that contains on average 96% alpha-GOS, consisting of 4% melibiose, 43% manninotriose, and 49% verbascotetraose (see Tables 5 and 6 in our response to Question #3).

Acid hydrolysis of sugars can produce degradation products, such as furfural and 5-hydroxyfurfural (5-HMF). Furfural is produced during the dehydration of five carbon sugars whereas 5-HMF is produced during the dehydration of six carbon sugars (Chen et al., 2013). Complete hydrolysis of AlphaGOS® to monosaccharides results in 99.98% glucose, galactose and fructose, 0.017% arabinose and no xylose or ribose. It is therefore reasonable to conclude that there are negligible amounts of five carbon sugars that could be dehydrated to furfural.

To remove monosaccharides and potential small molecular weight degradation products, from the finished product, Olygose subjects the pea soluble acid-treated hydrolysate to nanofiltration followed by polishing using a strong anionic ion-exchange resin (Purolite A510 purification ion exchange resin, described in Table 2. AlphaGOS® Processing Aids' Regulatory Compliance), which is a technique widely used in corn sweetener refining. The nanofiltration step, which is repeated seven times, removes molecules less than 150-300 Da, separating the alpha-linked oligosaccharides (molecular weights $\geq$ 342 Da) in the retentate from the smaller molecules in the filtrate.

To confirm that the nanofiltration and strong anionic ion-exchange resin steps remove 5-HMF from the production process, Olygose developed an internal high-performance liquid chromatography (HPLC) method that detects 5-HMF, glucose, galactose, fructose, and sucrose (see Figure 1). The limit of quantitation for 5-HMF is 2 ppm and the limit of detection is 1 ppm for AlphaGOS® syrup and between 1-2 ppm for AlphaGOS® powder.

To demonstrate that 5-HMF is not detectable in the finished products, representative chromatograms of AlphaGOS® syrup and powder are shown in Figures 2 and 3, respectively. Although very small amounts of the galactose, glucose and fructose were present, no 5-HMF was detected in either AlphaGOS® syrup or powder. Thus, there is reasonable certainty that consumers of AlphaGOS® are not exposed to 5-HMF that may result from the acid hydrolysis of the pea solubles.

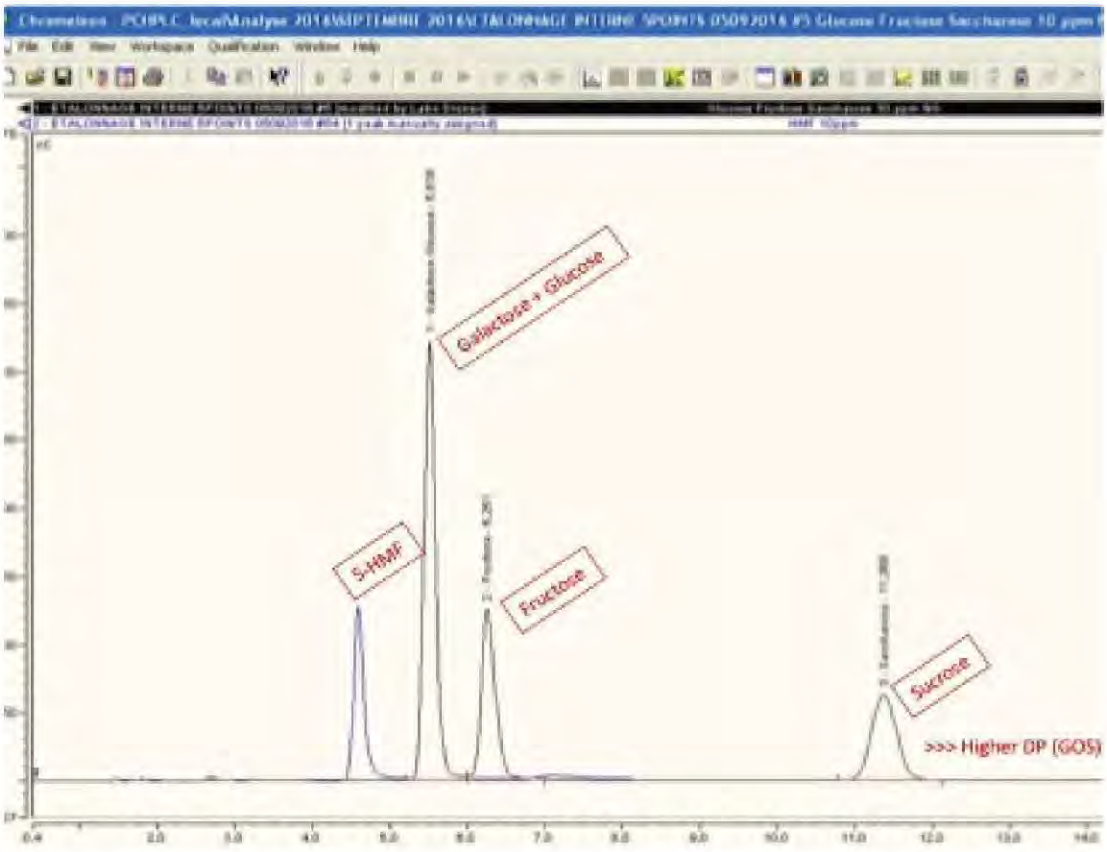


Figure 1. Representative chromatograph of the 5-hydroxymethyl furfural, galactose, glucose, fructose, and sucrose standards (10 ppm) in the HPLC method used for batch qualification.

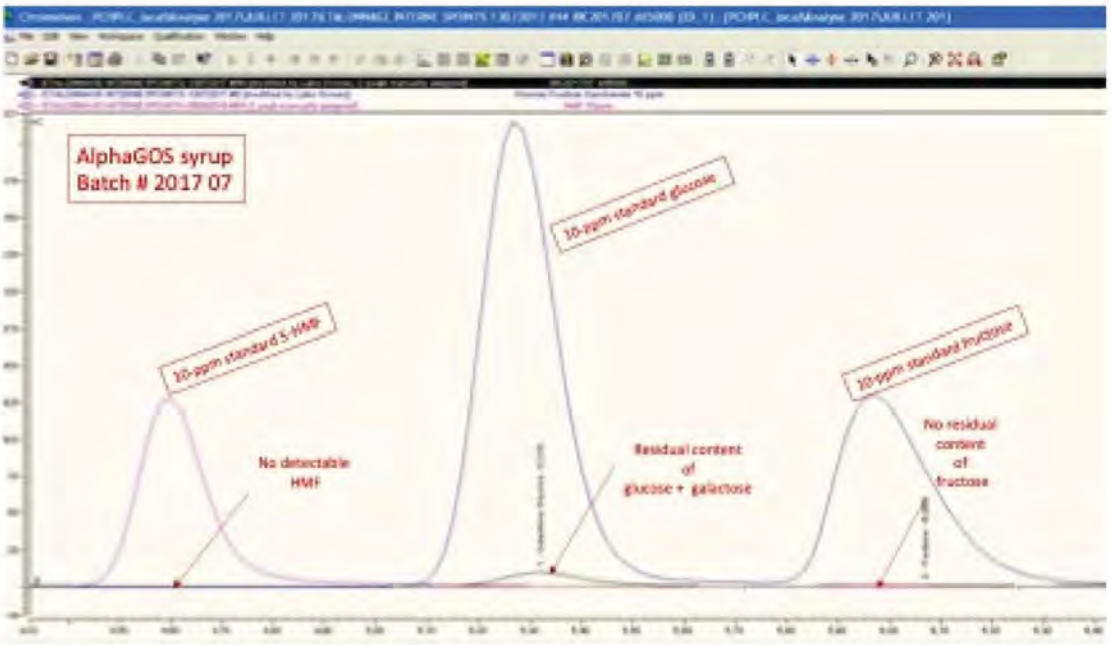


Figure 2. Representative chromatogram of AlphaGOS® syrup batch #2017 07, overlaid with 10 ppm 5-hydroxymethylfurfural, glucose, galactose, fructose standards. 5-hydroxymethylfurfural was not detected.

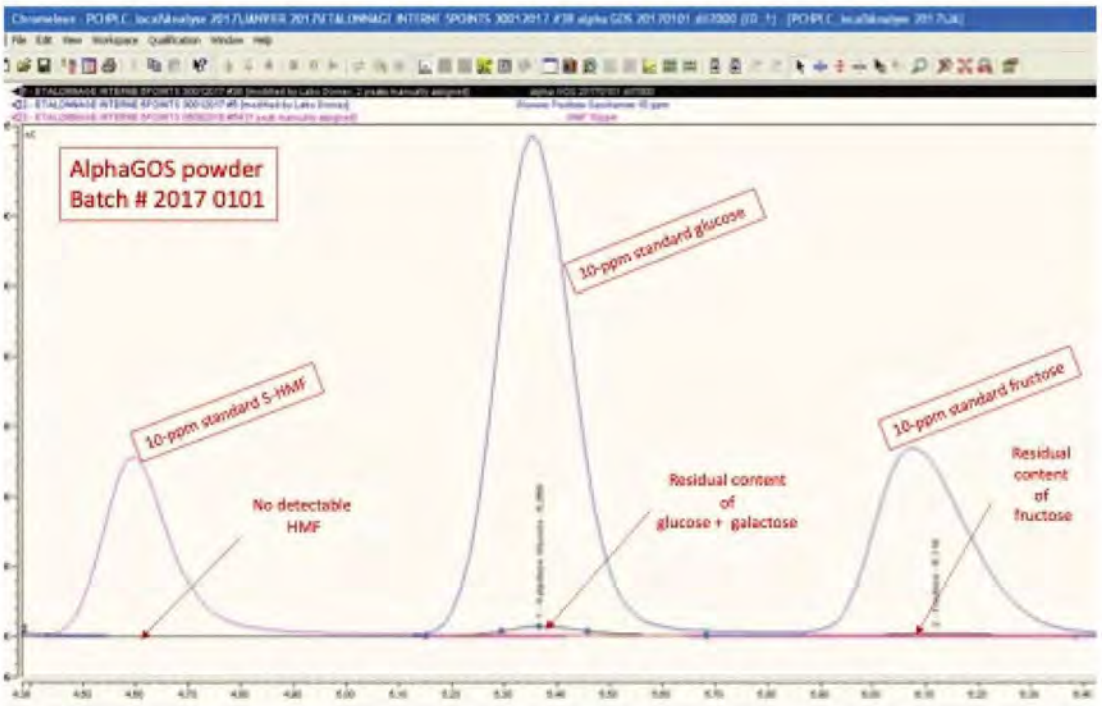


Figure 3. Representative chromatogram of AlphaGOS® powder batch #2017 01 01, overlaid with 10 ppm 5-hydroxymethylfurfural, glucose, galactose, fructose standards. 5-hydroxymethylfurfural was not detected.

7. *Olygose provides an exposure estimate for α-GOS for the proposed use (pp. 21-25). However, Olygose did not address the impact of the notified use on the cumulative exposure. If the notified use is not substitutional for the current uses, Olygose should address whether there would be an increase in the dietary exposure to GOS and provide a cumulative exposure to GOS.*

   AlphaGOS® is intended to be used as a sole source of galactooligosaccharides (GOS). To clarify this in the Notice, Section III.A. Intended Effect should read:

   "The intended effect of adding AlphaGOS® to powdered, ready-to-feed, and concentrated liquid versions of non-exempt term infant formulas and selected conventional foods is to increase oligosaccharide intake in formula-fed infants and the general population. AlphaGOS® is intended to be the sole source of GOS in any given food category."

8. *Please confirm that a literature search was performed and did not identify any clinical studies performed in infants using this ingredient.*

   A literature search was performed on April 21, 2020, and did not identify any clinical studies performed in infants using AlphaGOS®. Section VI.D.1. AlphaGOS® should now read:

   "AlphaGOS® has been studied in two published clinical trials, both described in Morel et al., (2015). Both of these trials reported that AlphaGOS® was well tolerated in human subjects with no test-article related adverse events recorded. AlphaGOS® has also been investigated in several unpublished clinical trials sponsored by Olygose. In these trials, AlphaGOS® was well tolerated and no serious adverse events were reported. A literature search on PubMed and Google Scholar performed on April 21, 2020 yielded no AlphaGOS® clinical studies performed in infant populations."

Should you need additional information, please feel free to contact me at 301-775-9476 or ckruger@spherixgroup.com.

Sincerely,

Claire L. Kruger, Ph.D. D.A.B.T.
Managing Partner

REFERENCES

Chen R, Wang YZ, Liao Q, Zhu X, Xu TF. Hydrolysates of lignocellulosic materials for biohydrogen production. BMB Rep. 2013 May;46(5):244-51.

Wu WH, Wen XS, Zhao Y. Optimization of manninotriose preparation from stachoyse. Zhong Yao Cai. 2007 Jul;30(7):848-51.

Twelve pages have been removed in accordance with copyright laws. The removed reference citations are:

Chen R, Wang YZ, Liao Q, Zhu X, Xu TF. Hydrolysates oflignocellulosic materials for biohydrogen production. BMB Rep. 2013 May;46(5):244-51.

Wu WH, Wen XS, Zhao Y. Optimization ofmanninotriose preparation from stachoyse. Zhong Yao Cai. 2007 Jul;30(7):848-51.

Viebrock, Lauren

From: kbrailer@spherixgroup.com
Sent: Thursday, April 30, 2020 3:51 PM
To: Viebrock, Lauren
Subject: RE: Question regarding GRN 000896

Dear Dr. VieBrock,

I would like to confirm that you received the response we sent yesterday afternoon. Please advise.

Best regards,

Kathy Brailer

From: kbrailer@spherixgroup.com <kbrailer@spherixgroup.com>
Sent: Wednesday, April 29, 2020 5:18 PM
To: Lauren.Viebrock@fda.hhs.gov
Cc: ckruger@spherixgroup.com; 'Dietrich Conze' <dconze@spherixgroup.com>; 'Jennifer Symonds' <jsymonds@spherixgroup.com>
Subject: FW: Question regarding GRN 000896

Dear Dr. VieBrock,

Attached please find our response to your questions regarding GRN 000896. Should you need any additional information, please don't hesitate to ask.

Best regards,

Kathy Brailer
Director of Administrative Services
Spherix Consulting Group, Inc.
11821 Parklawn Drive, Suite 310
Rockville, MD 20852
+1-301-557-0375
kbrailer@spherixgroup.com
www.spherixgroup.com

From: Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
Sent: Thursday, April 16, 2020 10:14 AM
To: ckruger@spherixgroup.com
Subject: Question regarding GRN 000896

Dear Dr. Kruger,

During our review of GRAS Notice No. 000896, we noted further questions that need to be addressed and are attached to this email.

We respectfully request a response within **10 business days**. If you are unable to complete the response within that time frame, please contact me to discuss further options.

If you have questions or need further clarification, please feel free to contact me. Thank you in advance for your attention to our comments.

Regards,
Lauren

Lauren VieBrock
Consumer Safety Officer/Microbiology Reviewer

Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration
Tel: 301-796-7454
lauren.viebrock@fda.hhs.gov

Viebrock, Lauren

From: kbrailer@spherixgroup.com
Sent: Friday, May 01, 2020 8:38 AM
To: Viebrock, Lauren
Subject: RE: Question regarding GRN 000896

Thank you for your confirmation.

From: Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
Sent: Friday, May 1, 2020 8:24 AM
To: kbrailer@spherixgroup.com
Subject: RE: Question regarding GRN 000896

Dear Ms. Brailer,

Thank you for your email with the responses to our questions regarding GRN 000896. This is to confirm receipt of the responses. We will be in touch as we proceed with our review. Please let me know if you have any questions.

Regards,
Lauren

From: kbrailer@spherixgroup.com <kbrailer@spherixgroup.com>
Sent: Thursday, April 30, 2020 3:51 PM
To: Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
Subject: RE: Question regarding GRN 000896

Dear Dr. VieBrock,

I would like to confirm that you received the response we sent yesterday afternoon. Please advise.

Best regards,

Kathy Brailer

From: kbrailer@spherixgroup.com <kbrailer@spherixgroup.com>
Sent: Wednesday, April 29, 2020 5:18 PM
To: Lauren.Viebrock@fda.hhs.gov
Cc: ckruger@spherixgroup.com; 'Dietrich Conze' <dconze@spherixgroup.com>; 'Jennifer Symonds' <jsymonds@spherixgroup.com>
Subject: FW: Question regarding GRN 000896

Dear Dr. VieBrock,

Attached please find our response to your questions regarding GRN 000896. Should you need any additional information, please don't hesitate to ask.

Best regards,

Kathy Brailer
Director of Administrative Services
Spherix Consulting Group, Inc.

11821 Parklawn Drive, Suite 310
Rockville, MD 20852
+1-301-557-0375
kbrailer@spherixgroup.com
www.spherixgroup.com

From: Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
Sent: Thursday, April 16, 2020 10:14 AM
To: ckruger@spherixgroup.com
Subject: Question regarding GRN 000896

Dear Dr. Kruger,

During our review of GRAS Notice No. 000896, we noted further questions that need to be addressed and are attached to this email.

We respectfully request a response within **10 business days**. If you are unable to complete the response within that time frame, please contact me to discuss further options.

If you have questions or need further clarification, please feel free to contact me. Thank you in advance for your attention to our comments.

Regards,
Lauren

Lauren VieBrock
Consumer Safety Officer/Microbiology Reviewer

Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration
Tel: 301-796-7454
lauren.viebrock@fda.hhs.gov

Viebrock, Lauren

From: kbrailer@spherixgroup.com
Sent: Wednesday, June 10, 2020 2:09 PM
To: Viebrock, Lauren
Cc: ckruger@spherixgroup.com; 'Dietrich Conze'; 'Jennifer Symonds'
Subject: FW: Follow up Questions regarding GRN 000896
Attachments: 2020_06_08 GRN 896 Follow up questions.pdf; Response to FDA Questions on GRN896 6-10-20.pdf

Follow Up Flag: Follow up
Flag Status: Flagged

Dear Dr. Viebrock:

Attached please find our response to your request for additional information regarding GRN 000896.

Best regards,

Kathy Brailer
Director of Administrative Services
Spherix Consulting Group, Inc.
11821 Parklawn Drive, Suite 310
Rockville, MD 20852
+1-301-557-0375
kbrailer@spherixgroup.com
www.spherixgroup.com

From: Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
Sent: Monday, June 8, 2020 2:17 PM
To: ckruger@spherixgroup.com
Subject: Follow up Questions regarding GRN 000896

Dear Dr. Kruger,

During our review of GRAS Notice No. 000896, we noted further questions that need to be addressed and are attached to this email.

We respectfully request a response within **10 business days**. If you are unable to complete the response within that time frame, please contact me to discuss further options.

If you have questions or need further clarification, please feel free to contact me. Thank you in advance for your attention to our comments.

Regards,
Lauren

June 8, 2020

GRN 896 Follow up questions

1. In your response to question #6, Olygose states "Olygose developed an internal high-performance liquid chromatography (HPLC) method that detects 5-HMF, glucose, galactose, fructose, and sucrose."

Olygose provided HPLC chromatograms to demonstrate that 5-HMF is not detected in the finished products. However, please clarify the detection system (i.e., detector, wavelength) used for your HPLC analysis. Further, Olygose should state that all analytical methods, including this HPLC method, are validated for their respective purpose.

2. In your response to question #7, Olygose states "The intended effect of adding AlphaGOS® to powdered, ready-to-feed, and concentrated liquid versions of non-exempt term infant formulas and selected conventional foods is to increase oligosaccharide intake in formula-fed infants and the general population. AlphaGOS® is intended to be the sole source of GOS in any given food category."

   We considered that AlphaGOS® will be used as an alternative to lactose-based GOS (galactooligosaccharides) as they do not contain any trace of dairy products (such as other GOS products obtained from lactose through a trans-galactosylation process) and is another source of GOS that may have applications in infant formulas and other foods. However, Olygose responded that AlphaGOS® will be used as the sole source of GOS in the diet (i.e., intended to replace the entire daily GOS intake). Therefore, we consider that the notified use is not a substitute for currently-marketed sources of GOS and its use will result in exposure beyond that estimated in previous GRAS notices."

We request that Olygose address the impact of the notified use on the cumulative exposure to GOS.

June 8, 2020

GRN 896 Follow up questions

1. In your response to question #6, Olygose states "Olygose developed an internal high-performance liquid chromatography (HPLC) method that detects 5-HMF, glucose, galactose, fructose, and sucrose."

Olygose provided HPLC chromatograms to demonstrate that 5-HMF is not detected in the finished products. However, please clarify the detection system (i.e., detector, wavelength) used for your HPLC analysis. Further, Olygose should state that all analytical methods, including this HPLC method, are validated for their respective purpose.

2. In your response to question #7, Olygose states "The intended effect of adding AlphaGOS® to powdered, ready-to-feed, and concentrated liquid versions of non-exempt term infant formulas and selected conventional foods is to increase oligosaccharide intake in formula-fed infants and the general population. AlphaGOS® is intended to be the sole source of GOS in any given food category."

   We considered that AlphaGOS® will be used as an alternative to lactose-based GOS (galactooligosaccharides) as they do not contain any trace of dairy products (such as other GOS products obtained from lactose through a trans-galactosylation process) and is another source of GOS that may have applications in infant formulas and other foods. However, Olygose responded that AlphaGOS® will be used as the sole source of GOS in the diet (i.e., intended to replace the entire daily GOS intake). Therefore, we consider that the notified use is not a substitute for currently-marketed sources of GOS and its use will result in exposure beyond that estimated in previous GRAS notices."

We request that Olygose address the impact of the notified use on the cumulative exposure to GOS.

Viebrock, Lauren

From: ckruger@spherixgroup.com
Sent: Friday, September 18, 2020 12:20 PM
To: Viebrock, Lauren
Cc: 'Jennifer Symonds'; 'Dietrich Conze'; 'Kathy Brailer'
Subject: RE: GRN 000896

Dear Lauren,
Thank you for bringing this to our attention. I can confirm that the dose tested in the piglet safety study discussed in the notice was 8 g/L and that the use level in non-exempt infant formulas is 8 g/L and not 7.8 g/L as stated on page 28. This was a typographical error. Please let us know if any additional clarification is needed.

Best regards,
Claire

Claire Kruger, Ph.D., DABT, CFS
Managing Partner
Spherix Consulting Group
11821 Parklawn Drive
Suite 310
Rockville MD 20852
+1-301-775-9476

From: Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
Sent: Thursday, September 17, 2020 1:08 PM
To: ckruger@spherixgroup.com
Subject: GRN 000896

Dear Dr. Kruger,

During our review of GRAS Notice No. 000896, we noted an additional point to be addressed.

Please confirm that the dose tested in the piglet safety study discussed in the notice was 8 g/L and that the use level in non-exempt infant formulas is 8 g/L and not 7.8 g/L as stated on page 28. ("….AlphaGOS® was determined to be safe and well tolerated at this level of ingestion and supports the proposed use in non-exempt infant formulas at 7.8 g/L.")

We respectfully request a response within **10 business days**. If you have questions or need further clarification, please feel free to contact me. Thank you in advance for your attention to our comments.

Regards,
Lauren

Lauren VieBrock
Regulatory Review Scientist/Microbiology Reviewer

Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration
Tel: 301-796-7454
lauren.viebrock@fda.hhs.gov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