Article

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Safety evaluation of α-galacto-oligosaccharides for use in infant formulas investigated in neonatal piglets

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Abstract

Galacto-oligosaccharide (GOS), comprising galactoses with a glucose or sucrose, is a family of nondigestible oligosaccharides. The present study evaluates the safety of an α-GOS product (P-GOS® P) in a neonatal piglet model for 3 weeks. Three days after birth, neonatal piglets were divided into control and treated groups and provided with swine milk replacers in the absence and presence of 8 mg/mL—of the α-GOS product, respectively. An increase in the weight of the large intestines in treated males was noted, which is a common finding in studies of animals fed nondigestible oligosaccharides. There were no α-GOS product-related adverse effects in the piglets in terms of clinical signs, body weights, feed consumption, clinical chemistry, hematology, organ weights, or histopathology. The study demonstrated that formula supplemented with 8 mg/mL of P-GOS P is safe and well tolerated in neonatal piglets and supports the safe use of P-GOS P in infant formulas.

Keywords

Galacto-oligosaccharide, safety, piglet, infant formula, prebiotic, nondigestible carbohydrate

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Introduction

Galacto-oligosaccharides (GOSs) are a group of nondigestible oligosaccharides consisting of galactose units linked via glycosidic bonds to galactose, a terminal glucose, or a sucrose. The linkages are not digested by human and animal pancreatic or intestinal enzymes.[1–4] Based on the configuration of the linkages, GOS exists in the form of α- or β-GOS. β-GOS is a β-linked sugar, which is usually derived from enzymatic hydrolysis of lactose.[5,6] The raffinose family oligosaccharides (RFOs), including raffinose, stachyose, and verbascose, are a group of α-GOS that are naturally present in grains and legumes, ranging from 5% to 8% of dry matter (DM).[7–10] They are linked by a galactose or galactoses and a sucrose via α-1,6-glycosic bonds (Figure 1) and are subject to fermentation processes by the gut microflora.[4] RFO has been shown to have prebiotic properties, due to their indigestible nature, which beneficially influence the composition of the gastrointestinal microflora.[11–13] Melibiose,[14] the nonfructosylated raffinose (Figure 1), has been found to be similar to raffinose in significantly increasing beneficial microbiota *Bifidobacterium* and decreasing fecal putrefactive products such as p-cresol, indole, and succinic acid. Similar to other nondigestible carbohydrates, RFO is an important factor in the production of flatulence caused by consuming legumes.[15] As a key bacteria in intestinal tract of humans, *Clostridium perfringens* have been shown in vitro to produce significant amount of gas when raffinose was a substrate. However,

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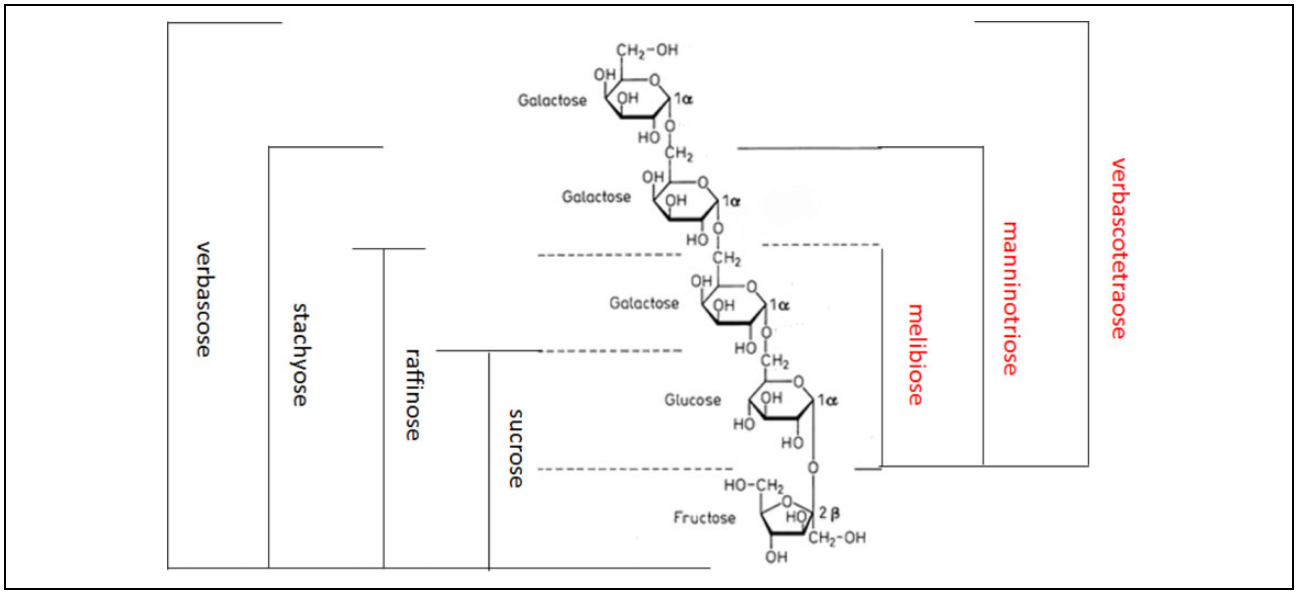

Figure 1. Structure of α-linked GOS. GOS: galacto-oligosaccharide.

melibiose did not promote gas formation, suggesting that the fructose moiety present in raffinose was responsible for the gas production.[16]

In addition to melibiose, the nonfructosylated raffinose family of α-GOS includes manninotriose and verbascotetraose (Figure 1). This group of carbohydrates has been found naturally in foods, such as cocoa beans[17] and raw and processed soybeans,[4,8,18,19] and some plants consumed for health benefits.[20,21] European Food Safety Authority (EFSA) has concluded that this group of α-GOS is resistant to hydrolysis and absorption in the small intestine and therefore does not contribute to postprandial glycemic responses as compared to sugar.[22]

Raffinose has been used in infant diets in Japan for many years including regular (http://www.hagukumi.ne.jp/eng/products/hagukumi/hagukumi.shtml, accessed 6 February 2017), peptide (http://www.hagukumi.ne.jp/eng/products/ebaby/ebaby.shtml, accessed 6 February 2017), and hypoallergenic lactose-free (http://www.hagukumi.ne.jp/products/specialmilk/newma1.shtml, accessed 6 February 2017) infant formulas. The Organization for Economic Cooperation and Development (OECD) has reported that the 90-day rodent toxicology study of α-GOS provides pivotal safety information.[23] The neonatal piglet, however, has been accepted as the best preclinical model of infant development,[24,25] and it has been used in the investigation of β-GOS.[26] This model has been accepted by various government agencies (the US Food and Drug Administration (FDA), US Environmental Protection Agency, and the OECD) in support of infant formula ingredient safety. Therefore, a study of the nonfructosylated α-GOS in neonatal piglets corroborates the safety and tolerance of this product for ingestion by human infants.

In the current study, both female and male 3-day-old piglets were fed swine milk replacer in the absence and presence of an α-GOS product, a mixture of nonfructosylated α-GOS 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.

Materials and 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 killed 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).

Animals and husbandry

Farm piglets (Yorkshire crossbred) were employed in the current study for α-GOS since this species has been identified to be an appropriate neonatal model for safety evaluation of ingredients in infant formula.[27] 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

Table 1. Diets design for all animals.

Number of males	Number of females	Description	Dose concentration (mg/mL)	Dose volume (mL/kg/day)
6	6	Control	0	500
6	6	α-GOS	8	500

GOS: galacto-oligosaccharide.

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 the actual body weights were recorded. Each animal was acclimated to the feeding containers at arrival and as needed until eating satisfactorily on its own. 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 25°C (77°F) and a relative humidity range of approximately 30–70%. Lighting controls were set to maintain a 12-h light/12-h dark cycle.

Diets

All animals were offered swine milk replacer (the milk replacer was prepared from mixing 1.8 pounds of Solustart® II in 1 gallon of warm water. All the formulations were prepared the day prior to dosing and stored refrigerated overnight and were allowed to warm under ambient conditions for at least 30 min prior to administration; Solustart II; Land O' Lakes Animal Milk Products Co, Shoreview, Minnesota, USA) alone or supplemented with an α-GOS product for three consecutive weeks at a dose volume of 500 mL/kg/day (Table 1). 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. Thereafter, a commercially available automatic feeding system was employed to provide the animals with the required daily volume of dosing formula. The control group received base formula alone, while test group received the same formula supplemented with 8 mg/mL of an α-GOS product (P-GOS® P (P-GOS P contains minimum 95% DM and 90.25% α-GOS including 95% DP3 (manninotriose) + DP4 (verbascotetraose), maximum 0.5% (% DM) other sugar, 0.5% (% DM) crude protein, and 0.5% (% DM) ash.); Olygose, Venette, France). The level of α-GOS product used in the current study is based on the levels used in other infant clinical studies[28,29] and proposed levels of β-GOS products in several Generally Recognized as Safe (GRAS) determinations that received "no questions" letters from the FDA[30,31,32] 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).

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.

Feed efficiency

$$\text{Percent FE} = \frac{\text{Mean BWG (g)}}{\text{Total mean FC (g)}} \times 100 \qquad (1)$$

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

Compound consumption

$$\text{CC (mg/kg/day)} = \frac{\text{Total mean FC (g)} \times \text{Conc. (mg/mL)}}{\text{Interval} \times \text{density (g/mL)} \times \text{mean BW (kg)}} \qquad (2)$$

where CC is the compound consumption (mg/kg/day) also designated as dose level; FC is the feed consumption (g)—total amount of feed consumed

during study period; Conc. is the concentration (mg/mL)—test article in the milk replacer with α-GOS product (8 mg/mL); interval is the duration of administration (21 days); density is the density of test article formula (g/mL) and milk replacer with α-GOS product (1.0465 g/mL) measured at preparation; and BW is the body weight (kg)—mean body weight for the 21-day study period.

Blood collection

On day 22, blood for clinical chemistry and hematology was collected via the brachiocephalic trunk just prior to the scheduled necropsy. Hematology and clinical chemistry parameters measured are listed below:

	Parameters
Hematology	Red blood cell (RBC) count, white blood cell (WBC) count, 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), and automated differential leukocyte count (absolute and relative) including neutrophil (NEUT), lymphocyte (LYMPH), monocytes (MONO), eosinophils (EOS), large unstained cell (LUC), basophils (BASO), prothrombin time (PT), activated partial thromboplastin time (aPTT), and fibrinogen (F)
Clinical chemistry	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), and triglycerides (TRIGs)

Postmortem

All surviving 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 Fatal Plus® (Vortech Pharmaceuticals, Dearborn, Michigan, USA) at a dose level of 100 mg/kg 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). Tissues marked with asterisk (*) in the tissue processing section were weighed at scheduled necropsy, and organ to body and organ to brain weight ratios were calculated. Paired organs were weighed together. In addition, 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.

Safety evaluation of galacto-oligosaccharides tissue processing	
Adrenals*	Parathyroids* (when available)
Brain* (forebrain, midbrain, and cerebellum)	Small intestine (stomach to cecum)
Gross lesions	Large intestine (cecum and colon)
Kidneys*	Spleen*
Liver*	Testes*
Lymph nodes (mesenteric)	Thymus*
	Thyroid*

Histopathology

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

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 is 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. If a variance-stabilizing transformation was not found, another suitable test was performed. Experimental results are expressed as means with their standard deviations. Analyses were performed using Systat® (Systat, San Jose, California, USA).

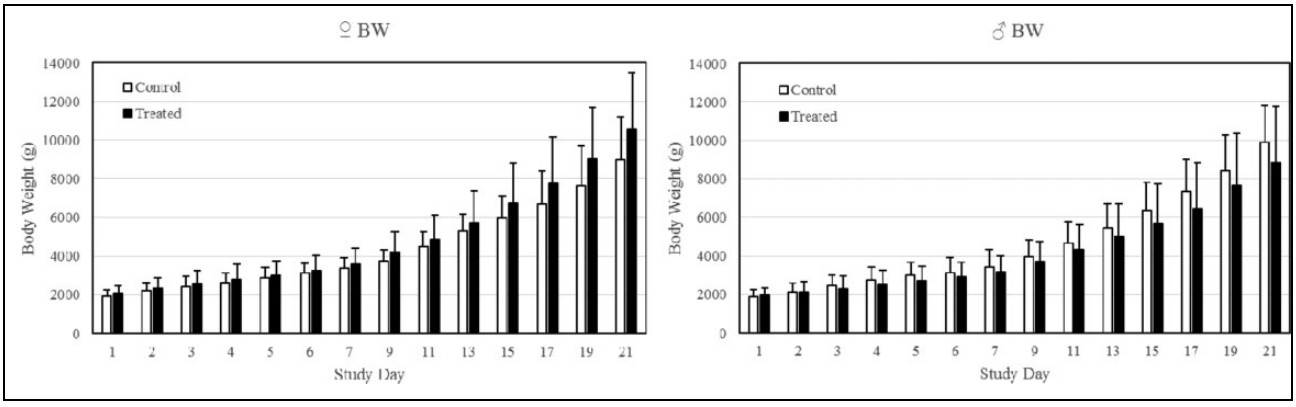

Figure 2. Female (above) and male (below) mean BW during study. BW: body weight.

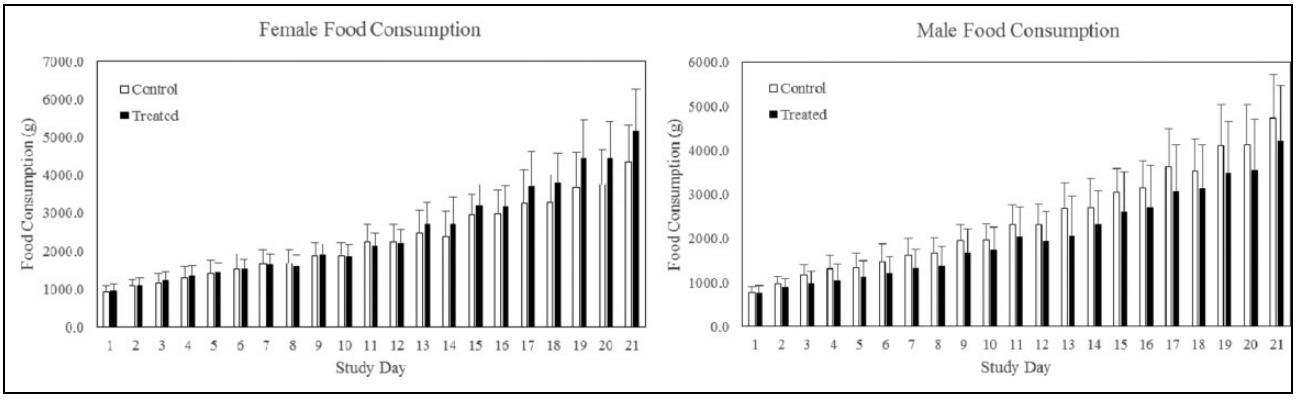

Figure 3. Female (above) and male (below) mean feed consumption during study.

Results

Clinical observations and viability

All animals were normal at study initiation with an 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 administration; however, in the control females, it was noted up to 2 weeks into the dosing period and ranging 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 administration. 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 administration.

Body weight, feed consumption, and feed efficiency

The mean body weights of female and male in the α-GOS-treated group were slightly higher and lower, respectively, than in the control group (Figure 2); 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 3).

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 α-GOS-treated piglets (15.8%).

α-GOS consumption

The mean α-GOS product (P-GOS P) consumption for male and female piglets was 3697 and 3900 mg/kg/day,

Table 2. Summary of clinical chemistry.

	Male		Female	
Parameters	Group 1, control (0 mg/mL)	Group 2, α-GOS (8 mg/mL)	Group 1, control (0 mg/mL)	Group 2, α-GOS (8 mg/mL)
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[a]	426 ± 150.1	437 ± 72.6
ALT (U/L)	24 ± 2.1	20 ± 1.9[a]	20 ± 1.7	23 ± 3.9
AST (U/L)	32 ± 5.0	22 ± 2.9[a]	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 ± 67.0	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[a]	25.6 ± 7.3	25.0 ± 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[a]	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 ± 0.3	4.5 ± 0.4	4.7 ± 0.3	4.9 ± 0.4
TRIG (mg/dL)	32 ± 5.2	51 ± 13.0[a]	31 ± 19.6	48 ± 15.9
BUN (mg/dL)	17 ± 2.2	14 ± 2.3[a]	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 ± 1.0	5.7 ± 0.8	6.5 ± 0.6
Cl (mmol/L)	105 ± 1.5	104 ± 1.9	105 ± 1.5	105 ± 1.9

GOS: galacto-oligosaccharide; 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.
[a]Significantly different from the control ($p < 0.05$).

respectively, and the consumption of α-GOS was 3336 and 3520 mg/kg/day, respectively.

Hematology and clinical chemistry

All the clinical chemistry parameters are listed in Table 2, and those that were statistically significantly different between control and treated males include alkaline phosphatase (ALKP) 491 ± 47.9 versus 420 ± 37.9 U/L, alanine aminotransferase (ALT) 24 ± 2.1 versus 20 ± 1.9 U/L, aspartate aminotransferase (AST) 32 ± 5.0 versus 22 ± 2.9 U/L, gamma glutamyl transferase (GGT) 30.8 ± 6.1 versus 22.5 ± 6.1 U/L, lactate dehydrogenase (LDH) 669 ± 86.1 versus 531 ± 40.1 U/L, TRIG 32 ± 5.2 versus 51 ± 13.0 mg/dL, and blood urea nitrogen (BUN) 17 ± 2.2 versus 14 ± 2.3 mg/dL, respectively. No statistically significant changes were noted between females in the control and treated groups.

In the treated males, a significant change in the hematocrit (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. No statistically significant changes in hematology parameters were noted in females between the control and treated groups.

Organ weights

Organs with a statistically significant difference between control and treated animals are shown in Table 3. Administration of α-GOS resulted in an increase in intestinal weight, particularly large intestines; this finding is expected because of the undigestible nature of the test article. In addition, the absolute adrenal weight (0.89 ± 0.14 g in control v.s 1.04 ± 0.08 g) and organ to brain weight ratio (1.73 ± 0.23 in control vs. 1.98 ± 0.13) in the treated males were statistically increased compared to controls; however, the organ to body weight ratio (0.008 ± 0.001 in control vs. 0.012 ± 0.004) for the adrenals was not statistically different.

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 are neonatal animals. There were a few findings in all groups that are considered incidental and not related to the test articles. One control male showed pigmentation in the left kidney. One treated male had minimal diffuse

Table 3. Summary of absolute and relative organ weights of intestines and adrenals.[a]

Sex	Tissue	Organ weight	Group 1, control (0 mg/mL)	Group 2, α-GOS (8 mg/mL)	Percentage 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 t (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[b]
		% 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[b]
		% Body wt	0.008 (±0.001)	0.012 (±0.004)	50
		% Brain wt	1.73 (±0.23)	1.98 (±0.13)	14[b]
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

GOS: galacto-oligosaccharide; Wt: weight; Abs: absolute; SD: standard deviation.
[a]Mean values (±SD).
[b]Significantly different from control ($p < 0.05$).

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 the kidney.

Discussion

Neonatal farm piglets (Yorkshire crossbred) were selected as test species in current study because they provide an opportunity to observe the interaction between nutrient requirements and metabolic immaturity, a situation relevant to the assessment of safety and tolerability for the human infants.[33] However, there are some differences between neonatal piglets and human infants such as organ weight ratios.[34] Therefore, the faster growth rate of organs in piglets than in human infants may increase the sensitivity of the piglet to any potential adverse effects of test article on organ growth, composition, and function.

All animals grew normally throughout the entire study period. The incidence of diarrhea in the first week of the study is a common finding because the young animals are acclimating to the milk replacers and new environment.[35,36] The slightly higher feed efficiency in females from the treatment group and unchanged feed efficiency in males from the treatment group indicate that the α-GOS product was well tolerated and piglets grew normally throughout the study period. In a 3-week study,[37] feeding young growing castrated male pigs dietary fructo-oligosaccharides and β-GOS caused a reduction in body weight and feed conversion and tended to reduce feed intake in the first week, yet all the parameters of the treated groups were not significantly different from control groups during the reminder time of the study. It is also noted in rat studies that feeding oligosaccharides up to 10% of the diets did not affect the body weight and feed intake, although it resulted in significantly increased cecal weights[38–40].

The α-GOS product did not produce treatment-related adverse findings in clinical chemistry or hematology. There were some statistically significant reductions in selected clinical chemistry parameters between control and treated males. However, toxicity is associated with elevation in these values[41,42]; therefore, the decreases noted in ALKP, ALT, AST, GGT, and LDH are not considered toxicologically or clinically meaningful. The reduction in BUN value may be due to the increased nitrogen utilization by a larger microbial population induced by oligosaccharide intake.[43] 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. Additionally, no statistically significant changes in chemistry parameters were noted in females from the treated group compared to controls.

In the treated males, significant changes in the hematocrit, relative MONO, and relative EOS were noted compared to controls. 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.[36, 44,45] Therefore, the findings are most likely associated with normal biological variation rather than an adverse treatment-related effect. No statistically significant changes in these hematology parameters

Table 4. Summary of intestinal contents pH.

Sex	Description	Group 1, control (0 mg/mL)	Group 2, α-GOS (8 mg/mL)
Male	Cecum pH	6.23 ± 0.19	5.87 ± 0.25
Male	Colon pH	6.69 ± 0.29	6.11 ± 0.25
Female	Cecum pH	6.39 ± 0.18	6.21 ± 0.51
Female	Colon pH	6.79 ± 0.28	6.54 ± 0.57

GOS: galacto-oligosaccharide.

were noted in treated females compared to control. 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 α-GOS-treatment-related effects and were most likely attributed to normal biological variation.

A significant increase in weight of the large intestine relative to body weight was noted in treated males compared to controls. This finding is commonly seen with substances that are incompletely digested and poorly absorbed in small intestine and thus are subjected to microbial metabolism in both cecum and colon. The effect of oligosaccharides on intestines has been studied extensively in the rat model, and it is common to see weight increase and/or enlargement of intestines, particularly in the large intestine (e.g. cecum and/or colon).[35–37,46,47] Nondigestible oligosaccharides increase microbial fermentation and result in the production of osmotically active by-products, for example, short-chain fatty acids, which can cause soft stools and cecal weight increase/enlargement.[48] However, the functional or morphological changes are reversible when diets are returned to normal.[47] 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.[49] 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 shown in Table 4 also corroborates the effect of GOS on the microbial metabolic products.

In males, the absolute and relative to brain weights of the adrenal glands were statistically elevated compared to controls; however, the organ to body weight ratio for the adrenals was not statistically different in treated compared to control animals. The absolute adrenal weight for the control males was slightly lower than that for the control female (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 adrenal cortex and 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.

Histopathological evaluation did not reveal any adverse changes that could be attributed to the treatment with the α-GOS product.

In conclusion, infants are exposed to endogenous RFO at low levels when they are consuming soy-based infant formulas,[50,51] but the intake level is not known, although RFO has been historically used in infant formulas on the Japan market (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). β-GOS has been detected in human milk from lactating women,[52] and the intended use level of 7.2-g β-GOS/L infant formulas was proposed in several GRAS determinations, which received "no questions" letters from the FDA[30–32] and was approved by the EU commission (http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32016R0127, accessed 17 March 2017).

α-GOS product is another source of oligosaccharides that may have application in infant formulas and general foods. The current study demonstrated that formula supplemented with 8 mg/mL of P-GOS P is safe and well tolerated in neonatal piglets and supports the safe use of P-GOS P in infant formulas.

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