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Safety evaluation of galacto-oligosaccharides: Subchronic oral toxicity study in Sprague-Dawley rats

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Abstract

Galacto-oligosaccharides (GOS) have been added to infant formulas and conventional foods as prebiotics all over the world. The present study was conducted to assess the subchronic toxicity of a GOS syrup (VITAGOS™) when administered orally by gavage daily at 0, 1020, 2041, and 4082 mg GOS syrup/kg/day to male and female Sprague-Dawley rats to deliver doses of 0, 500, 1000, and 2000 mg GOS/kg/day for 90 days. Throughout the entire treatment period, no abnormal clinical signs or mortalities were observed. Similarly, no test article-related toxicologically adverse findings were seen in body weight, feed consumption, ophthalmological findings, hematology, coagulation, clinical chemistry, urinalysis, organ weights, and gross pathology or histopathology. Significant increases in the cecum weight of males and females treated with 2000 mg GOS/kg/day were associated with mucosal hypertrophy/hyperplasia; no changes in the cecum were noted at lower doses. The organ weight and histopathological changes noted in the cecum are consistent with findings in rats administered other poorly digestible and fermentable substances; thus, this is considered to be an adaptive rather than toxic response. The No-Observed-Adverse-Effect-Levels for VITAGOS™ is 4082 mg GOS syrup/kg body weight/day or 2000 mg GOS/kg body weight/day.

Keywords

Galacto-oligosaccharide, food ingredient, safety, sub-chronic toxicity

Introduction

Galacto-oligosaccharides (GOS) are also known as oligogalactosyllactose, oligogalactose, oligolactose, transgalactosylated oligosaccharide, and transgalacto-oligosaccharide. GOS are produced from lactose by β-galactosidases. There is no globally adopted definition of GOS; however, they are usually defined in the literature as a mixture of oligosaccharides derived from lactose, comprising between 2 and 8 saccharide units, with one of these units being a terminal glucose and the remaining saccharide units being galactose, and disaccharides comprising two units of galactose.[1,2]

Typically, the oligosaccharides in synthetic GOS preparations are linked via β-(1→3), β-(1→4), or β-(1→6) glycosidic bonds, which are determined by the type of β-galactosidase used during manufacturing and the manufacturing conditions.[3,4] Although tri- to hexa-saccharides with 2–5 galactose units (degree of polymerization (DP) of 3–6) tend to be the main components of GOS-containing products, disaccharides (DP2) consisting of galactose and glucose with β-glycoside bonds different from lactose are also present and, because these disaccharides have physiological characteristics that are similar to longer GOS, they are considered GOS.[5–7] These β-linked oligosaccharides are difficult to digest by human and animal pancreatic or intestinal enzymes but are subject to fermentation processes by the gut microflora.[8–11] Studies have shown that adding

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manufactured GOS to infant formula may promote the growth of beneficial bacteria, including but not limited to bifidobacteria and lactobacillus.[12,13]

GOS has been used extensively in infant formulas and general foods in Japan, European Union, United States, Australia, New Zealand, and China. In the United States, the intended use level of GOS is up to 7.8 g/L in reconstituted infant formulas and up to 11 g/serving in conventional foods, which were proposed in several Generally Recognized As Safe (GRAS) Determinations that received "no questions" letters from the US Food and Drug Administration (US FDA[14–17]). In the European Union, GOS is approved for use in infant and follow-on formulas in combination with fructo-oligosaccharides (FOS) at levels up to 8 g (90% GOS and 10% FOS)/L 7.2 g GOS and 0.8 g FOS/L)[18] Similarly, GOS is permitted in Australia and New Zealand in infant and follow-on formulas at levels up to 290 mg/100 kJ or approximately 8 g/L.[19]

Two published 90-day studies in Sprague-Dawley rats of two GOS products (Vivinal®, Mead Johnson Nutritionals; Oligomate, Yakult Pharmaceutical Industry) have been reported. When administered by gavage, GOS syrup was determined to have No-Observed-Adverse-Effect-Levels (NOAELs) of 2000 mg GOS syrup/kg/day (Oligomate[20]) and 5000 mg GOS syrup/kg/day (Vivinal®[21]), resulting in NOAELs of 825 and 2250 mg GOS/kg, respectively. In addition, an unpublished 90-day study estimated the NOAEL of Vivinal® to be 6900 mg GOS/kg/day when it was included in the diets of Wistar rats.[22] These two GOS products were both manufactured from lactose by using β-galactosidases but the sources of the enzymes were different. The β-galactosidases used for the manufacture of Oligomate were derived from *Sporobolomyces gingularis* and *Kluyveromyces lactis*[15] while the sole enzyme used for the production of Vivinal® was derived from *Bacillus circulans.*[23]

VITAGOS™ is a GOS-containing product manufactured using lactose and β-galactosidases derived from *Aspergillus oryzae* and *K. lactis* in a manner similar to other GOS-containing products and essentially equivalent with respect to product specifications with other GOS-containing products that have received "no questions" letters for GRAS Determinations Notified to the US FDA. Beta-galactosidases derived from *A. oryzae* and *K. lactis* were used at optimized conditions to maximize the synthesis of GOS and hydrolysis of lactose. Because the combination of β-galactosidases derived from these two microorganisms is unique to the production of this GOS product, and to ensure the safety of a food ingredient through the GRAS process, a 90-day toxicology study was conducted in Sprague-Dawley rats to corroborate the safety and establish the NOAEL of VITAGOS™.

Materials and methods

The study of GOS product VITAGOS™ was conducted per OECD Guideline No. 408 for Testing of Chemicals, "Repeated Dose 90-Day Oral Toxicity Study in Rodents" adopted on September 21, 1998. The study was performed in an Association for Assessment and Accreditation of laboratory Animal Care accredited laboratory, and all procedures were in compliance with the Committee for Purpose of Control and Supervision of the Experiments on Animals guidelines of India.

Test article

VITAGOS™ GOS syrup provided by Vitalus Nutrition Inc. contained 75% solids and was stored at a refrigerated temperature (2–8%). The syrup contains approximately 65.8% GOS, 14% lactose, and 18% glucose on a dry basis.

Test animal

Sprague-Dawley rats from Envigo (Harlan Laboratories; Placentia, CA, USA) were examined for good health and the suitability for the study and acclimatized for 5 days before the start of the treatment. All animals were housed (2/cage) with a temperature of 21–24°C, relative humidity of 65–67%, and 12-h light and 12-h dark cycle. All animals were fed *ad libitum* with a standard diet (Teklad Certified (2014C) Global 14% Protein Rodent Maintenance Diet – Pellet (Certified); Envigo, Madison, WI) and filtered water. At the commencement of the treatment, the weight variation of rats did not exceed $\pm 20\%$ of the mean body weight in each sex and group. These 6- to 8-weeks-old rats were randomly distributed to groups as noted in Table 1 by the body weight stratification method using Provantis™ software (version 8.7.3; Instem LSS, Staffordshire ST15OSD, UK). Rats were dosed with control (Milli-Q water) or VITAGOS™ GOS syrup by gavage at the dose volumes shown in Table 1 for 90 consecutive days. The dose volume was calculated for individual animals on the first day of the treatment period (Day 1) and was adjusted according to the most recent body weights recorded during the treatment period. Volumes of VITAGOS™ GOS syrup were calculated to deliver doses of 500, 1000, or 2000 mg GOS/kg/day.

Observations

Clinical examination. All rats were observed once daily for changes in appearance, behavior, clinical/toxic signs, and neurological changes and twice daily for morbidity and mortality. Detailed clinical examination was done prior to the test article administration on day 1 and at weekly intervals thereafter during treatment period.

Ophthalmological examination. Ophthalmological examination for all rats was carried out with a direct ophthalmoscope before start of treatment and at the end of the treatment period. Mydriasis was induced before examination using a solution of 1% Tropicamide (Unimed Technologies LTD., India).

Table 1. Design of GOS syrup dose formulation.

Group no.	Group	GOS dose (mg/kg/day)	GOS syrup dose (mg/kg/day)	GOS syrup dose volume (mL/kg)[a]	GOS concentration (mg/mL)	Number of rats	
						Male	Female
G1	Control	0	0	2.9[b]	0	10	10
G2	Low dose	500	1020	0.7	686	10	10
G3	Mid dose	1000	2041	1.5	686	10	10
G4	High dose	2000	4082	2.9	686	10	10

GOS: galacto-oligosaccharides.
[a]GOS syrup has a density of 1400 mg/mL.
[b]Milli-Q water was administered in place of GOS formulation in control group.

Body weight and feed consumption

Individual body weight was recorded prior to test article administration on day 1 and at weekly intervals thereafter except for week 13 when the animals were weighed on day 5 of that week. Fasting body weight was recorded prior to termination. The feed consumption was measured on the same day as body weights were recorded except for day 1. Feed efficiency was calculated as body weight gain divided by feed consumption during the same period.

Clinical pathology

All rats were fasted overnight before blood collection on day 91. The blood was collected for all groups by retro-orbital plexus puncture with the help of a fine capillary tube under isoflurane (Abbott Laboratories, Chicago, IL) anesthesia. The hematological, coagulation, and clinical chemistry parameters listed in Table 2 were determined using the ADVIA 2120 hematology system (Bayer HealthCare LLC, Pittsburgh, PA), Start-4 coagulation analyzer (Diagnostica stago, 92600 Asnieres, France) and Dimension RxL Max clinical Chemistry System (Dade Behring Inc., Newark, DE).

Urinalysis

Urine was collected from all rats at the end of the treatment period in urine collection tubes. On day 90, each rat was placed in a specially fabricated cage overnight (water allowed) and next morning, the collected urine was sent for analysis. Specific gravity was analyzed using refractometry method (Refractometer-PAL-10S; Atago, Japan). Nitrite, proteins, glucose, ketone bodies, urobilinogen, and bilirubin were analyzed by using Multistix 10 SG strips in Clinitek status analyzer (Bayer Healthcare LLC, UK). Gross appearance (color and clarity), pH, and total volume were recorded. Urine was also subjected to microscopic examination for sediments such as crystals, epithelial cells, erythrocytes, leukocytes, and casts.

Table 2. Parameters evaluated in hematology, coagulation, and clinical chemistry.

	Parameters
Hematology	Hematocrit (Hct)
	Hemoglobin (Hgb)
	Mean Corpuscular Hemoglobin (MCH)
	Mean Corpuscular Hemoglobin Concentration (MCHC)
	Mean Corpuscular Volume (MCV)
	Mean Platelet Volume (MPV)
	Platelets (Plat)
	Red Blood Cells (RBC)
	White Blood Cells (WBC)
	Differential Leukocyte Counts[a] (DLC)
	Reticulocytes (Retic)
Coagulation	Prothrombin Time (PT)
	Activated Partial Thromboplastin Time (APTT)
Clinical chemistry	Alanine Aminotransferase (ALT)
	Albumin (Alb)
	Alkaline Phosphatase (ALP)
	Albumin/Globulin ratio (A/G)
	Aspartate Amino Transferase (AST)
	Gamma Glutamyl Transpeptidase (GGT)
	Blood Urea Nitrogen (BUN)
	Creatinine (Creat)
	Creatine kinase (CK)
	Calcium (Ca)
	Chloride (Cl)
	Globulin (Glob)
	Glucose (Glu)
	Inorganic phosphorous (Pi)
	Potassium (K)
	Sodium (Na)
	Total cholesterol (T.Chol)
	Total Plasma Protein (T.Pro)
	Total Bilirubin (T.Bil)
	Triglycerides (Trig)

[a]Differential leukocyte parameters and their respective abbreviations are: Neutrophils (Neut), Lymphocytes (Lymp), Monocytes (Mono), Eosinophils (Eosi), and Basophils

Necropsy

After the last treatment on day 90, all animals were fasted overnight and on day 91, they were all weighed and exsanguinated under isoflurane anesthesia and

subjected to detailed necropsy. The organs and tissues listed in Table 3 from all rats were collected and fixed using 10% neutral buffered formalin (monosodium phosphate dihydrate, Disodium hydrogen phosphate, and formaldehyde; Rankem, India). The organs marked with X were weighed. The paired organs were weighed together, and combined weight was presented. The organ weight ratios as percentage of body and brain weight were calculated based on the fasted body weight and brain weight.

Histopathological examination was carried out on all the preserved organs and tissues of vehicle control (G1; 0 mg GOS/kg/day) and high dose (G4; 2000 mg GOS/kg/day) group rats. In addition, all gross lesions from all the animals were examined microscopically. Furthermore, the cecum from the lower dose groups (G2; 500 mg GOS/kg/day and G3; 1000 mg GOS/kg/day) was examined, as test article-related histopathological changes were observed in the high dose group.

Statistical analysis

All data were captured using Provantis and analyzed using the software's built-in statistical tests. The data were analyzed using analysis of variance (ANOVA), after testing for homogeneity for intragroup variance using Levene's test. Where intragroup variances were heterogeneous, ANOVA was performed after suitable transformation of data. Dunnett's pairwise comparison of the treated group means with the control group mean was performed, when the group differences were found significant. Feed efficiency was calculated and analyzed by EXCEL using one-way ANOVA and t test. All analysis and comparisons were evaluated at the 5% ($p < 0.05$) level.

Results

Clinical observations

All animals survived until scheduled anesthesia on day 91, and no clinical signs were observed in all animals throughout the treatment period.

Ophthalmological examination

No eye abnormalities were found during ophthalmological examination conducted during the acclimatization and at the end of the treatment period.

Body weight

When compared to the control group, body weight was unaffected at 500 mg/kg/day (G2) in males and in females at all doses. The body weight change in all animals is shown in the growth curve (Figure 1). In male rats, starting from day 43 until the end of treatment, the animals in group G3 (1000 mg/kg/day) and G4 (2000 mg/kg/day)

Table 3. Organ/tissue collection and preservation.

Tissue	Organ weighing
Adrenal glands	X
Aorta	
Axillary lymph nodes	
Bone marrow smear[a]	
Brain (cerebrum, cerebellum, medulla/pons)	X
Cecum[b]	X
Colon	
Duodenum	
Epididymides	X
Esophagus	
Eyes (with optic nerve)[c]	
Gross lesions	
Femur bone with distal joint[d]	
Femoral muscle (Skeletal Muscle)	
Heart	X
Harderian glands	
Ileum with Peyer's Patch	
Jejunum	
Kidneys	X
Lacrimal glands	
Larynx	
Liver	X
Lungs[e]	X
Mesenteric lymph node	
Mandibular lymph node	
Mammary gland	
Nerves, sciatic	
Ovaries	X
Oviduct	
Pancreas	
Pharynx	
Pituitary[f]	X
Prostate[g]	X
Rectum	
Salivary glands (submandibular, sublingual, and parotid)	
Seminal vesicles and coagulating glands[h]	X
Skin	
Spinal cord (cervical, thoracic, and lumbar)	
Spleen	X
Sternum with marrow[e]	
Stomach	
Testes[h]	X
Thymus	X
Thyroid with Parathyroids[g]	X
Tongue	
Trachea	
Urinary bladder	
Ureters	
Uterus with cervix	X
Vagina	

[a]Bone marrow smears were prepared from femur marrow and stained using Giemsa stain.
[b]Weighted with and without content.
[c]Eyes were collected in Davidson's fluid (Isopropyl alcohol and glacial acetic acid was from Rankem, India and Spectrochem, India, respectively).
[d]Decalcified prior to sectioning.
[e]Inflated with 10% neutral buffered formalin before fixation.
[f]Weighed after formalin fixation.
[g]Prostate + Seminal vesicles with coagulating glands were weighed as a whole; subsequently prostate was separated and weighed. The derived weight was presented for the seminal vesicles and coagulating glands.
[h]Collected in modified Davidson's fluid.

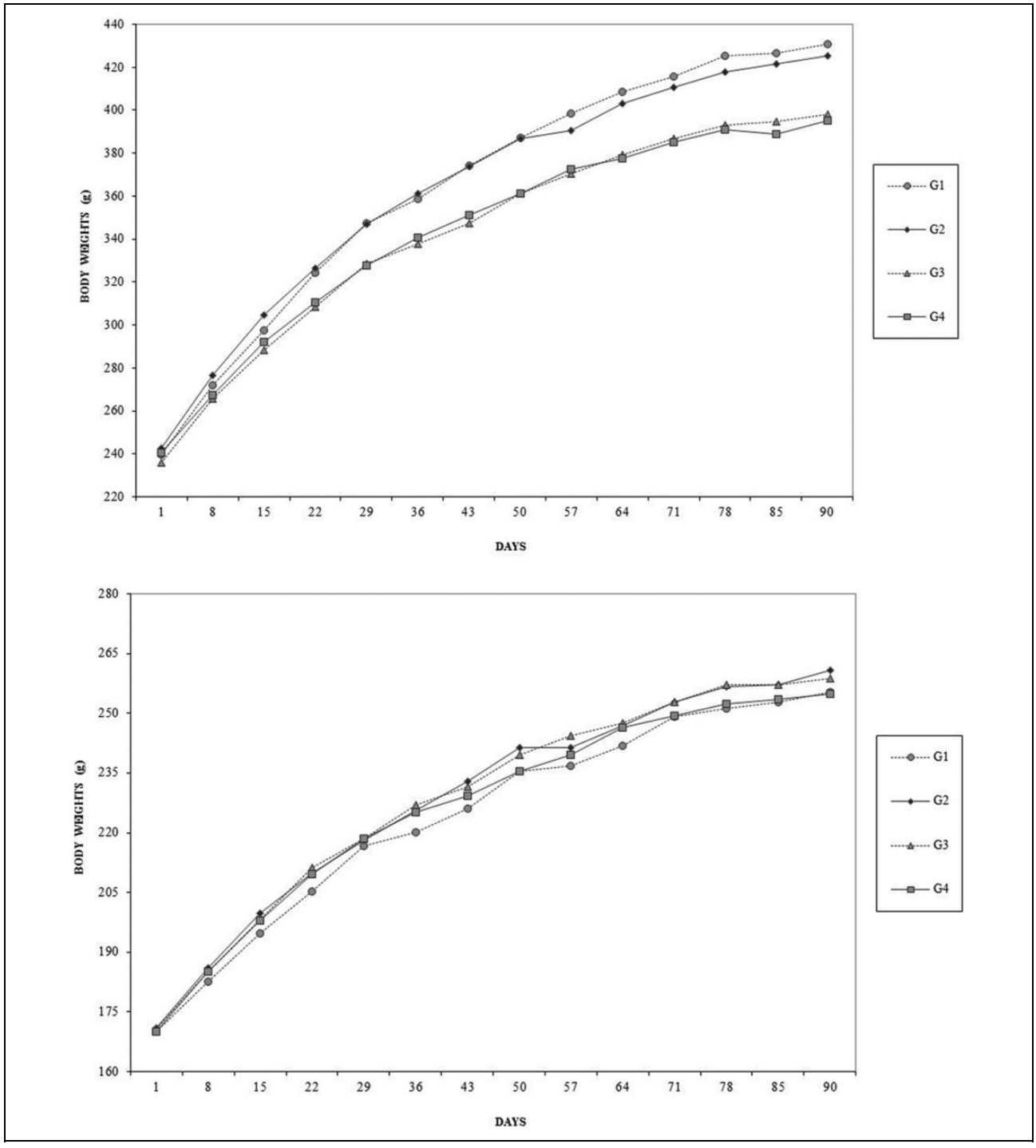

Figure 1. Growth curves of male (top) and females (bottom) in terms of mean body weight. G1, G2, G3, and G4 represent 0, 500, 1000, and 2000 mg GOS/kg/day, respectively.

had significantly lower body weight than the controls, ranging from 7% to 8% and 6% to 9% in reduction, respectively ($p < 0.05$). However, the body weight gain of animals of both sexes was not significantly different ($p < 0.05$) among all groups throughout the treatment period (data not shown).

Feed consumption

When compared to the control group, feed consumption was unaffected at 500 mg/kg/day (G2) in males and at all the dose levels in females. In males, significant changes in feed consumption occurred during the following days at doses of 1000 and 2000 mg/kg/day with a reduction of 6–10% and 7–12%, respectively ($p < 0.05$).

G3 (1000 mg/kg/day): Day 8–15, 15–22, 36–43, 43–50, 57–64, 71–78, and 78–85
G4 (2000 mg/kg/day): Day 8–15, 15–22, 22–29, 36–43, 43–50, 50–57, 57–64, 64–71, 71–78, 78–85, and 85–90

Feed efficiency

Feed efficiency of all animals is shown in Table S1 in supplementary material. With an exception of males during says 15 and 22 and females at the 500 mg/kg/day group during days 50–57, the feed efficiency of male and female rats at all dose levels was not significantly different than controls ($p < 0.05$).

Clinical pathology

Hematology and coagulation. Hematology and coagulation data are presented in Table 4. There are occasional sporadic findings of significant differences in hematology parameters in GOS-treated animals compared to controls including decreased absolute and relative reticulocyte count in females treated with 1000 mg/kg/day dose, decreased white blood cell in males at 1000 mg/kg/day, increased mean platelet volume in females at 2000 mg/kg/day dose, and decreased absolute neutrophil count in all treated males. In the coagulation parameters, decreased prothrombin time values in all treated males and increased activated partial thromboplastin time values in all treated females were noted.

Clinical chemistry

Values of clinical chemistry parameters are presented in Table 5. Significant differences in the following parameters from GOS-treated rats compared to controls included: increases in blood urea nitrogen in males treated with 1000 and 2000 mg/kg/day and females treated with 1000 mg/kg/day, decreases in total bilirubin of males treated with 500 mg/kg/day and all treated females, decreases in triglycerides of females treated with 500 mg/kg/day and males treated with 2000 mg/kg/day, increases in albumin of males treated with 1000 and 2000 mg/kg/day, decreases in calcium and increases in sodium of all treated rats, and increases in chloride of animals treated with the highest dose.

Urinalysis

There were no test article-related changes in the urinalysis parameters in treated rats compared to controls.

Organ weights

Absolute and relative organ weights of all animals are presented in Tables S2 and S3 in supplementary material; organ weights with statistically significant changes when compared to the control group are shown in Table 6. The mean absolute and relative cecum weight to body weight and brain weight (with or without content) were significantly increased in both sexes in the 2000 mg/kg/day group. In male rats, mean brain weight relative to body weight in the 1000 (+7.7%) and 2000 (+7.6%) mg/kg/day groups was significantly higher than the control group; mean absolute liver weight (–11%) and liver weight relative to brain weight (–11%) from the 1000 mg/kg/day group were significantly lower than the control group; mean absolute liver weight (–15%) and liver weight relative to body weight (–7.4%) and brain weight (–14%) from the 2000 mg/kg/day group were significantly lower than those of the control group; other significant observations were incidental findings. No other significant differences were noted in GOS-treated females.

Gross pathology

All tissues and organs were examined for gross pathology. Dilated uterus with cervix was noted (2/10 in control, 3/10 in 500 mg/kg/day, and 2/10 in 2000 mg/kg/day) in female rats.

Histopathology

Histopathology results from control and high dose (2000 mg/kg/day) groups are listed in Table 7. Mucosal hypertrophy/hyperplasia was noted in the cecum of both male and female animals treated with the highest dose level (2000 mg/kg/day), but no polyps were noted in any of the cecum. Furthermore, the cecum from the lower dose groups (G2; 500 mg/kg/day and G3; 1000 mg/kg/day) was examined, as test article-related histopathological change was observed in the high dose group. No abnormal findings were noted in the cecums of these two lower dose groups. Other findings not related to test article administration include single incidences in one dose group (adrenal glands, epididymides, harderian gland, liver, mammary glands, and thymus), incidences mainly in one sex (lungs, spleen, and mandibular lymph nodes), and incidences occurred in both control and GOS-treated groups at similar rates (kidneys, lacrimal glands, thyroid gland, and uterus with cervix).

Discussion

Sprague-Dawley rats (10 per sex per group) were treated with VITAGOS™ to deliver GOS at doses of 0, 500, 1000, and 2000 mg/kg/day for 90 consecutive days.

There were no deaths, relevant clinical signs, or abnormal ophthalmological findings reported at any dose level in this study. Both body weight and feed consumption were reduced at 1000 and 2000 mg/kg/day doses in males but not

Table 4. Mean hematology and coagulation values.

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

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

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

Table 5. Values of clinical chemistry.

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

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

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

Table 6. Organ weight.

Organ	G1, 0 mg/kg/day			G2, 500 mg/kg/day			G3, 1000 mg/kg/day			G4, 2000 mg/kg/day		
	Abs	Relative to body weight	Relative to brain	Abs	Relative to body weight	Relative to brain	Abs	Relative to body weight	Relative to brain	Abs	Relative to body weight	Relative to brain
Male rats												
Terminal fasting body weight (g)	410.35 ± 21.17	–	–	404.99 ± 25.30	–	–	379.82 ± 21.09[b]	–	–	375.45 ± 24.13[b]	–	–
Brain (g)	1.9856 ± 0.0668	0.4846 ± 0.0214	–	1.9346 ± 0.0947	0.4783 ± 0.0187	–	1.9774 ± 0.0593	0.5220 ± 0.0314[a]	–	1.9543 ± 0.0879	0.5216 ± 0.0258[a]	–
Cecum with content (g)	3.8003 ± 0.7685	0.932 ± 0.215	192.205 ± 42.587	3.7213 ± 0.9320	0.921 ± 0.229	192.922 ± 49.343	4.6803 ± 0.5799	1.235 ± 0.162[a]	236.806 ± 29.825	5.1308 ± 1.2295[a]	1.372 ± 0.336[a]	262.866 ± 63.005[a]
Cecum without content (g)	1.0938 ± 0.1264	0.266 ± 0.024	55.077 ± 5.999	1.1419 ± 0.1563	0.282 ± 0.032	59.053 ± 7.775	1.2433 ± 0.1897	0.328 ± 0.049[a]	63.031 ± 10.446	1.3541 ± 0.1385[a]	0.361 ± 0.029[a]	69.357 ± 7.074[a]
Heart (g)	1.3026 ± 0.0650	0.3177 ± 0.0140	65.6208 ± 2.9566	1.2680 ± 0.0900	0.3132 ± 0.0153	65.5345 ± 3.3326	1.1975 ± 0.1062[b]	0.3156 ± 0.0249	60.5204 ± 4.4850[b]	1.2107 ± 0.1000	0.3223 ± 0.0127	61.9179 ± 3.8052
Liver (g)	10.3398 ± 0.7257	2.5192 ± 0.1039	520.8712 ± 34.8687	10.1220 ± 1.0965	2.4973 ± 0.1820	522.6148 ± 41.4108	9.1957 ± 0.7975[b]	2.4202 ± 0.1452	465.0234 ± 37.1198[b]	8.7667 ± 0.9153[b]	2.3332 ± 0.1627[b]	448.0115 ± 35.0781[b]
Spleen (g)	0.7646 ± 0.0674	0.1863 ± 0.0125	38.5305 ± 3.4031	0.7233 ± 0.0828	0.1791 ± 0.0221	37.4411 ± 4.4660	0.6675 ± 0.0915	0.1755 ± 0.0192	33.7264 ± 4.1850[b]	0.6714 ± 0.1010	0.1786 ± 0.0214	34.2927 ± 4.2832
Female rats												
mTerminal fasting body weight (g)	242.04 ± 8.42	–	–	246.45 ± 11.25	–	–	245.45 ± 9.10	–	–	239.05 ± 11.10	–	–
Brain (g)	1.8070 ± 0.0633	0.7471 ± 0.0283	–	1.8323 ± 0.0580	0.7449 ± 0.0435	–	1.8188 ± 0.0862	0.7417 ± 0.0386	–	1.8229 ± 0.0589	0.7635 ± 0.0318	–
Cecum with content (g)	2.6505 ± 0.7224	1.098 ± 0.308	146.786 ± 39.974	3.4878 ± 0.7394[a]	1.424 ± 0.337	190.992 ± 45.009	2.9198 ± 0.5337	1.192 ± 0.227	160.835 ± 29.653	3.7521 ± 1.3186[a]	1.557 ± 0.499[a]	206.204 ± 75.136[a]
Cecum without content (g)	0.8540 ± 0.1004	0.353 ± 0.046	47.368 ± 6.382	0.8592 ± 0.0722	0.349 ± 0.036	46.983 ± 4.859	0.9251 ± 0.0973	0.377 ± 0.038	51.000 ± 6.243	1.0666 ± 0.1426[a]	0.446 ± 0.059[a]	58.515 ± 7.828[a]
Heart (g)	0.8616 ± 0.0421	0.3562 ± 0.0173	47.6993 ± 2.0664	0.8498 ± 0.0622	0.3452 ± 0.0276	46.3510 ± 2.6502	0.8770 ± 0.0559	0.3574 ± 0.0200	48.2600 ± 2.9492	0.8337 ± 0.0467	0.3491 ± 0.0199	45.7354 ± 2.1560
Liver (g)	5.7410 ± 0.4011	2.3714 ± 0.1360	317.5273 ± 15.9323	5.6597 ± 0.3005	2.2977 ± 0.1058	309.0160 ± 16.1269	5.7593 ± 0.4224	2.3452 ± 0.1249	316.9453 ± 22.9956	5.5773 ± 0.3855	2.3333 ± 0.1194	306.3632 ± 25.5879
Spleen (g)	0.5664 ± 0.0568	0.2338 ± 0.0204	31.3153 ± 2.7053	0.5619 ± 0.0550	0.2278 ± 0.0167	30.6945 ± 3.0667	0.5442 ± 0.0321	0.2217 ± 0.0107	30.0023 ± 2.5960	0.5348 ± 0.0752	0.2230 ± 0.0232	29.2953 ± 3.5905

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

females. The reductions in body weight were not considered clinically adverse since they were less than 10%. The reductions in feed consumption were considered a test article-related nonadverse finding, as the lower feed consumption did not result in any clinical signs during the in-life phase of the treatment period. Most importantly, no test article-related effect was seen on feed efficiency. Therefore, the reductions in body weight and feed consumption may be due to poor acceptability rather than less efficient utilization of the diets.

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

The absolute and relative weight of cecum with and without contents at 2000 mg/kg/day dose in males and females was considered a test article-related effect, as it was microscopically associated with mucosal hypertrophy/hyperplasia (males, 8/10 hypertrophy/hyperplasia with 5 minimal, 3 mild; females, 7/10 hypertrophy/hyperplasia with 4 minimal, 3 mild). It was noted that the brain weight relative to body weight in 1000 and 2000 mg/kg/day groups of males were significantly increased when compared to the control group, but the increase was not dose dependent. Because the absolute weight of brains in these two groups was not different from the control group, the reduced terminal body weight of these two groups may have been the cause of the reductions in the brain relative to body weight. Thus, it was an incidental change and not associated with any microscopic changes in the histopathological examination. In addition, absolute and relative to brain weights of liver in males at 1000 mg/kg/day and absolute and relative

Table 7. Incidence of selected histopathological findings.

	Males, $n = 10$		Females, $n = 10$	
	Control	2000 mg/kg/day	Control	2000 mg/kg/day
Adrenal glands				
Cortical vacuolation, minimal	0/10	1/10	0/10	0/10
Cecum				
Mucosal hypertrophy/hyperplasia, minimal	0/10	5/10	0/10	4/10
Mucosal hypertrophy/hyperplasia, mild	0/10	3/10	0/10	3/10
Epididymides				
Cellular debris in duct lumen, bilateral	1/10	0/10	–	–
Harderian gland				
Inflammatory focu(i), minimal	0/10	2/10	0/10	0/10
Kidneys				
Basophilic tubules, minimal	1/10	1/10	0/10	0/10
Mineralization, minimal	0/10	0/10	2/10	0/10
Proteinaceous material in tubules, minimal	4/10	4/10	6/10	3/10
Lacrimal glands				
Harderian gland alteration	6/10	4/10	1/10	3/10
Liver				
Inflammatory focus(i), minimal	0/10	1/10	0/10	0/10
Necrosis, focal, minimal	1/10	0/10	0/10	0/10
Lungs				
Mineralization-pulmonary vessels, minimal	1/10	4/10	0/10	1/10
Mammary glands				
Glandular hyperplasia, mild	0/10	0/10	0/10	1/10
Mandibular lymph nodes				
Sinus erythrocytosis, minimal	1/10	1/10	0/10	0/10
Prostate				
Lymphocytic infiltration, mild	1/10	1/10	–	–
Spleen				
Hemosiderosis, minimal	0/10	0/10	3/10	2/10
Thymus				
Lymphoid necrosis, focal, mild	0/10	0/10	1/10	0/10
Thyroid gland				
Ultimobrachial cysts	2/10	1/10	1/10	1/10
Uterus with cervix				
Dilatation	–	–	2/10	3/10

to body and brain weights of liver in males at 2000 mg/kg/day were significantly reduced when compared to the control. No histopathological findings were seen in the livers of all males except one minimal necrosis in the control and one minimal inflammatory focus in the 2000 mg/kg/day group. In addition, no adverse findings were noted in these two groups of male rats in terms of clinical chemistry parameters that are related to compromised liver function, such as T. Bil, ALT, AST, ALP, and GGT.[24] No changes in liver weights were noted in treated females. Incidental reduction in liver weight was also seen in females given 2500 mg GOS/kg/day in a study of another GOS product.[21] Thus, the reductions of liver weight in males at 1000 and 2000 mg/kg/day dose from this study were not considered to be of toxicological significance. Other statistically significant changes in organ weights were not considered to be test article-related adverse effects because they were single incidences.

Because the cecum is an area of significant bacterial fermentation, cecal hypertrophy/hyperplasia is thought to occur because of the increased amounts of short-chain fatty acids that are produced by bacterial fermentation after large amounts of nonadsorbed carbohydrate and dietary fiber enter the caecum and colon.[25–27] Increased concentrations of short chain fatty acids and enhanced ion absorption due to decreased pH can alter the osmotic balance of the GI tract and enhance the fluid volume of enterocytes. Therefore, in the current study, the histological changes seen in the cecum of high-dose animals, although related to test article administration, is considered an adaptive rather than toxic response.

Cecal enlargement is a common finding in toxicology studies of GOS products. An oral dose of 2000 mg Oligomate/kg/day (825 mg GOS/kg/d) in Sprague-Dawley rats increased cecum weight over 90 days.[20] Cecal enlargement was also seen in male and female Wistar rats when feeding GOS syrup in diets at levels of 1600, 3200, and 6100 mg/kg/day (684, 1368, and 2608 mg GOS/kg/day),and 1800, 3600, and 6900 mg/kg/day (770, 1539, and 2959 mg GOS/kg/day) in female and male rats, respectively.[22] In general,

feeding GOS at levels from 2.96% to 20% in the diets of rats will result in an increased cecum weight.[8,28–36] Chonan and Watanuki[32] also found cecal hypertrophy after administering approximately 650 mg GOS/kg/day to Wistar for 30 days. Feeding a diet containing 2% GOS has a similar effect on cecal weight increase in pigs.[37] GOS-related effects reported in studies of GOS products (cecal weight increase) are well-established physiological effects that are consistent with the transport of resistant sugars/carbohydrates to the colon and are widely recognized as not being toxicologically relevant to humans.[38]

The cecum is not found in humans, although in rats, it is the site of fermentation for non-digestible substances. Therefore, cecal enlargement along with mucosal hypertrophy and hyperplasia has been observed as a response in several rodent species to food ingredients other than GOS such as modified starches, polyols, some fibers, and lactose; these ingredients share the feature of being poorly absorbed and osmotically active.[39] Many studies have demonstrated that consumption of pectin,[40] malitols, glucomannan, cellulose,[41–43] fructans,[44] and wheat bran[45] can cause mucosal hyperplasia/hypertrophy of caecum/colon in rats. It is noted that enhanced colonic mucosal growth (hypertrophy/hyperplasia) was found in rats fed dietary fiber cellulose and wheat bran that have been previously shown to inhibit the development of genotoxin-induced colonic neoplasia in rats.[46] It has also been reported that mucosal hypertrophy in rodents represents a physiological adaptation to increased osmotic forces when high doses of undigestible substances are consumed; the effect is reversible after test article is withdrawn from the diet.[39,47,48]

The histopathologic features of the cecal enlargement noted in the current study are indicative of an adaptive response. Mucosal hypertrophy/hyperplasia was characterized by an increase in cell density and mitotic activity within the crypts involving diffuse areas of the mucosa and/or by the presence of elongated mucosal glands with increased height of surface columnar cells. The cytoplasm of epithelial cells had increased basophilia with slightly elongated or vesicular nuclei. Importantly, in the current study, there were no polyps observed in the cecum. These pathologic features are produced by other nondigestible substances that produce characteristic increases in cecal crypt depth, circumference, number of crypts, and number of cells per crypt in the cecum.[27]

Therefore, hypertrophy/hyperplasia without atypical cellular features represents a compensatory and adaptive response to a large amount of GOS, consistent with the effects seen with other poorly absorbable carbohydrates.[47] Thus, the observed cecal hypertrophy/hyperplasia, without evidence of polyps, is considered compensatory and not preneoplastic and, although test article-related, is not considered to be a toxic response.

In conclusion, the NOAEL for VITAGOS™ following oral gavage is estimated to be 4082 mg/kg body weight/day and the NOAEL for GOS following gavage of VITAGOS™ is estimated to be 2000 mg/kg body weight/day under the test conditions employed.

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Supplemental material

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