Food and Chemical Toxicology 46 (2008) 3132–3139

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Safety evaluation of oligofructose: 13 Week rat study and *in vitro* mutagenicity

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ARTICLE INFO

Article history:
Received 27 March 2008
Accepted 25 June 2008

Keywords:
Oligofructose
Safety
Prebiotics
Bifidobacteria
Ames test
CHO

ABSTRACT

Oligofructose (OF), comprised of fructose oligomers with a terminal glucose unit, is a family of oligosaccharides derived from the hydrolysis of inulin. Consumption of OF in animals and humans increases colonic bifidobacteria levels. The present study evaluates the safety of OF in both a 13 week rat feeding study and using *in vitro* mutagenicity tests. Fecal bifidobacteria levels were also determined by *in situ* hybridization to assess a biological function of OF. Rats received either a control diet or diets containing one of four doses of OF. Total, HDL, and LDL-cholesterol levels were significantly lower at several time points during the study in groups receiving OF compared to controls with the largest effects occurring in the high dose male animals. Weight gain in the male high dose group was significantly lower at early time points compared to controls but not significantly different at the end of study. As expected, cecal weights increased in a dose-related manner and fecal bifidobacteria levels also demonstrated a dose-related increase. There were no consistent differences in gross pathology or histopathology related to dietary OF. OF did not induce a positive response in the Ames test or chromosomal aberration test with CHO cells. These results demonstrate no adverse effects of OF.

1. Introduction

Prebiotics are non-digestible, fermentable food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of potentially beneficial colonic bacteria, such as bifidobacteria and lactobacilli (Gibson and Roberfroid, 1995). Some studies have reported a concomitant decrease in potentially pathogenic bacteria such as clostridia or coliforms (Gibson, 1999; Losada and Olleros, 2002). Like other soluble fibers, non-digestible oligosaccharides may also reduce constipation, reduce plasma cholesterol and triglyceride concentrations (Davidson and Maki, 1999; Jackson et al., 1999; Pereira and Gibson, 2002; Williams and Jackson, 2002) and modulate glucose metabolism (Luo et al., 1996; Yamashita et al., 1984; Rumessen et al., 1990). Additional reported effects of dietary oligosaccharides include stimulation of the immune system, increased mineral absorption, and antimutagenesis (Morohashi et al., 1998; Kaur and Gupta, 2002; Bouhnik et al., 1996; Teitelbaum and Walker, 2002).

Complex oligosaccharides are a major component of human milk, which contains 5–8 g oligosaccharides per liter (Gnoth et al., 2000; Kunz et al., 2000). These complex oligosaccharides are diverse in structure, with more than 100 different species being present in human milk. Some of these structures mimic the oligosaccharide profile of enterocytes and bind to pathogens, thus blocking the binding and subsequent internalization of pathogens across the intestinal barrier (Kunz et al., 2000). In addition to directly neutralizing pathogens, human milk supports a favorable intestinal flora population, dominated by a bifidobacteria and lactobacilli microbial pattern that is beneficial for the infant's developing intestinal and immune systems (Aggett et al., 2003). It will be extremely difficult to mimic the complex oligosaccharide composition of human milk, however, it is possible that less complex oligosaccharides will have at least some beneficial activities if added to infant formula. Some studies have demonstrated that infants receiving formulas without non-digestible oligosaccharides have fecal bifidobacteria and lactobacilli levels similar to breast-fed infants, (Balmer et al., 1989; Bettler and Kullen, 2007) while other studies have shown that some formulas do not support this type of bacterial population. Addition of non-digestible oligosaccharides may improve the bacterial profile in such cases. Several investigators reported that infants fed oligosaccharide supplemented

Abbreviations: AIN, American Institute of Nutrition; ANOVA, analysis of variance; CFR, code of federal regulations.

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doi:10.1016/j.fct.2008.06.079

infant formula had higher stool counts of bifidobacteria and lactobacilli. Despite the extensive use of prebiotic-supplemented formulas in Japan and their recent addition to formulas in Europe (Ghisolfi, 2003), few safety studies have been published. Since limited preclinical safety data are available (Carabin and Flamm, 1999; Tokunaga et al., 1986), we evaluated the safety and efficacy of OF in rats fed diets containing up to 9.9% OF for 13 weeks.

2. Materials and methods

2.1. Study design

OF was administered to male and female rats for 13 weeks to evaluate its safety profile. Purified diets containing four levels of OF were compared to the same diet containing no OF. An additional group that received standard rodent chow was included for reference to laboratory data. The study was conducted at Wyeth-Ayerst Research, Drug Safety Evaluation, Chazy, New York, USA, in accordance with the Food and Drug Administration *Good Laboratory Practice for Nonclinical Laboratory Studies* (Part 58 of 21 CFR and amendments). This study met the guidelines for The Food and Drug Administration *Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Food*, *"Redbook II"*. All procedures were conducted in compliance with Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals, and the Office of Laboratory Welfare Act.

2.2. Test materials

The OF (Raftilose 95; Orafti, Tienen, Belgium), derived from chicory root inulin by enzymatic hydrolysis, contained 95% dry matter, and was composed of glucose and fructose (0.1%), sucrose (5.6%) and oligofructose (94.3%). The degree of polymerization (DP) ranges from 2 to 7 fructose units/oligomer with an average DP of 4–5 fructose units/oligomer (Carabin and Flamm, 1999).

2.3. Animals

Five week-old Sprague–Dawley CD® rats, purchased from Charles River Canada (Quebec, Canada), were fed standard rodent chow (Certified Rodent Diet #5002, PMI Feeds, Inc., Richmond, IN, USA) ad libitum for a 2-week pretest acclimation period. At study initiation, animals were seven weeks old, and ranged in weight from 191 to 287 g (males) and from 155 to 197 g (females). These animals were randomly assigned to a test diet group that contained 0% OF, or one of four doses of OF. A reference group continued to receive the standard rat chow for the duration of the study. There were 20 animals per sex in each diet group, and males and females were randomized separately. Animals were housed individually in stainless steel cages. The rooms were controlled for temperature (22 °C ± 2 °C), relative humidity (50% ± 10%) and lighting (12 h light/dark cycle). Water and diets were provided ad libitum. The diets were fed for 13 weeks, and were replaced each week.

2.4. Test diets

Bio-Serv, Inc. (Frenchtown, New Jersey, USA) prepared the purified test diets, which were stored at 6 ± 4 °C. All diets were fed in the powdered form. The AIN-93G mineral mix and AIN-93 vitamin mix were modified to replace sucrose with cornstarch to permit accurate quantitation of dietary OF, an assay based on the determination of fructose after enzymatic hydrolysis of OF. Cornstarch, the primary source of carbohydrate for the test diets, was replaced with increasing quantities of OF. Test diets A through E provided 0%, 0.55%, 1.65%, 4.96% and 9.91% OF (w/w), respectively. Table 1 provides the composition of the diets. The targeted dose of OF for addition to infant formula was 3 g/l and the levels of OF studied represented 1, 3, 9 and 18 times the targeted dose.

To measure uniformity, OF concentrations were measured in samples taken from the top, middle and bottom of each batch of test diet. OF stability and content were measured in samples taken from the first and last containers of each test diet, and from samples of test diets stored at room temperature for one week. Analyses of OF content were performed by Covance Laboratories (Madison, WI, USA) in accordance with good laboratory practice (GLP).

Table 1
Composition of test diets (g/kg)

Diet ingredients	0% OF A	0.55% OF B	1.65% OF C	4.96% OF D	9.91% OF E
Casein	200	200	200	200	200
Cornstarch	497.5	492	481	447.9	398.4
Dextrinized cornstarch (Lo-dex 10)	132	132	132	132	132
Fiber	50	50	50	50	50
OF	–	5.5	16.5	49.6	99.1
AIN 93G modified mineral mix[a]	35	35	35	35	35
AIN 93G modified vitamin mix[a]	10	10	10	10	10
Soy oil	70	70	70	70	70
Choline bitartrate	2.5	2.5	2.5	2.5	2.5
L-cystine	3.0	3.0	3.0	3.0	3.0

[a] Sucrose replaced with cornstarch.

2.5. Ante-mortem observations

From the pretest period (weeks −2 and −1) through week 13, all rats were observed at least twice daily for mortality, and at least once daily for gross motor and behavioral activity, and for changes in appearance. Individual food consumption and body weight were measured daily from week −1 through week 13. Individual water consumption was measured daily for three consecutive days during weeks −1, 6 and 12. Once each week from the pretest period through week 13, a detailed physical examination was performed on all animals. Ophthalmoscopic examinations were performed on all rats during week −2, and during weeks 5 and 11. Fecal samples (approximately 2 g) were collected from the first 10 males of each of the OF diet groups during week −1 and week 12. The samples were collected into plastic cryovials and were stored at −80 °C.

Blood samples for hematology and serum clinical chemistry measurements were collected from the retro-orbital sinus of 10 male and 10 female animals from each diet group during weeks −1, 1, 6 and 13. Hematology measurements included red blood cell count, reticulocyte count, hemoglobin, hematocrit, mean cell volume, mean cell hemoglobin, mean cell hemoglobin concentration, red cell distribution width, differential, platelet count, white blood cell, lymphocyte, monocyte, eosinophil and basophil counts. Clinical chemistry measurements included alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, glucose, blood urea nitrogen, creatinine, blood urea nitrogen/creatinine ratio, total protein, albumin, total globulin, albumin/globulin ratio, total bilirubin, direct bilirubin, indirect bilirubin, cholesterol, high-density lipoproteins (HDL), low-density lipoproteins (LDL), LDL/HDL ratio, triglycerides, sodium, potassium, chloride, calcium and inorganic phosphorous. Coagulation measurements, prothrombin time (PT), activated partial thromboplastin time (APTT) and fibrinogen (FBGN), were determined on blood samples collected at necropsy.

2.6. Post-mortem observations

At study termination, all rats were anesthetized with carbon dioxide and were exsanguinated. A complete necropsy was performed that included examination of the external surface of the body, all orifices, the cranial, thoracic and abdominal cavities, adrenal glands, aorta (thoracic), bones and joints (femoral–tibial joint), bone marrow (sternum), brain, cecum, cervix, colon, duodenum, epididymides, esophagus, eyes, heart, ileum, jejunum, kidneys, liver, lungs, lymph nodes (cervical and mesenteric), mammary gland, ovaries, pancreas, parathyroid glands, peripheral nerve (sciatic), pituitary gland, prostate gland, salivary gland, seminal vesicles, skeletal muscle (thigh), skin, spinal cord (cervical and lumber), spleen, stomach (squamous and glandular), testes, thymus, thyroid glands, tongue, trachea, urinary bladder, uterus, and vagina. All tissue lesions were examined. The adrenal glands, brain, heart, kidneys, liver, ovaries, pituitary gland, testes, thyroid/parathyroid glands and cecum (empty) were weighed.

Representative samples from each animal for all of the above listed organs and tissues were fixed in 10% neutral buffered formalin. Fixed organs and tissues from the 0% OF and highest dose OF Diet group (Groups A and E) were embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined microscopically. Any tissue from animals in groups B, C, and D, which showed gross abnormalities, was examined microscopically. Histopathologic lesions were classified with standard pathological nomenclature.

2.7. Hybridization probe

The *Bifidobacterium* genus-specific probe, Bif 164 (Langendijk et al., 1995), was used to quantify the bifidobacterial content of the fecal samples. The probe was commercially synthesized, and was labeled with the fluorescent dye Cy3 Indocarbocyanin (Eurogentec Nederland b.v., Maastricht, The Netherlands). The total bacterial content of each sample was measured using the nucleic acid stain 4′, 6-diamidino-2-phenylindole (DAPI; Porter and Feig, 1980).

2.8. Fluorescent in situ hybridization (FISH) methodology

Fecal samples were homogenized 1/10 (w/v) in phosphate buffered saline (PBS) and centrifuged (1500 rpm, 2 min) to remove particulate matter. Bacterial cells were fixed overnight using 4% paraformaldehyde solution (1/3 [v/v]), then were washed twice in 1 ml of filtered PBS (13,000 rpm, 5 min) and resuspended in 150 μl of filtered PBS. An equal volume of 96% ethanol was added to the cell

solution and mixed thoroughly. Sixteen microliters of sample were mixed with 64 μl of pre-warmed HPLC water and 200 μl of pre-warmed, filtered 2 × hybridization buffer containing SDS to form the hybridization mixture, which was maintained at 50 °C (hybridization temperature). Ninety microliters of the hybridization mixture were hybridized overnight with 10 μl of Bif 164 probe (50 ng/μl). A portion of the hybridized sample (between 20 and 100 μl) was then washed in 5 ml of filtered, pre-warmed hybridization buffer (without SDS) containing 20 μl of DAPI. The wash mixture was incubated at 50 °C for 30 min to allow staining of all bacterial cells by DAPI. The sample was then applied to an Isopore membrane filter (Millipore Corp., Billerica, MA, USA), placed on a glass microscope slide, and covered with a drop of Slow Fade and a cover slip. Slides were stored in the dark at 4 °C to minimize fading, prior to examination using a Nikon Microphot EPI fluorescent microscope and Fluor 100 lens. Fifteen fields of view were randomly selected for each slide, and bacterial counts were obtained for each field. The total bacterial content of each sample was determined by DAPI staining, visualized under UV light with a DM455 excitation filter. Bifidobacterial cells, hybridized with fluorescently labeled Bif 164 probe, were visualized and enumerated using a DM510 excitation filter.

The overall total bacterial and bifidobacterial loads of week −1 and 12-week fecal samples for the 0–9.91% OF groups were calculated by averaging the bacterial populations in samples collected from five rats in each dietary group. Pretest counts of total bacteria and bifidobacteria were consistent across all dietary groups. There was substantial variability in bacterial populations within each dietary group, with variations of as much of 1.5 logs in bifidobacterial numbers in samples collected from rats within the same dietary group.

2.9. Mutagenicity: Ames test

The mutagenic potential of OF (or its metabolites) was evaluated by measuring its ability to induce reverse mutations at selected loci of *Salmonella typhimurium* tester strains TA98, TA100, TA1535 and TA1537 (Ames et al., 1975), and at the tryptophan locus of *Esherichia coli* tester strain WP2 *uvr*A in the presence and absence of Aroclor 1254-induced rat liver S9 (Maron and Ames, 1983). For the mutagenicity assay (initial and confirmatory assays) a minimum of five dose levels (100, 333, 1000, 3333, 5000 μg/plate) of OF, along with appropriate vehicle and positive controls, were plated with tester strains TA98, TA100, TA1535, TA1537 and WP2 *uvr*A in the presence and absence of rat liver S9 activation. In the preliminary toxicity assay, the maximum dose tested was 5000 μg per plate. All dose levels of test article, vehicle controls and positive controls were plated in triplicate. The test system was exposed to the test article via the plate incorporation method described by Ames et al. (1975) and updated by Maron and Ames (1983). In the confirmatory assay, the test system was exposed to the test article via the preincubation methodology described by Yahagi et al. (1977).

2.10. In vitro chromosomal aberration test with CHO cells

The clastogenic potential of OF was evaluated based upon its ability to induce chromosome aberrations in Chinese hamster ovary (CHO) cells in both the absence and presence of an Aroclor-induced S9 activation system. The initial chromosome aberration assay was conducted using standard procedures (Evans, 1976) by exposing cultures of CHO cells to five concentrations of OF as well as to positive and solvent controls. The dose levels of OF tested were 313, 625, 1250, 2500, and 5000 μg/mL. The dose solution concentrations were adjusted in order to compensate for the purity. In the non-activated test system, treatment was for 4 h and for 20 h; in the S9 activated test system, treatment time was for 4 h. The confirmatory assay was conducted by exposing CHO cells to OF at dose levels of 1250, 2500, 5000 μg/mL, and to positive and solvent controls continuously for 20 h in the absence of S9 activation only. To ensure evaluation of first division metaphase cells, the dividing cells were arrested in the metaphase and were harvested for microscopic evaluation of chromosome aberrations at approximately 20 h (1.5 normal cell cycles) after the initiation of treatment. The clastogenic potential of OF was measured by its ability to increase structural aberrations in a dose–responsive manner compared to the solvent control group. OF was also assessed for its ability to induce numerical chromosome aberrations.

2.11. Statistical analyses

Statistical analyses did not include the reference group of animals fed standard rat chow. Body weight, food intake, water intake, and hematology and clinical chemistry measurements were compared among the OF diet groups at the last pretest sampling time, and at each of the subsequent sampling times using a nonparametric one-way analysis of variance and a one-sided nonparametric trend test. Similar analyses were performed for the amount of change during each time period. Analyses were done separately for each sex.

For organ weights, dosage groups were compared using a nonparametric one-way analysis of variance and a one-sided nonparametric trend test. A nonparametric one-way analysis of covariance, and a one-sided nonparametric covariance trend test where body weight was the covariate, were also performed. Analyses were done separately for each sex. Statistical analysis was not conducted on histology results.

Statistical analysis of the percent aberrant cells was performed using the Fisher's exact test with Bonferroni's adjustment. Fisher's test with Bonferroni's adjustment was used to compare pairwise the percent aberrant cells of each treatment group with that of the solvent control. In the event of a positive Fisher's test at any test article dose level, the Cochran–Armitage test was used to measure dose–responsiveness.

Statistical significance of differences in the changes in fecal bifidobacteria (Bif 164) and total bacterial (DAPI) counts were determined by a one-way ANOVA with Student–Newman–Keuls comparison test ($p = 0.05$).

3. Results

3.1. Diets

Top, middle and bottom analyses for the test diets demonstrated consistent OF concentrations in each batch. Stability studies demonstrated that the diets were stable during an entire week at room temperature, and for the duration of the study at 6 ± 4 °C.

3.2. Body weight

During the study, mean body weights of male rats fed the 9.91% OF diets were significantly lower than those fed the 0% OF diet. The mean body weights of the male rats fed 4.96% OF was significantly lower at weeks 2 through 5 (Fig. 1). The lower weights were due primarily to decreased weight gains during the first few weeks of the study; body weight gain from weeks 6 through 13 was not statistically different between groups. There were no statistically significant differences in the body weights of female rats in the different diet groups (Fig. 2).

3.3. Food, water, and OF consumption

During the first half of the study, food consumption was significantly lower in animals fed the highest levels of OF. During study week 1, male rats fed the 4.96% and 9.91% OF consumed 17% and 28% less food, respectively, than those fed 0% OF, while female rats fed the 4.96% and 9.91% OF consumed 11% and 21% less (Figs. 3 and 4). There were no biologically meaningful differences in water consumption among diet groups during the study.

In all of the OF-fed groups dietary OF concentrations remained constant during the study and as a result, the amount of OF consumed per kg body weight decreased throughout the 13 week feeding period. In males, OF intake during the first week of the study in the lowest and highest dose groups were 410 mg/kg bw/day and 5730 mg/kg bw/day respectively. At week 13, the intakes were 200 mg/kg bw/day and 3630 mg/kg bw/day at week 13. For females, these intake levels were 450 mg/kg bw/day and 6130 mg/kg bw/day at week 1 whereas they were 270 mg/kg bw/day and 5310 mg/kg bw/day at week 13.

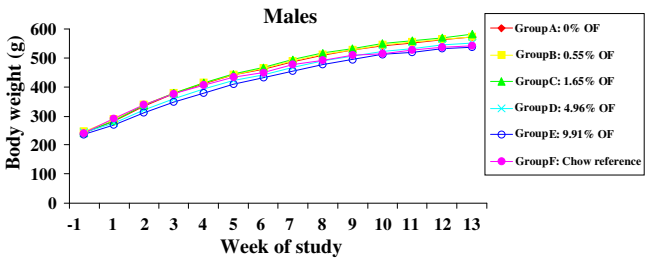

Fig. 1. Body weights of male rats fed diets containing 0–9.91% OF, or reference rodent chow from baseline (week −1) through week 13. *Group D significantly different ($p < 0.05$) from Group A during weeks 2 through 5. **Group E significantly different ($p < 0.05$) from Group A during weeks 1–13.

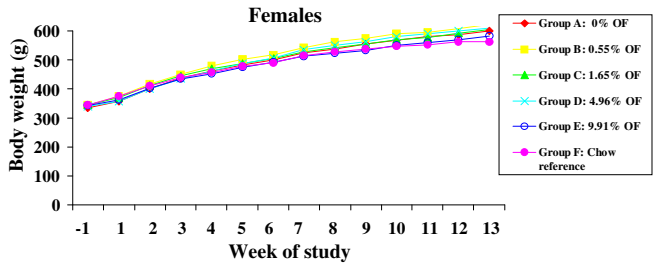

Fig. 2. Body weights of female rats fed diets containing 0–9.91% OF, or reference rodent chow from baseline (week −1) through week 13.

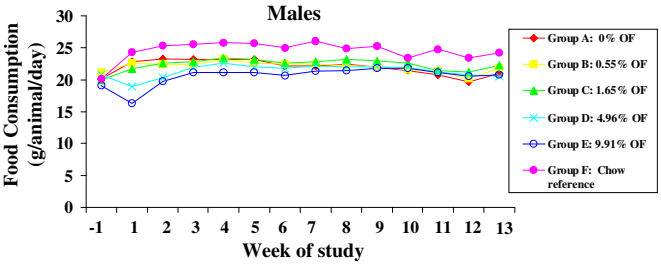

Fig. 3. Food consumption from baseline (week −1) through week 13 for male rats fed diets containing 0–9.91% OF, or reference rodent chow. *Group D significantly different ($p = 0.01$) from Group A during weeks 1 and 2. **Group E significantly different ($p = 0.01$) from Group A during weeks 1, 2 and 4.

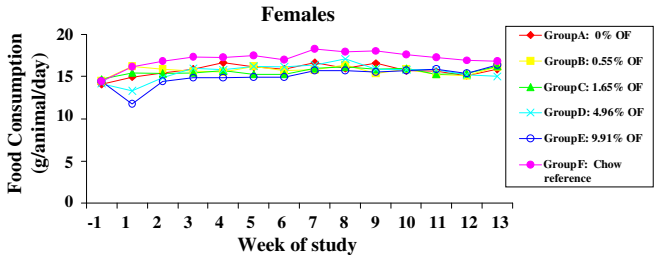

Fig. 4. Food consumption from baseline (week −1) through week 13 for female rats fed diets containing 0–9.91% OF, or reference rodent chow. *Group D significantly different from Group A during week 1. **Group E significantly different from Group A during week 1.

3.4. Clinical observations

There were no toxicologically relevant clinical observations related to the administration of OF.

3.5. Hematology and clinical chemistry

There were no changes in hematology measurements related to the administration of OF. Total, HDL- and LDL-cholesterol concentrations, and the LDL/HDL ratio were significantly lower at several time points in animals fed OF compared to animals receiving 0% OF, particularly among male rats fed the highest levels of OF (Table 2). At week 6, males fed the two highest doses of OF had significantly lower triglyceride levels (data not shown). Lower lipid levels among OF-fed rats were observed in males throughout the study, while lower levels in females were mainly evident during week 1.

Most male and female groups fed the OF-supplemented diets had significant increases in serum levels of inorganic phosphate (up to 36%) compared to those fed the 0% OF diet at weeks 1, 6 and 13 (males) and at weeks 1 and 13 (females) The values in the OF groups were similar to the chow reference group (data not shown). There were no correlations between this finding and any other clinical pathology or histopathology changes, and the finding was not considered toxicologically relevant. There were no significant differences between groups for other clinical chemistry parameters or the coagulation studies.

3.6. Organ weights, gross pathology and mortality

There was a significant dose-related increase in cecal weights in male and female rats fed the OF-supplemented diets (trend $p = 0.01$) (Fig. 5). The increase in cecal weight relative to the casein control (0% OF) ranged from 16% to 159% in the males, and from 14% to 136% in the females. There were no biologically meaningful differences among diet groups in the weights of other organs.

Among males, hepatic discoloration was present in all diet groups, ranging from 45% of animals in the casein control group to 55% of animals in the 1.65% OF group. Among the females, hepatic discoloration was present in the casein control group (15%), 0.55% and 1.65% OF groups and chow reference group (5%). Hepatic discoloration was not seen in the 4.96% and 9.91% groups. There was no dose response pattern seen, and the incidences were similar or lower in the OF-supplemented compared with the 0% OF group. Distended cecae were found in 15% of males in the 9.91% group, and none were found in any of the other groups. Gross findings were uncommon in females, and they did not follow a dose response pattern.

One male rat in the 9.91% OF group was found dead on day 79. At necropsy, the cecum was distended to about 1.5 times normal size compared to controls, in agreement with the increased cecal weights found in this group. In addition, a discolored prostate were found. Histologically, the cecum was normal, and a moderate, multifocal prostatitis was observed. The incidence and severity of prostatitis were not different among the diet groups, and the death was not considered to be related to the OF.

3.7. Histopathology

Ninety-five percent to 100% of males and females in both the 0% OF and 9.91% OF groups had hepatic fatty changes. In the other groups of males, the incidence ranged from 20% (chow reference) to 60% (1.65% OF); there was no dose response pattern. Hepatic findings were rare in the other groups of females. The average severity of fatty change did not follow a dose response pattern in males or females.

Renal tubular mineralization was not present in any males. In females, slight tubular mineralization was found in 4/20 and 2/20 of animals in the 0% OF and 9.91% OF groups, respectively. Slight renal tubular basophilia was present in male rats (4/20 in the 0% OF group, and 6/20 in the 9.91% OF group) and in female rats (1/20 in the 0% OF group, and 3/20 in the 9.91% OF group).

The distended cecae in males and females were microscopically normal, thus the increased cecal weights in the higher OF groups lacked a histological correlate. All other histological findings were considered incidental.

3.8. Fecal bacterial populations

Fig. 6 presents the changes in bifidobacterial and total bacterial populations in fecal samples from baseline (week −1). There were significantly greater increases in bifidobacteria levels in animals fed diets containing ⩾4.96% OF (diets D and E) versus 0% OF, and there were significantly greater increases in total bacterial load in animals fed diets containing ⩾1.65% OF (diets C through E) versus 0% OF. In general, the changes in the bifidobacterial population levels were twice as large as the concurrent changes in total bacterial load.

Table 2
Serum cholesterol measurements in rats fed diets containing 0–9.91% OF, or standard rodent chow (reference)

	Males						Females					
	0% OF A	0.55% OF B	1.65% OF C	4.96% OF D	9.91% OF E	Chow (reference) F	0% OF A	0.55% OF B	1.65% OF C	4.96% OF D	9.91% OF E	Chow (reference) F
Cholesterol mg/dl												
Wk −1	88.4 ± 12.45	81.6 ± 8.68	78.3 ± 8.43	73.3 ± 11.98	75.0 ± 11.47	75.0 ± 10.69	71.8 ± 11.74	71.7 ± 7.70	76.1 ± 10.50	72.7 ± 8.12	75.1 ± 13.15	74.0 ± 9.15
Wk 1	101.4 ± 15.15	85.4 ± 10.59[a]	80.8 ± 12.95[a]	69.5 ± 7.60[a]	70.5 ± 10.90[a]	70.7 ± 14.05	85.1 ± 16.97	81.1 ± 18.69	90.2 ± 8.23	69.6 ± 10.27[a]	62.3 ± 13.42[a]	73.9 ± 14.18
Wk 6	114.2 ± 16.53	97.0 ± 17.72[a]	101.2 ± 13.60	77.4 ± 10.77[a]	79.5 ± 13.18[a]	73.9 ± 12.66	84.8 ± 24.53	82.3 ± 14.83	89.0 ± 10.02	79.5 ± 12.97	72.9 ± 14.18	76.2 ± 17.04
Wk 13	131.8 ± 21.19	115.6 ± 20.33	112.1 ± 14.07	86.5 ± 12.19[a]	97.2 ± 16.60[a]	80.8 ± 21.06	94.9 ± 22.98	89.4 ± 14.24	96.5 ± 13.49	92.6 ± 14.55	87.0 ± 16.03	79.3 ± 14.45
HDL mg/dl												
Wk −1	56.4 ± 7.00	51.22 ± 7.98	52.5 ± 6.29	49.2 ± 8.39	49.4 ± 8.14	47.8 ± 4.27	54.7 ± 9.42	53.7 ± 5.01	56.3 ± 4.3	54.2 ± 5.57	55.4 ± 7.79	55.6 ± 6.28
Wk 1	70.2 ± 10.92	57.5 ± 12.49[a]	57.3 ± 9.03[a]	48.7 ± 5.72[a]	48.2 ± 8.92[a]	47.3 ± 7.26	68.1 ± 12.33	62.8 ± 13.55	69.5 ± 5.42	55.7 ± 6.58[a]	50.7 ± 8.33[a]	60.5 ± 9.68
Wk 6	74.8 ± 11.44	63.5 ± 14.39[a]	68.4 ± 8.57	55.8 ± 5.45[a]	59.7 ± 9.13[a]	51.0 ± 8.37	64.5 ± 15.67	65.5 ± 7.74	66.3 ± 6.90	61.4 ± 6.65	60.3 ± 8.56	58.0 ± 10.13
Wk 13	86.2 ± 11.55	75.5 ± 14.14[a]	76.1 ± 10.48[a]	63.9 ± 7.98[a]	69.9 ± 10.05[a]	55.2 ± 10.94	70.0 ± 13.37	67.0 ± 10.49	67.5 ± 6.40	68.2 ± 9.66	68.8 ± 10.12	63.3 ± 10.10
LDL mg/dl												
Wk −1	32.0 ± 6.38	30.3 ± 6.65	25.8 ± 8.07	24.1 ± 4.58	25.6 ± 6.75	27.3 ± 8.45	17.1 ± 3.73	18.0 ± 4.52	19.8 ± 6.71	18.5 ± 3.24	19.7 ± 7.36	18.4 ± 4.60
Wk 1	31.2 ± 6.12	27.9 ± 8.88	23.5 ± 6.95[a]	20.8 ± 5.18[a]	22.3 ± 5.74[a]	23.4 ± 8.17	17.0 ± 5.10	18.3 ± 7.13	20.7 ± 9.21	13.9 ± 5.11	11.6 ± 6.22[a]	13.4 ± 5.30
Wk 6	39.4 ± 8.90	33.5 ± 6.40	32.8 ± 14.89[a]	21.6 ± 6.69[a]	19.8 ± 5.25[a]	22.9 ± 7.32	20.3 ± 9.7	16.8 ± 7.80	22.7 ± 11.91	18.1 ± 9.31	12.6 ± 7.79	18.2 ± 10.71
Wk 13	45.6 ± 15.66	40.1 ± 12.70	36.0 ± 14.40	22.6 ± 7.89[a]	27.3 ± 10.31[a]	25.6 ± 11.66	24.9 ± 11.22	22.4 ± 7.44	29.0 ± 13.71	24.4 ± 9.16	18.2 ± 8.51	16.0 ± 6.18
L/H ratio												
Wk −1	0.57 ± 0.08	0.59 ± 0.19	0.50 ± 0.19	0.49 ± 0.09	0.52 ± 0.14	0.56 ± 0.16	0.32 ± 0.06	0.33 ± 0.07	0.34 ± 0.10	0.34 ± 0.05	0.36 ± 0.13	0.35 ± 0.07
Wk 1	0.44 ± 0.08	0.53 ± 0.33	0.41 ± 0.10	0.43 ± 0.11	0.48 ± 0.20	0.49 ± 0.11	0.26 ± 0.05	0.29 ± 0.09	0.31 ± 0.14	0.24 ± 0.08	0.21 ± 0.11	0.21 ± 0.07
Wk 6	0.54 ± 0.13	0.57 ± 0.14	0.50 ± 0.30[a]	0.39 ± 0.09[a]	0.33 ± 0.07[a]	0.46 ± 0.14	0.31 ± 0.11	0.24 ± 0.11	0.37 ± 0.21	0.29 ± 0.15	0.21 ± 0.09	0.31 ± 0.17
Wk 13	0.53 ± 0.18	0.54 ± 0.20	0.48 ± 0.22	0.34 ± 0.13[a]	0.40 ± 0.16	0.47 ± 0.13	0.36 ± 0.13	0.34 ± 0.13	0.44 ± 0.24	0.37 ± 0.13	0.26 ± 0.12	0.26 ± 0.08

Data represent the mean ± S.D.
[a] $p < 0.05$ compared with 0% OF.

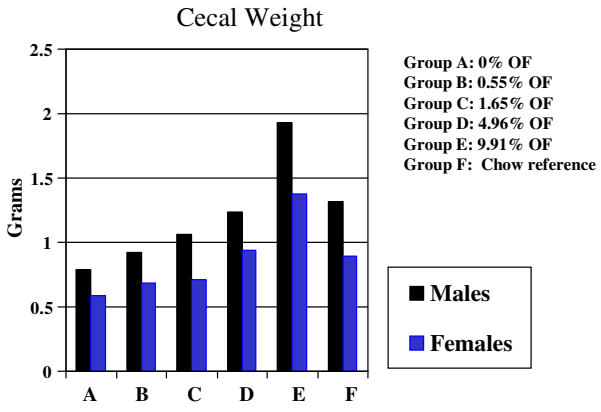

Fig. 5. Cecal weights of male and female rats fed diets containing 0–9.91% OF, or a standard rodent chow (reference) for 13 weeks. There was a significant dose-related increase in cecal weights in male and female rats fed the OF-supplemented diets (trend $p = 0.01$).

3.9. Mutagenicity: Ames test

OF did not induce a positive response at levels up to 5000 μg per plate in the presence and absence of S9 activation with any of the tester strains used (data not shown).

3.10. In vitro chromosomal aberration test with CHO cells

In the initial and confirmatory assay no toxicity, as measured by a >50% reduction in cell growth relative to the solvent control, was observed at any dose level tested in both the absence and presence of S9 activation. The dose levels selected for evaluation of chromosomal aberrations in all treatment groups of the initial assay were 1250, 2500, and 5000 μg/mL. Either no statistically significant increases in structural or numerical chromosome aberrations, relative to the solvent control, were observed in the non-activated or S9 activated groups, regardless of dose level.

4. Discussion

OF has been the subject of numerous studies in animals as well as adult humans and has a history of safe use (Spiegel et al., 1994; Crittenden and Playne, 1996; Ziemer and Gibson, 1998; Williams, 1999; Kaur and Gupta, 2002; Delzenne, 2003), although only a limited number of formal animal safety evaluation studies have been reported (Carabin and Flamm, 1999; Tokunaga et al., 1986). In the present study, we assessed an OF derived from inulin hydrolysis. There were no unexpected adverse events associated with feeding OF in the present study.

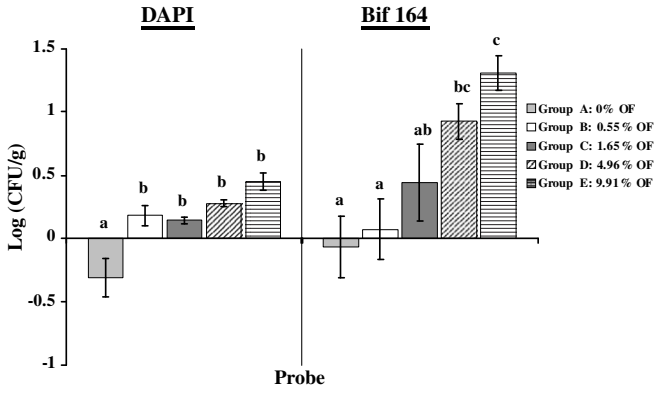

Fig. 6. Changes in fecal bifidobacteria (Bif 164) and total bacterial (DAPI) counts from baseline (week −1) to week 13 in animals fed diets containing 0–9.91% OF mean ± S.E. Groups with the same letter not significantly different, $p < 0.05$.

The mean body weights of male rats fed diets containing the highest level of OF were slightly lower than control animals throughout the study. These changes were not considered toxicologically relevant. Carabin and Flamm (1999) found transiently reduced weight gain in rats receiving 5% and 10% synthetic OF while Tokunaga et al. (1986) reported significantly lower weight gains in rats fed 20% OF, but not 10% OF. In contrast Clevenger et al. (1988) reported no dose-related effects on growth on rats. While growth was reportedly normal in most studies in human infants (Bettler and Euler, 2006; Boehm et al., 2002; Moro et al., 2002), Schmelzle et al. (2003) reported that infants gained less weight during the first six weeks of prebiotic feeding (part of the prebiotic mix was OF). The velocity differences, however, were not maintained throughout the 12-week study.

Total, HDL- and LDL-cholesterol concentrations were significantly lower at several time points in animals fed OF, particularly in male rats fed the highest levels. The reason for the difference in OF effects on blood lipids between male and female rats is unclear. However, gender-specific effects of diet on lipids have been reported previously, which may relate to the influence of sex hormones on lipid metabolism (Robins et al., 1995; Wilson et al., 1999; Blair et al., 2002). Lipid-lowering effects of OF in rodents have been demonstrated by other investigators. Fiordaliso et al. (1995) reported that daily administration of a 10% (w/w) OF-supplemented diet to normolipidemic male rats resulted in a significant decrease in plasma triglycerides, phospholipids and cholesterol. Other investigators report reduced serum triglycerides in animals fed OF or inulin, with variable effects on cholesterol (e.g. Tokunaga et al., 1986; Kok et al., 1998; Delzenne et al., 2002; Juśkiewicz et al., 2007). Dietary OF effects on serum lipids appears to be mediated by a reduction in hepatic *de novo* triglyceride synthesis. Reduced hepatic lipogenic enzyme activity (Agheli et al., 1998; Kok et al., 1996) and mRNA levels (Delzenne et al., 2002) have been reported in studies of OF-supplemented rats.

There are fewer studies regarding OF or inulin effects on lipids in humans. Jackson et al. (1999) reported that daily consumption of inulin resulted in reduced fasting insulin concentrations and lower plasma triglyceride levels in middle-aged adults, while Brighenti et al. (1999) reported significantly decreased plasma total cholesterol and triglycerides in adult males fed inulin-supplemented cereal. Other investigators report no effect on serum lipids in normal adults (Luo et al., 1996; Pedersen et al., 1997). Factors that may impact dietary OF effects on blood lipids include the dose of OF used, the duration of the study, the levels of fat and carbohydrate in the background diet, and the inclusion of hyperlipidemic subjects (Pereira and Gibson, 2002; Williams and Jackson, 2002).

An increase in plasma inorganic phosphate occurred in most OF groups. OF and inulin increase the absorption of many minerals (Scholz-Ahrens and Schrezenmeir, 2007) which may be due to increased colonic pH and solubility. Data are generally lacking for phosphorous due to the absence of usable isotopes for tracer studies.

Three alternations in the histopathology assessment of the animals were found: hepatic fatty changes, renal tubular mineralization and tubular basophilia. We have previously observed all of these changes in a study which compared two purified rat diets (AIN 76A and AIN 93G) to rat chow, and these changes appear to be due to the nutrient and fiber balance of the diets themselves (Lien et al., 2001).

In the present study, there was a positive relationship between dietary OF intakes and bifidobacterial concentrations in fecal samples. These results are in agreement with those from previous

studies. Dietary OF increases the stool concentrations of bifidobacteria in rats (Howard et al., 1995; Kleessen et al., 2001) and humans (Brighenti et al., 1999; Bouhnik et al., 1996; Gibson, 1999). Studies in term (Moro et al., 2002; Schmelzle et al., 2003) and preterm (Boehm et al., 2002) infants also demonstrated that feeding galacto-oligosaccharide (GOS) and OF-supplemented formula significantly increases stool bifidobacteria counts. The results in term infants are difficult to interpret, since no breast-fed infants were involved in these studies. Softer and more frequent stools (Boehm et al., 2002; Moro et al., 2002; Schmelzle et al., 2003; Moore et al., 2003) and lower fecal pH (Moro et al., 2002) were also reported.

Increased levels of cecal bifidobacteria are associated with a decrease in the levels of pathogenic bacterial species in rats (Campbell et al., 1997) and humans (Gibson et al., 1995). The mechanism of inhibition relates to the extensive fermentation of OF to short chain fatty acids (SCFA). The resulting decrease in pH prevents enteric colonization of potentially pathogenic microorganisms and the growth of putrefactive bacteria (Gibson, 1999; Losada and Olleros, 2002; Campbell et al., 1997). However, the acid pH does not appear to be the only mechanism of pathogen inhibition. In vitro, *bifidobacterium* inhibit the growth of *E. coli* and *Clostridium perfringens* at a neutral pH, and excrete an anti-microbial substance with a broad spectrum of activity (Gibson and Wang, 1994).

Colonocytes are dependent on SCFA derived from bacterial fermentation as a fuel source, with butyrate oxidation providing more than 70% of the oxygen consumed by colonic tissue (Roediger, 1980). Campbell et al. (1997) speculate that OF plays a role in maintaining mucosal cell differentiation and gastrointestinal mucosa by serving as an indirect energy source for the bowel. A trophic effect of dietary OF is suggested by the significantly higher cecal weights in rats fed each of the OF doses in the present study. Other investigators also reported increased cecal total weights and wall weights in OF-fed rats; these effects are not considered to be adverse effects but rather a physiologic responses (Tokunaga et al., 1986; Younes et al., 1995; Campbell et al., 1997; Genta et al., 2005; Juśkiewicz et al., 2007).

OF appears to have no genotoxic or carcinogenic effects, and may, in fact, be antimutagenic (Spiegel et al., 1994; Crittenden and Playne, 1996; Ziemer and Gibson, 1998; Williams, 1999; Kaur and Gupta, 2002; Delzenne, 2003). In humans and rats, dietary oligosaccharides suppress fecal activities of carcinogen-metabolizing enzymes. Animals fed OF have fewer aberrant crypt foci in the colon, which may be due to bifidobacteria's limited capacity to convert precancerous substances into carcinogens (Clevenger et al., 1988; Bouhnik et al., 1996; Teitelbaum and Walker, 2002). Colonic butyrate generated from OF fermentation may also be antimutagenic (Kleessen et al., 2001; Kaur and Gupta, 2002). In the present study, dietary OF did not induce any positive responses in the Ames test, and the *in vitro* chromosomal aberration test indicated no toxicity.

Exact dose calculation for OF in humans is challenging. A number of studies in adult humans have evaluated the effect of OF dose on gastrointestinal bacteria levels (Fuller and Gibson, 1997; Gibson and Roberfroid, 1995). OF consumption in humans of 4–15 g/day results in increases of both bifidobacteria and lactobacilli (Ziemer and Gibson, 1998). Since intake was not measured in relationship to body weight, an actual dose (g/kg/day) cannot be calculated. Very substantial differences in body weights would result in large variations in intake on a weight basis. For example, a 50 kg woman consuming 4 g/day of OF would receive 0.08 g/kg/day, while a 90 kg man would receive only 0.044 g/kg/day. Human milk is a rich source of complex oligosaccharides, with concentrations up to 10 g/l (Kunz et al., 2000). Certain oligosaccharides, such as OF, GOS or pectin oligosaccharides, are not found in human milk, but the effects of formulas fortified with concentrations from 1.5 g/l to 8 g/l have been evaluated in infants (Euler et al., 2005; Fanaro et al., 2005; Moro et al., 2002; Kunz et al., 2000). Prebiotics such as these may also be of interest in the diets of older infants and young children, since the intestinal microbial populations during this time of life undergoes modulations in response to dietary changes, such as introduction of weaning foods (Stark and Lee, 1982). In pediatric populations, consumption considerations are even more difficult to assess than in adults due to rapid growth rates and the substantial decrease in energy consumption on a body weight basis during the first year of life. For example, energy intake of breast-fed male infants falls from 115 kcal/kg/day at one month of age to 92 kcal/kg/day at 12 months (Fomon, 1993). Females have a similar, but slightly lower caloric intake. Assuming a formula concentration of 3 g OF/liter and a caloric density of 670 kcal/l, the OF intake would range from 0.51 g/kg/day at one month of age to 0.41 g/kg/day at 12 months (in infants who receive all calories from formula). The highest dose group in the present study received an intake (g/kg/day) approximately 10 fold higher than this.

In conclusion, the OF evaluated in this study demonstrated an excellent safety profile, both in a 13-week *in vivo* study and in a variety of *in vitro* analyses. Based on these data, the no adverse effect observed level (NOAEL) of oligofructose in the diet is 9.91%. Although oligosaccharides such as OF are not found in human milk, there may be functional benefits of addition to infant formula or products for use in early childhood.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Sources of funding: Eric L. Lien: Wyeth Laboratories. Anne L. McCartney: Wyeth Nutrition funded the FISH analysis of the samples from this study. Researchers of Wyeth were responsible for the study design, running of the study and collection of samples. FISH analysis was performed and interpretation of FISH data done by the research team at the University of Reading. The manuscript was prepared in collaboration with all parties.

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