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Safety assessment of biotechnologically produced 2′-Fucosyllactose, a novel food additive

Damien van Berlo[a,*], Alinde E. Wallinga[a], Frederique A. van Acker[a], Dianne J. Delsing[b]

[a] Triskelion BV, Utrechtseweg 48, 3700 AV Zeist, The Netherlands
[b] FrieslandCampina, Amersfoort, The Netherlands

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1. Introduction

Breastfeeding is advocated by health authorities as the ideal feeding practice for infants (WHO, 2003; Center for Disease Prevention and Control, 2007), especially during the first 6 months of their lives. Breastfeeding is not always possible, however, and in certain sub-optimal circumstances (e.g. maternal malnutrition) it is not sufficiently nutritious. In such cases, infant formula is the best alternative to provide or complement nutrition for the infant. The third most prevalent component of human breast milk is the oligosaccharide fraction (Kunz and Rudloff, 2006; Bode et al., 2004) known as human milk oligosaccharides (HMO). The oligosaccharide fraction is much smaller in animal milk (Tao et al., 2009; Meyrand et al., 2013). Therefore, infant formula based on cow's milk currently lacks the oligosaccharide structures that are prevalent in human milk.

HMO content in human milk is considered a very important factor for the development of intestinal microbiota of infants (Kunz and Rudloff, 2006) and has been found to protect against infant diarrhea (Morrow et al., 2004). When HMO are ingested in relatively large amounts (multiple grams per day), they have been shown to prevent pathogen adhesion to the intestinal epithelium (Bode et al., 2004; Newburg and Ruiz-Palacios, 2005), which reduces the chance of infection and facilitates pathogen excretion. Although the HMO content in human milk shows considerable variation depending on geographical differences (McGuire et al., 2017) and stage of lactation (Austin et al., 2016; Sumiyoshi et al., 2003), the most abundant HMO on average is 2′-Fucosyllactose (2′-FL) (e.g. Erney et al., 2000; Asakuma et al., 2008; Castanys-Muñoz et al., 2013). Several epidemiological studies suggest an important role for 2′-FL in the health of infants. The level of 2′-FL in human milk has been shown to be inversely correlated with risk of diarrhea (Morrow et al., 2004). In addition, infants breastfed with milk containing 2′-FL show enhanced establishment of bifidobacteria in their gut microbiota, as compared to infants fed with milk lacking 2′-FL (Lewis et al., 2015). Furthermore, survival of HIV-exposed Zambian infants was found to be correlated to the 2′-FL content in the breastmilk they received (Kuhn et al., 2015).

Two recent clinical trials show the first evidence for both good tolerability and potential efficacy of 2′-FL supplementation to infant formula (Marriage et al., 2015; Puccio et al., 2017). Supplementation of infant formula with the HMOs 2′-FL and Lacto-N-neotetraose reduced respiratory infections and medication use (Puccio et al., 2017). In the other study, 2′-FL supplementation reduced plasma pro-inflammatory cytokines to resemble more closely the levels found in breastfed infants (Goehring et al., 2016). So far, the first clinical results on the benefits of 2′-FL show great promise for addition of 2′-FL to infant formula. Moreover, there is evidence suggesting that 2′-FL supplementation

Abbreviations: BN, Binucleated cells; b.w., Body weight; CP, Cyclophosphamide; CPBI, Cytokinesis-block proliferation index; DP, Degree of polymerization; EFSA, European food safety authority; FDA, Food and drug administration; 2′-FL, 2′-fucosyllactose; FOB, Functional observational battery; GALT, Gut associated lymphoid tissue; GD, Gestation day; GLP, Good laboratory practice; GRAS, Generally recognized as safe; HMO, Human milk oligosaccharide; HPLC, High performance liquid chromatography; MAA, Motor activity assessment; MNBN, Micronucleated binucleated cells; NOAEL, No observed adverse effect level; OECD, Organization for economic Co-Operation and development; PBS, Phosphate-buffered saline; PND, Post-natal day; VB, Vinblastin sulphate; WHO, World health organization

* Corresponding author.
E-mail addresses: damien.vanberlo@triskelion.nl (D. van Berlo), alinde.wallinga@triskelion.nl (A.E. Wallinga), frederique.vanacker@triskelion.nl (F.A. van Acker), dianne.delsing@frieslandcampina.com (D.J. Delsing).

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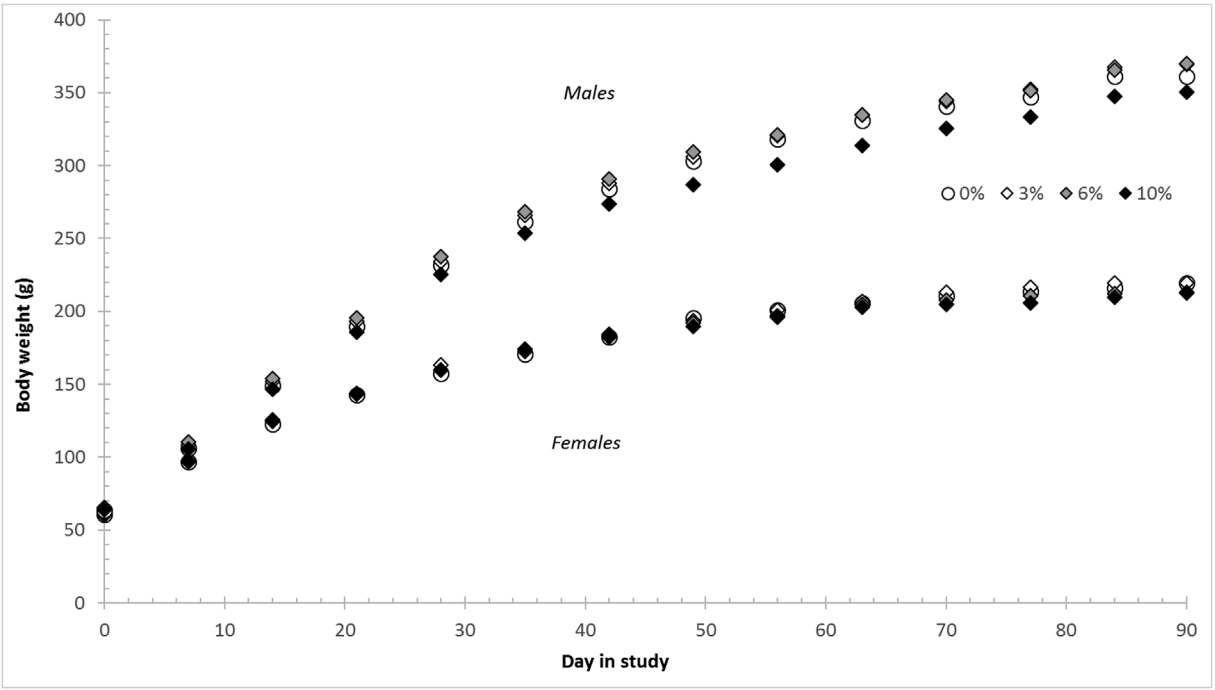

Fig. 1. Growth curve of rats fed 2′-FL for 90 days.

might also be beneficial for adults. In healthy adults, supplementation of up to 20 g/day of 2′-FL was tolerated well and promoted growth of beneficial bifidobacteria in the gut (Elison et al., 2016).

2′-FL is a trisaccharide; its structure consists of the monosaccharide L-fucose and the disaccharide D-lactose, which are linked by an α-(1 → 2) (Fig. 1). It is a non-digestible carbohydrate which can be produced by chemical synthesis from the raw materials L-fucose and D-lactose, or can be biotechnologically produced by genetically engineered bacteria.

Thus far, a number of studies have been performed to assess the potential toxicity of 2′-FL. In three in vitro genotoxicity assays, an Ames test (Coulet et al., 2014; OECD test guideline 471), a mouse lymphoma assay (Coulet et al., 2014; OECD test guideline 476) and an in vitro micronucleus test in human lymphocytes (unpublished study described in EFSA NDA Panel, 2015; OECD test guideline 487), chemically synthesized 2′-FL was found to be non-mutagenic. In a sub-chronic (13-weeks) oral toxicity study, juvenile rats (exposure started at post-natal day (PND) 7) were exposed to chemically synthesized 2′-FL at doses of 0, 2, 5 and 6 g/kg/day by oral gavage (OECD test guideline 408) (Coulet et al., 2014). In this study, the NOAEL was placed at 5 g/kg/day. In another study, the sub-acute (3-week) oral toxicity of biotechnologically produced 2′-FL was investigated in neonatal piglets. Starting at day 2 of lactation, crossbred farm piglets received a liquid diet containing 0, 200, 500 or 2000 mg/L 2′-FL. After 3 weeks of exposure, no exposure-related toxicity was observed; thus, the NOAEL was placed at the high dose level (2000 mg/L; 292 mg/kg/day for males and 299 mg/kg/day for females) (Hanlon and Thorsrud, 2014). It is important to emphasize at this point, that 2′-FL can be produced using different techniques; chemical synthesis results in a slightly different product, with different purity and impurities present, as compared to biotechnologically produced 2′-FL which can be produced by various genetically engineered *E.coli* strains. Regulatory authorities such as the European Food Safety Authority (EFSA) or the US Food and Drug Administration (US FDA) require safety testing of each specific product before a product can be introduced on the market.

To assess the safety of biotechnologically produced 2′-Fucosyllactose (2′-FL) using a genetically engineered *E. coli* K12 strain as a processing aid, its genotoxic potential was investigated in an Ames test (OECD test guideline 471) and micronucleus test (OECD test guideline 487) while repeated-dose toxicity was investigated in a sub-chronic (13-weeks) oral toxicity study in rats (OECD test guideline 408).

2. Material and methods

The studies were conducted in accordance with the OECD Principles of Good Laboratory Practice (OECD, 1998a). The welfare of the animals was maintained in accordance with the general principles governing the use of animals in experiments of the European Communities (European Council Directive, 1986 86/609/EEC) and the Netherlands legislation (Experiments on Animals Act, 1977; revised in 2014).

2.1. Test material

2′-Fucosyllactose (2′-FL; chemical formula: $C_{18}H_{32}O_{15}$; molecular weight: 488.44 Da; CAS No 41263-94-9) was produced and provided by FrieslandCampina (Amersfoort, the Netherlands); the purity was 94%, as determined by high performance liquid chromatography (HPLC). The test material was produced through fermentation by genetically modified *E. coli* K12 GI724/ATCC 55151 bacteria, followed by purification. The physico-chemical characteristics are provided in Table 1:

Table 1
Analysis of the composition of 2′-FL.

Physical/chemical characteristic	Value
2′-Fucosyllactose	94%
3-Fucosyllactose	< 1%
Difucosyllactose	< 1%
Fucose	< 1%
Lactose	< 1%
Glucose	< 1%
Protein	0.002%
Moisture	3%
Sulphated ash	0.06%

2.2. Genotoxicity studies

The potential genotoxicity of 2′-FL was assessed by two in vitro tests; 1) the bacterial reverse mutation test (Ames test) and 2) the in vitro mammalian micronucleus test in cultured human lymphocytes. Appropriate positive and negative control substances were included in the design of both tests. The Ames test was performed in the presence and absence of Aroclor 1254-induced rat liver post-mitochondrial fraction (S9 mix; Trinova Biochem, Giessen, Germany) for metabolic activation. The studies met the acceptance criteria for validity as described in the applicable OECD test guidelines; the results for the positive and negative control substances were within their respective historical ranges.

2.2.1. Bacterial reverse mutation (Ames) test

The bacterial reverse mutation test (Ames test) was performed in accordance with OECD testing guideline No. 471 (OECD, 1997). The standard plate incorporation method with the histidine-requiring *S. typhimurium* strains TA 1535, TA 1537, TA 98, TA 100 (Trinova Biochem, Giessen, Germany; originally from Moltox Molecular Toxicology Incorporated, Boone, USA) and the tryptophan-requiring *E. coli* strain WP2 *uvrA* (Trinova Biochem, Giessen, Germany) was used. 2′-FL was dissolved in PBS to achieve a stock solution of 50 mg 2′-FL/ml PBS (taking into consideration the test item purity of 94%) which was clear and colorless. From the filter-sterilized stock solution, five test concentrations were prepared ranging from 62 to 5000 μg/plate, with 5000 μg/plate being the recommended limit dose for this test system. Cytotoxicity was assessed by visual inspection of the bacterial background lawn.

Bacteria were cultured in nutrient broth for 10-16 h at 37 °C before treatment was started. Treatment was started by adding fully grown bacterial culture and either the test item treatment solution, the positive control substance or the vehicle to the molten top agar. To investigate the effect of metabolic activation, S9 mix was added to half of the culture plates, with the other half receiving 100 mM sodium phosphate. This mixture was poured on a minimal glucose agar plates and plates were incubated for 48-72 h at 37 °C; triplicate plates were used for each bacterial strain and each concentration. Following incubation, the number of His^+ and Trp^+ revertants was counted. A mutagenic response was defined as a more than 2-fold and/or dose-related increase in the mean number of revertant colonies compared to the concurrent negative controls.

2.2.2. Micronucleus test in cultured human lymphocytes

The in vitro micronucleus test was performed in accordance with OECD testing guideline No. 487 (OECD, 2016). Cells were treated with the actin polymerization inhibitor cytochalasin B after (pulse treatment) or during (continuous treatment) 2′-FL treatment to generate binucleate cells; this allows for micronucleus analysis in only those cells that have completed one mitosis. The ability of 2′-FL to induce micronuclei was investigated in cultured binucleated human lymphocytes, in both the absence and presence of a metabolic activation system (S9-mix). Cytotoxicity was determined from the Cytokinesis-Block Proliferation Index (CBPI); three concentrations were selected based on the level of cytotoxicity induced at a certain treatment concentration, with the highest level of cytotoxicity deemed acceptable was 55 ± 5%. Two independent experiments were conducted for which blood was obtained from two different donors. Culture medium (RPMI1640) was used as a solvent for the test substance. The final concentrations of the test substance in the cultures ranged from 3.9 to 2000 μg/ml. In both experiments, the maximum final concentration in the culture medium was 2000 μg/ml, based on the purity of the test substance, which is the recommended limit dose for this test system. Duplicate cultures were used in both experiments. In the first experiment, in the presence and absence of S9-mix, the treatment/recovery time was 4/20 h (pulse treatment). In the second experiment (continuous treatment group), the treatment/recovery time was 24/0 h. Solvent control and positive controls were run in parallel. At least two thousand binucleated cells per concentration (1000 per culture) were examined for the presence of micronuclei. The response was considered clearly positive if all of the following criteria were met: 1) at least one of the test concentrations exhibits a statistically significant increase compared to the concurrent negative control, 2) the increase is dose-related in at least in one experimental condition when evaluated with an appropriate trend test and 3) any of the results are outside the distribution of the historical solvent control data. A response was considered clearly negative if all of the following criteria were met: 1) none of the test concentrations exhibits a statistically significant increase compared to the concurrent negative control, 2) there is no dose-related increase when evaluated with an appropriate trend test and 3) all results are inside the distribution of the historical negative control data.

2.3. Ninety-day oral toxicity study in rats

2.3.1. Animals and maintenance

Time-mated female Wistar Han IGS rats (Crl:WI(Han)) were obtained from a colony maintained under SPF-conditions at Charles River Deutschland, Sulzfeld, Germany and acclimatized to the laboratory conditions. The animals, 16 time-mated females, were at gestation day 15 (GD15) of pregnancy when they arrived at the test facility. All pregnant females delivered pups; these were weaned and weighed at post-natal day 21 (PND21) and all rats were checked for overt signs of ill health and anomalies. Out of 82 males and 87 females, 40 males and 40 females were allocated to experimental groups by manual randomization, taking into account lineage, sex and individual body weight (by controlling that all body weights were within ± 20% of the mean weight of each sex).

The experimental animals were 25 days old when treatment was commenced. This is intended to be equivalent to the immune development of the human neonate; the exposure ended on PND 115 when immune and sexual maturity is attained in rats. The age of first exposure is in accordance with recommendations in OECD test guideline 408, in which is stated that animals should preferably be under 9 weeks old when first exposed.

The mean body weight was 64.4 g for males (range 52.2–75.8 g) and 60.9 g for females (range 48.2–71.7 g). Animals were housed under conventional conditions in Macrolon cages (five rats/cage), in a controlled environment (temperature of 20–24 °C, relative humidity between 40 and 70%, 12-h light/dark cycle, about 10 air changes/h). Wood shavings were provided as bedding material while a wooden block and strips of paper were present as environmental enrichment. Chow and tap water were provided *ad libitum*.

The welfare of the animals was maintained in accordance with the general principles governing the use of animals in experiments of the European Communities (Directive, 2010/63/EU) and Dutch legislation (The revised Experiments on Animals Act, 2014).

2.3.2. Experimental design

The study was conducted in accordance with OECD test guideline 408 (OECD, 1998b), and included four experimental groups of 10 rats per sex. The experimental diets were prepared by adding 2′-FL to the animals' feed (VRF1 finely ground, cereal-based rodent diet, SDS Special Diets Services, Witham, England) at levels of 0% (controls), 3%, 6% and 10% (w/w), and was thus administered for 13 consecutive weeks. Fresh batches of experimental diets were prepared once per month and were stored as portions in sealed plastic bags in a freezer (< −18 °C). Twice weekly, diet that remained in the animal feeders was replaced by fresh portions from the freezer.

2.3.3. Diet analysis

Experimental diets were analyzed for 2′-FL with respect to stability, homogeneity and concentration. From all three batches of diets prepared in the study (ca. 1 batch per month), samples were taken and analyzed. 2′-FL was assayed using Ultra-Performance Liquid Chromatography – tandem Mass Spectrometry (UPLC-MS/MS) on an

Acquity UPLC Glycan BEH amide column, held at 40 °C and 5 mM ammonium formate in water (mobile phase A) and 10 mM ammonium formate in acetonitrile and water (mobile phase B) as the mobile phase. The following analyses were conducted during the study:

- Homogeneity and content of the test substance at each dose level (5 samples per dose level, covering top, middle, left, right and bottom; one control sample) in the first batch.
- Content of the test substance at each dose level in all three of the prepared batches (first batch: average of 5 samples per dose level and one control sample; second and third batch: one sample per dose level and one control sample).
- Stability of the test substance under experimental conditions (one sample per dose level and one control sample of the first batch, after storage for 4 days in the animal room and after storage for at least 5 weeks in the freezer (≤-18 °C).

All samples were measured in duplicate.

2.3.4. Observations

General clinical observations: animal condition and behavior was monitored twice daily. Neurobehavioral testing, which comprised detailed clinical observations outside of their cage, was performed once weekly after exposure commenced. Furthermore, 12 weeks after initiation of the exposure, additional behavioral endpoints (Functional Observation Battery) as well as motor activity assessment were performed in all animals. Ophthalmoscopic changes were assessed using an ophthalmoscope after atropine phosphate-induced mydriasis in the last week of the exposure period (animals from the control and high-dose groups). Because no treatment-related ocular changes were observed in the high-dose group, eye examination was not extended to the animals of the intermediate-dose groups at the end of the study.

Body weight: the body weight of all pups was measured at PND 21 to enable allocation to the groups. The body weight of each allocated rat was recorded at initiation of treatment (day 0), and once weekly thereafter. All animals were weighed on their scheduled necropsy date in order to calculate the correct organ to body weight ratios.

Feed and water consumption: feed consumption was measured per cage by weighing the feeders over successive periods of 3 or 4 days (i.e. twice weekly). Water consumption was measured per cage, by weighing the drinking bottles daily, during 5-day periods in weeks 1, 6 and 12.

Hematology and clinical chemistry: for hematology and clinical chemistry, blood samples were collected at necropsy from the *a. abdominalis* of all animals, after overnight fasting. Citrate (for prothrombin time), EDTA (other hematological endpoints) or heparin (clinical chemistry) were used as anticoagulants. *Hematology*: blood was assessed for hemoglobin, packed cell volume, red blood cell count, reticulocytes, total white blood cell count, differential white blood cell counts, thrombocyte count (Advia 2120i Hematology Analyzer; Siemens N.V. the Netherlands) and prothrombin time (Neoplastine CI Plus; Stago, the Netherlands); the indices mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were calculated. *Clinical chemistry*: the following endpoints were measured in plasma: alkaline phosphatase activity, aspartate aminotransferase activity, alanine aminotransferase activity, gamma glutamyl transferase activity, total protein, albumin, ratio albumin to globulin (calculated), urea, creatinine, (fasting) glucose, total bilirubin, total cholesterol, triglycerides, phospholipids, calcium, sodium, potassium, chloride and inorganic phosphate (Olympus AU-400 analyzer, Beckman Coulter BV, the Netherlands).

Renal concentration test and urinalysis: in week 13 of the study, urine was individually collected from all animals. Before urine collection, rats were deprived of water (24 h) and feed (16 h). Urine was analyzed for volume (weighing), appearance (visual inspection), density (specific gravity, measured using a Sysmex refractometer; ATAGO, Japan), pH, glucose, occult blood, ketones, protein, bilirubin, urobilinogen (all assessed using Clinitek STATUS test strips; Siemens N.V. the Netherlands). In addition, urinary sediment was microscopically examined for red blood cells, white blood cells, epithelial cells, amorphous material, crystals, casts, bacteria, worm eggs and sperm cells.

Necropsy and (histo-)pathology: after overnight fasting, rats were sacrificed by exsanguination from the *a. abdominalis* under CO_2/O_2 anesthesia 13 weeks post-exposure in such a sequence that the average time of killing was approximately the same for each group. Macroscopic examination was performed at necropsy, organ weights were determined as soon as possible after their dissection to avoid their dehydration. The weights of the following organs was recorded and related to the terminal body weight: adrenals, brain (three areas examined: brain stem, cerebrum and cerebellum), cecum (full and empty), epididymides, heart, kidneys, liver, ovaries, prostate, seminal vesicles (with coagulating glands), spleen, testes, thymus and uterus. Samples of the following tissues and organs were obtained from all animals and were preserved in a neutral aqueous phosphate-buffered 4% solution of formaldehyde: adrenals, aorta, axillary lymph nodes, brain, cecum, colon, epididymides, esophagus, eyes, gut associated lymphoid tissue (GALT, including Peyer's patches), heart, kidneys, liver, lungs, mammary gland (females), mesenteric lymph nodes, ovaries, oviducts (=fallopian tubes), pancreas, parathyroid, parotid salivary glands, pituitary, prostate, rectum, sciatic nerve, seminal vesicles with coagulating glands, skeletal muscle (thigh), skin, small intestine (duodenum, ileum, jejunum), spinal cord (three levels retained in the vertebral column), spleen, sternum with bone marrow, stomach (glandular and non-glandular), sublingual salivary glands, submaxillary salivary glands, testes, thymus, thyroid, trachea/bronchi, urinary bladder, uterus, vagina and any other tissue showing gross lesions. All tissues that were to be examined microscopically were embedded in paraffin wax, sectioned at 5 µm and stained with hematoxylin and eosin. Histopathological examination (by light microscopy) was performed on all samples from the control group (1) and the high-dose group (4). On animals that died intercurrently, a full microscopic examination was performed. Because no treatment-related changes were observed in the high-dose group, histopathology was not extended to the intermediate-dose groups.

2.3.5. Statistical analysis

2.3.5.1. Bacterial reverse mutation (Ames) test. No statistical analysis was performed.

2.3.5.2. Micronucleus test in cultured human lymphocytes. The frequencies of micronuclei in 2′-FL-treated cultures and positive control cultures were compared with those of the concurrent solvent control using the Chi-square test (one-sided). The results were considered statistically significant when the p-value of the Chi-square test was less than 0.05.

2.3.5.3. Ninety-day oral toxicity study in rats. Consumption of feed and water were analyzed using Dunnett's multiple comparison test.

Body weights were evaluated by one-way analysis of covariance (ANCOVA; with body weight at initiation of treatment as covariate) followed by Dunnett's multiple comparison test. The Levene test was used to check for homogeneity of variances while the Shapiro–Wilks test was used to assess normality of data distribution. If variances were not homogeneous or data were not normally distributed, the data were stepwise log or rank transformed prior to the analysis of variance.

Hematological and clinical chemistry measurements, urinary volume and density and organ weights, were evaluated by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests. To check for homogeneity of variances and normality of data distribution, the same procedure was followed as described above for body weight.

Semi quantitative urinalysis results were evaluated by Kruskal–Wallis test on rank transformed data followed by Dunnett's multiple comparison test.

Neurobehavioral data were analyzed by ANOVA followed by Dunnett's multiple comparison test (continuous FOB data, total distance

Table 2
Reference mutagens used as positive controls.

Strain	- S9 mix	+ S9 mix
TA 1535	sodium azide: 1.0 μg/plate	2-aminoanthracene: 2.0 μg/plate
TA 1537	9-aminoacridine: 80 μg/plate	benzo(a)pyrene: 4.0 μg/plate
TA 98	2-nitrofluorene: 2.0 μg/plate	2-aminoanthracene: 2.0 μg/plate
TA 100	sodium azide: 1.0 μg/plate	2-aminoanthracene: 2.0 μg/plate
WP2 *uvrA*	N-ethyl-N-nitrosourea: 100 μg/plate	2-aminoanthracene: 80 μg/plate

moved), repeated measures ANOVA on time blocks (habituation of motor activity), Kruskal–Wallis non-parametric analysis of variance followed by multiple comparison tests (rank order FOB data), or Pearson chi-square analysis (categorical FOB data).

Histopathological changes were assessed using Fisher's exact probability test.

All analyses were two-sided. Standard deviation is used to indicate variation between animals or cultures. Probability values of $p < 0.05$ were considered significant.

3. Results

3.1. Genotoxicity studies

3.1.1. Bacterial reverse mutation (Ames) test

2′-FL was non-toxic to all of the strains tested (*S. typhimurium* TA 1535, TA 1537, TA 98, TA100 and *E. coli* WP2 UvrA), which was reflected by (1) an absence of clearing of the background lawn of bacterial growth compared to the negative controls, (2) the lack of a decrease in the mean number of revertants and (3) the absence of pinpoint colonies. The numbers of revertant colonies are shown in Table 3. In both the absence and presence of S9-mix, the test substance did not induce a more than 2-fold and/or dose related increase in the mean number of revertant colonies compared to the background spontaneous reversion rate observed with the negative control. The mean numbers of His+ (*S. typhimurium*) and Trp+ (*E. coli*) revertant colonies of the negative (vehicle) controls were within the acceptable range in all strains, and the positive controls gave the expected increase in the mean numbers of revertant colonies. Therefore, the test was considered valid .

3.1.2. Micronucleus test in cultured human lymphocytes

The potential clastogenic and/or aneugenic effects of 2′-FL, were investigated using the in vitro micronucleus test in which cells were treated with cytochalasin B prior to mitosis. Cultured lymphocytes were treated with 2′-FL at a maximum final concentration of 2 mg/ml, based on the purity, as recommended in OECD test guideline 487. After either pulse treatment (with and without S9 mix) or continuous treatment (without S9 mix), no cytotoxicity was observed at any concentration tested, either with or without S9 mix. The maximally observed cytotoxicity was 13% and 18% after pulse treatment and continuous treatment, respectively. The induction of micronucleus formation was assessed for the treatment concentrations 500, 1000 and 2000 μg/ml 2′-FL and was compared to the solvent control; results are shown in Table 4.1(pulse treatment) and 4.2 (continuous treatment). No statistically significant, dose-dependent increase in the number of binucleated cells containing micronuclei was

Table 3
Number of revertants counted in the bacterial reverse mutation test.

Dose (μg/plate)	TA 1535		TA 1537		TA 98		Ta 100		WP2 *uvrA*	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
0	28 ± 7	25 ± 2	12 ± 6	15 ± 3	36 ± 5	49 ± 5	154 ± 10	169 ± 9	51 ± 7	56 ± 9
62	28 ± 5	22 ± 5	16 ± 4	24 ± 3	33 ± 12	47 ± 8	148 ± 14	185 ± 25	60 ± 8	55 ± 7
185	25 ± 6	24 ± 1	15 ± 6	18 ± 2	40 ± 4	54 ± 9	147 ± 11	188 ± 2	53 ± 0	55 ± 1
556	28 ± 1	26 ± 7	16 ± 2	19 ± 4	32 ± 4	49 ± 9	160 ± 17	191 ± 11	58 ± 4	66 ± 8
1667	25 ± 9	22 ± 4	12 ± 3	24 ± 5	32 ± 4	55 ± 5	166 ± 9	165 ± 20	60 ± 19	66 ± 4
5000	30 ± 9	27 ± 3	16 ± 3	22 ± 5	36 ± 5	45 ± 9	174 ± 18	194 ± 16	67 ± 10	68 ± 16
PC[a]	855 ± 31	284 ± 20	2932 ± 1269	213 ± 29	2065 ± 97	1906 ± 39	927 ± 82	2722 ± 192	483 ± 69	576 ± 38

[a] Positive control; specified in Table 2.

Table 4
1. Pulse treatment with and without metabolic activation.

2′-FL treatment (μg/ml)	+ S9 mix		- S9 mix	
	% Viability (CPBI)	% MNBN/BN	% Viability (CPBI)	% MNBN/BN
0	100	0.70	100	0.85
500	90	0.65	100	0.85
1000	87	0.65	100	0.75
2000	92	0.85	100	0.75
CP[#] (20 μg/ml)	41	2.55***	–	–

2′-FL treatment (μg/ml)	- S9 mix	
	% Viability (CPBI)	% MNBN/BN
0	100	0.85
500	100	0.95
1000	82	0.90
2000	100	0.95
VB[#] (0.0125 μg/ml)	18	6.35***

[#]Cyclophosphamide; CPBI, Cytokinesis-Block Proliferation Index; BN, binucleated cells; MNBN, micronucleated binucleated cells; ***$p < 0.001$.
[#]Vinblastin sulphate; CPBI, Cytokinesis-Block Proliferation Index; ***$p < 0.001$.

Table 5
Body weight, food consumption, water consumption and 2′-FL intake; mean values calculated over the 13-week exposure period.

Parameter	2′-FL concentration in diet (%)			
	0	3	6	10
Males				
Body weight (g)	261 ± 26.1	264 ± 24.5	266 ± 28.2	251 ± 30.2
Food consumption (g/rat/day)	18.6 ± 2.5	19 ± 2.4	18.7 ± 2.3	18 ± 2.3
Water consumption (g/rat/day)	21.2 ± 4.7	22.1 ± 4.9	21.5 ± 4.6	22.9 ± 5.1
2′-FL intake (g/kg b.w./day)	0 ± 0	2.17 ± 0.21	4.27 ± 0.48	7.25 ± 0.89
Females				
Body weight (g)	171 ± 11.4	173 ± 14.3	164 ± 21.3	169 ± 11.3
Food consumption (g/rat/day)	14.1 ± 1.3	14.1 ± 1.3	14.2 ± 1.2	13.1 ± 1.1
Water consumption (g/rat/day)	19.3 ± 3.3	18.2 ± 3.6	18.5 ± 3.4	19 ± 3.1
2′-FL intake (g/kg b.w./day)	0 ± 0	2.45 ± 0.20	5.22 ± 0.71	7.76 ± 0.51

Values are means ± SD for groups of 10 rats; **p < 0.01.

Table 6a
Hematology analysis in rats exposed for 13 weeks to 2′-FL; red blood cell parameters.

% 2′-FL in diet	RBC (10E12/l)	Hb (mmol/l)	PCV (l/l)	MCV (fl)	MCH (fmol)	MCHC (mmol/l)
Males						
0	8.984 ± 0.366	9.65 ± 0.30	0.4956 ± 0.0159	55.19 ± 1.22	1.075 ± 0.044	19.48 ± 0.44
3	9.007 ± 0.222	9.69 ± 0.37	0.5010 ± 0.0144	55.63 ± 1.15	1.076 ± 0.037	19.34 ± 0.32
6	8.875 ± 0.384	9.58 ± 0.31	0.4937 ± 0.0161	55.68 ± 1.98	1.081 ± 0.047	19.41 ± 0.22
10	8.951 ± 0.442	9.65 ± 0.48	0.5006 ± 0.0288	55.92 ± 1.07	1.078 ± 0.022	19.29 ± 0.25
Females						
0	8.334 ± 0.382	9.46 ± 0.30	0.4823 ± 0.0160	57.91 ± 1.46	1.136 ± 0.042	19.62 ± 0.33
3	8.360 ± 0.193	9.49 ± 0.34	0.4791 ± 0.0161	57.32 ± 1.67	1.135 ± 0.039	19.81 ± 0.31
6	8.397 ± 0.448	9.46 ± 0.38	0.4797 ± 0.0245	57.14 ± 1.10	1.127 ± 0.031	19.73 ± 0.45
10	8.184 ± 0.463	9.39 ± 0.36	0.4713 ± 0.0203	57.64 ± 1.62	1.149 ± 0.034	19.93 ± 0.31

RBC, red blood cell count; Hb, hemoglobin concentration; PCV, packed cell volume; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; Values are means ± SD for groups of 10 rats. Statistical analysis showed no significant differences between the 2′-FL-exposed groups and controls.

found when compared to the concurrent solvent cultures in both the pulse treatment (with and without metabolic activation) and the continuous treatment (without metabolic activation) groups. Moreover, the number of binucleated cells containing micronuclei were within the test facility's historical data range of all respective control groups. Thus, all three criteria for a clearly negative outcome were fulfilled.

3.2. Ninety-day oral toxicity study in rats

Intake of the test substance: Analysis of the prepared diets confirmed the stability, homogeneity and dose levels of the test substance throughout the study. Due to the decreased feed intake per kg body weight with increasing age of the rats, the 2′-FL intake per kg body weight gradually decreased in all groups. The overall mean intake of 2′-FL was 2.17, 4.27 and 7.25 g/kg body weight/day for males and 2.45, 5.22 and 7.76 g/kg body weight/day for females from the low-, mid- and high-dose group, respectively.

Clinical observations: no exposure-related mortality or clinical signs were observed. One female animal from the mid-dose group died in the 4th week post-exposure. Because a dose-response relationship was absent and no further mortalities were observed, this was considered to bear no toxicological relevance. The results of the detailed clinical observations, functional observational battery and motor activity assessment did not indicate any neurotoxic potential of 2′-FL in rats. Ophthalmoscopy did not reveal any exposure-related changes.

Growth, feed and water consumption: Growth curves of animals exposed to 2′-FL via their diet are shown in Fig. 1; mean values over the 13-week exposure period for the body weight, food consumption, water consumption and 2′-FL intake are shown in Table 5.

There were no statistically significant differences in body weights between the control group and the test groups. Thus, the animals' growth was not affected by the treatment.

There were no statistically significant differences in food consumption in male rats among the groups. In female rats of the high-dose group food consumption was slightly, but statistically significantly decreased; average food consumption over the 13-week exposure period is shown in Table 5.

There were no noticeable differences in water consumption among the groups (Tables 6a, 6b, 6c), except for an incidental increase in males of the high-dose group on day 35–36 and in females of the high-dose group on day 38–39.

Hematology and clinical chemistry: results for hematology and clinical chemistry are shown in Tables 6a, 6b, 6c and 7a, 7b, respectively. There were no statistically significant differences in red blood cell variables between the test groups and the controls. Thrombocytes were statistically significantly increased in high-dose females. This was considered as a chance finding because the difference with the controls

Table 6b
Hematology analysis in rats exposed for 13 weeks to 2′-FL; coagulation.

% 2′-FL in diet	Thrombocytes (10E9/l)	Prothrombin time (s)
Males		
0	826.2 ± 79.5	18.73 ± 0.89
3	858.0 ± 147.1	18.87 ± 0.93
6	851.3 ± 168.0	18.77 ± 0.81
10	804.5 ± 96.4	18.62 ± 0.73
Females		
0	742.0 ± 78.0	18.85 ± 0.63
3	802.9 ± 85.7	18.70 ± 0.54
6	809.8 ± 84.2	19.04 ± 1.01
10	855.6 ± 97.1*	18.66 ± 1.30

Values are means ± SD for groups of 10 rats. *p < 0.05.

Table 6c
Hematology analysis in rats exposed for 13 weeks to 2′-FL; absolute white blood cell counts.

% 2′-FL in diet	Leukocytes (10E9/l)	Lymphocytes (10E9/l)	Neutrophils (10E9/l)	Eosinophils (10E9/l)	Basophils (10E9/l)	Monocytes (10E9/l)
Males						
0	5.29 ± 1.37	4.00 ± 1.26	1.09 ± 0.19	0.058 ± 0.019	0.008 ± 0.006	0.111 ± 0.043
3	6.28 ± 1.84	4.87 ± 1.70	1.17 ± 0.32	0.071 ± 0.034	0.010 ± 0.007	0.119 ± 0.037
6	5.40 ± 1.54	4.01 ± 1.31	1.20 ± 0.56	0.057 ± 0.025	0.009 ± 0.005	0.100 ± 0.033
10	5.66 ± 1.87	4.35 ± 1.59	1.13 ± 0.26	0.051 ± 0.028	0.008 ± 0.006	0.091 ± 0.029
Females						
0	3.84 ± 1.15	2.99 ± 0.81	0.71 ± 0.41	0.042 ± 0.021	0.005 ± 0.003	0.077 ± 0.031
3	3.99 ± 1.02	3.10 ± 0.86	0.71 ± 0.21	0.061 ± 0.023	0.008 ± 0.006	0.086 ± 0.034
6	3.80 ± 1.43	2.96 ± 1.14	0.67 ± 0.31	0.044 ± 0.022	0.004 ± 0.004	0.091 ± 0.051
10	4.04 ± 0.80	3.16 ± 0.72	0.72 ± 0.14	0.054 ± 0.022	0.006 ± 0.005	0.082 ± 0.030

Values are means ± SD for groups of 10 rats. Statistical analysis showed no significant differences between the 2′-FL-exposed groups and controls.

Table 7a
Clinical chemical analysis in plasma from rats exposed for 13 weeks to 2′-FL.

% 2′-FL in diet	ALP (U/l)	ALAT (U/l)	ASAT (U/l)	Glucose (mmol/l)	Total protein (g/l)	Albumin (g/l)	albumin/globulin ratio	Urea (mmol/l)	creat. (μmol/l)
Males									
0	123.9 ± 36.3	46.9 ± 7.3	69.4 ± 9.2	7.706 ± 1.222	63.4 ± 2.4	34.4 ± 1.2	1.188 ± 0.059	6.16 ± 0.81	34.3 ± 4.1
3	135.1 ± 32.1	43.5 ± 5.1	67.8 ± 5.8	8.089 ± 1.872	63.9 ± 3.1	34.1 ± 1.2	1.148 ± 0.064	6.79 ± 0.60	33.0 ± 3.5
6	139.7 ± 31.9	43.0 ± 8.4	67.4 ± 5.4	7.996 ± 1.361	63.7 ± 2.5	34.6 ± 1.2	1.192 ± 0.062	7.03 ± 0.90*	34.7 ± 2.9
10	129.4 ± 31.9	41.6 ± 8.7	66.1 ± 8.1	7.676 ± 1.896	62.7 ± 3.4	33.9 ± 1.5	1.179 ± 0.046	7.18 ± 0.40**	34.3 ± 2.5
Females									
0	61.3 ± 18.8	38.1 ± 11.2	78.5 ± 5.8	5.638 ± 0.822	68.7 ± 3.0	38.0 ± 1.4	1.241 ± 0.062	6.92 ± 0.53	40.1 ± 4.0
3	59.9 ± 18.9	35.2 ± 5.7	74.8 ± 7.3	5.886 ± 0.603	68.2 ± 2.2	38.2 ± 1.7	1.274 ± 0.061	6.61 ± 0.65	37.5 ± 4.1
6	66.8 ± 18.5	37.9 ± 10.3	75.9 ± 7.3	5.474 ± 0.559	67.0 ± 4.0	37.1 ± 2.3	1.244 ± 0.074	6.82 ± 0.94	37.6 ± 2.6
10	60.5 ± 23.1	36.9 ± 13.8	75.4 ± 11.9	6.017 ± 1.482	66.9 ± 2.7	37.5 ± 1.8	1.277 ± 0.070	7.08 ± 0.90	37.2 ± 5.4

ALP, alkaline phosphatase; ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; creat., creatinine. Plasma g-glutamyl transferase activity was 0.0 U/l in all groups. Values are means ± SD for groups of 10 rats. *$p < 0.05$; **$p < 0.01$.

Table 7b
Clinical chemical analysis in plasma from rats exposed for 13 weeks to 2′-FL.

% 2′-FL in diet	Bilirubin (μmol/l)	Cholesterol (mmol/l)	Triglycerides (mmol/l)	Phospholipids (mmol/l)	P (mmol/l)	Ca (mmol/l)	K (mmol/l)	Na (mmol/l)	Cl (mmol/l)
Males									
0	1.36 ± 0.31	1.838 ± 0.276	1.156 ± 0.315	1.648 ± 0.180	2.581 ± 0.433	2.764 ± 0.062	5.82 ± 0.42	148.5 ± 1.1	100.1 ± 0.6
3	1.20 ± 0.28	1.764 ± 0.385	1.096 ± 0.469	1.649 ± 0.213	2.625 ± 0.415	2.769 ± 0.090	5.84 ± 0.45	147.9 ± 1.4	99.8 ± 1.0
6	1.15 ± 0.33	1.759 ± 0.335	1.140 ± 0.253	1.619 ± 0.131	2.624 ± 0.212	2.769 ± 0.043	5.87 ± 0.35	147.2 ± 2.0	100.6 ± 2.2
10	1.15 ± 0.21	1.619 ± 0.240	0.973 ± 0.341	1.537 ± 0.137	2.711 ± 0.486	2.757 ± 0.084	5.81 ± 0.48	147.9 ± 1.5	100.3 ± 1.9
Females									
0	1.19 ± 0.85	1.582 ± 0.430	0.980 ± 0.647	1.851 ± 0.445	2.271 ± 0.494	2.808 ± 0.067	5.65 ± 0.34	140.2 ± 0.6	97.1 ± 0.7
3	0.80 ± 0.61	1.385 ± 0.264	1.023 ± 0.281	1.668 ± 0.260	2.130 ± 0.445	2.828 ± 0.051	5.52 ± 0.40	141.2 ± 1.1	98.0 ± 1.7
6	0.97 ± 0.57	1.420 ± 0.381	0.867 ± 0.279	1.639 ± 0.363	2.349 ± 0.402	2.770 ± 0.056	5.62 ± 0.41	140.9 ± 1.6	98.1 ± 1.3
10	0.78 ± 0.75	1.420 ± 0.249	1.444 ± 0.688	1.770 ± 0.179	2.311 ± 0.422	2.820 ± 0.078	5.53 ± 0.30	140.3 ± 1.8	97.3 ± 2.3

Values are means ± SD for groups of 10 rats. Statistical analysis showed no significant differences between the 2′-FL-exposed groups and controls.

was only slight and occurred in one sex only. There were no statistically significant changes in total or differential white blood cell counts.

There were no statistically significant differences in clinical chemistry variables between the test groups and the controls, except for an increase in urea concentration in mid-dose and high-dose males. In the absence of this finding in females and any corroborative findings in males, this was considered as a chance finding.

Renal concentration test and urinalysis: The renal concentration test showed a statistically significantly decreased specific gravity in females of the high dose group. The decreased specific gravity was only very slight and ascribed to a higher (although not statistically significant) urinary volume excreted. Because these changes were very slight they do not point to impaired concentrating ability of the kidneys and therefore no toxicological significance was attached to this finding. Semi-quantitative (dipstick) urinary measurements and microscopic examination of the urinary sediment did not reveal any differences among the groups.

Organ weights: Absolute and relative organ weights are presented in Tables 8a and 8b, respectively. The relative weight of the liver was statistically significantly increased (by 8.25%) in males in the high-dose group. The absolute and relative weights of the filled and empty cecum were statistically significantly increased in the mid- and high-dose group in male and female rats. In low-dose males only the absolute weight of the filled cecum was statistically significantly increased.

Pathology: At necropsy no exposure-related macroscopic changes were observed. Microscopic evaluation did not reveal exposure-related histopathological changes. The histopathological changes observed were about equally distributed amongst the different treatment groups or occurred in one or a few animals only and are common findings in rats of this strain and age. As described in the clinical observations section, one female animal was found dead on day 24 of the study. Necropsy was performed, and collected tissues were microscopically examined; no cause of death could be established and therefore it was considered unrelated to the exposure.

Table 8a
Absolute organ weights (g/kg b.w.).

Parameter	2′-FL concentration in diet (%)			
	0	3	6	10
Males				
Adrenals	0.0552 ± 0.0098	0.0529 ± 0.0114	0.0549 ± 0.0088	0.0524 ± 0.0103
Brain	2.072 ± 0.113	2.065 ± 0.091	2.068 ± 0.095	2.023 ± 0.084
Cecum (full)	4.0987 ± 0.7466	5.2282 ± 0.9177*	6.8007 ± 1.6408**	7.8187 ± 1.1468**
Cecum (empty)	1.0207 ± 0.1800	1.2139 ± 0.1781	1.4565 ± 0.3894**	1.5951 ± 0.2303**
Epididymis	1.123 ± 0.101	1.154 ± 0.072	1.159 ± 0.083	1.125 ± 0.122
Heart	0.947 ± 0.099	0.974 ± 0.136	1.011 ± 0.129	0.939 ± 0.125
Kidneys	1.933 ± 0.197	2.025 ± 0.249	1.952 ± 0.211	1.932 ± 0.228
Liver	7.965 ± 1.112	8.384 ± 0.936	8.360 ± 1.249	8.254 ± 1.149
Prostate	0.911 ± 0.136	0.906 ± 0.151	0.923 ± 0.111	0.896 ± 0.166
Seminal vesicles	1.012 ± 0.151	1.008 ± 0.166	1.000 ± 0.111	0.995 ± 0.175
Spleen	0.5575 ± 0.0840	0.6082 ± 0.0509	0.6007 ± 0.0774	0.5876 ± 0.0833
Thymus	0.3322 ± 0.0514	0.3544 ± 0.0796	0.3694 ± 0.0906	0.3218 ± 0.0978
Testes	3.478 ± 0.223	3.439 ± 0.257	3.504 ± 0.306	3.394 ± 0.337
Females				
Adrenals	0.0710 ± 0.0134	0.0697 ± 0.0145	0.0683 ± 0.0070	0.0637 ± 0.0057
Brain	1.934 ± 0.067	1.926 ± 0.073	1.880 ± 0.041	1.903 ± 0.055
Cecum (full)	2.7851 ± 0.6912	3.2094 ± 0.5869	3.7067 ± 0.5819**	5.0184 ± 0.3805**
Cecum (empty)	0.7174 ± 0.1018	0.8223 ± 0.1247	0.9552 ± 0.1677**	1.2088 ± 0.1935**
Heart	0.667 ± 0.055	0.668 ± 0.93	0.674 ± 0.041	0.631 ± 0.046
Kidneys	1.315 ± 0.110	1.322 ± 0.131	1.298 ± 0.067	1.290 ± 0.080
Liver	5.254 ± 0.488	5.028 ± 0.448	5.336 ± 0.536	5.233 ± 0.378
Ovaries	0.0939 ± 0.0130	0.0885 ± 0.0130	0.0977 ± 0.0179	0.0912 ± 0.0143
Spleen	0.3925 ± 0.0503	0.4029 ± 0.0497	0.04026 ± 0.0694	0.4362 ± 0.0302
Thymus	0.2776 ± 0.0477	0.2929 ± 0.0438	0.3034 ± 0.0764	0.2939 ± 0.0403
Uterus	0.8214 ± 0.5800	0.6552 ± 0.3163	0.6536 ± 0.4208	0.7189 ± 0.3751

Values are means ± SD for groups of 10 rats; *p < 0.05; **p < 0.01.

Table 8b
Mean terminal body weight (g) and relative organ weight (g/kg b.w.)

Parameter	2′-FL concentration in diet (%)			
	0	3	6	10
Males				
Terminal body weight	345.25 ± 38.51	351.60 ± 36.27	351.76 ± 39.74	331.16 ± 42.87
Adrenals	0.1603 ± 0.0257	0.1502 ± 0.0273	0.1568 ± 0.0226	0.1587 ± 0.0270
Brain	6.046 ± 0.512	5.917 ± 0.520	5.926 ± 0.490	6.175 ± 0.577
Cecum (full)	11.91 ± 2.11	14.93 ± 2.69	19.20 ± 3.52**	23.71 ± 2.58**
Cecum (empty)	2.98 ± 0.56	3.47 ± 0.54	4.13 ± 0.98**	4.85 ± 0.70**
Epididymis	3.272 ± 0.323	3.304 ± 0.287	3.315 ± 0.244	3.412 ± 0.229
Heart	2.749 ± 0.159	2.765 ± 0.195	2.878 ± 0.217	2.838 ± 0.157
Kidneys	5.609 ± 0.282	5.765 ± 0.477	5.558 ± 0.280	5.863 ± 0.533
Liver	23.03 ± 1.23	23.85 ± 1.16	23.73 ± 1.81	24.93 ± 1.50*
Prostate	2.645 ± 0.343	2.583 ± 0.374	2.634 ± 0.264	2.720 ± 0.465
Seminal vesicles	2.967 ± 0.550	2.863 ± 0.339	2.880 ± 0.473	3.056 ± 0.710
Spleen	1.617 ± 0.184	1.741 ± 0.173	1.720 ± 0.239	1.778 ± 0.162
Thymus	0.965 ± 0.142	1.006 ± 0.194	1.046 ± 0.215	0.964 ± 0.241
Testes	10.155 ± 0.963	9.837 ± 0.845	10.015 ± 0.799	10.297 ± 0.617
Females				
Terminal body weight	206.80 ± 14.21	208.66 ± 16.68	202.67 ± 6.71	200.64 ± 11.83
Adrenals	0.3427 ± 0.0539	0.3331 ± 0.0580	0.3375 ± 0.0359	0.3178 ± 0.0260
Brain	9.376 ± 0.436	9.264 ± 0.525	9.283 ± 0.303	9.515 ± 0.629
Cecum (full)	13.56 ± 3.64	15.38 ± 2.56	18.26 ± 2.66**	25.07 ± 2.13**
Cecum (empty)	3.46 ± 0.34	3.94 ± 0.54	4.71 ± 0.81**	6.02 ± 0.89**
Heart	3.230 ± 0.234	3.196 ± 0.292	3.330 ± 0.212	3.147 ± 0.184
Kidneys	6.367 ± 0.463	6.333 ± 0.306	6.409 ± 0.370	6.433 ± 0.251
Liver	25.40 ± 1.54	24.11 ± 1.22	26.38 ± 3.22	26.12 ± 1.76
Ovaries	0.4558 ± 0.0677	0.4265 ± 0.0745	0.4817 ± 0.0849	0.4547 ± 0.0680
Spleen	1.899 ± 0.218	1.937 ± 0.255	1.994 ± 0.388	2.185 ± 0.246
Thymus	1.343 ± 0.210	1.414 ± 0.259	1.499 ± 0.378	1.469 ± 0.213
Uterus	3.948 ± 2.765	3.142 ± 1.519	3.264 ± 2.237	3.587 ± 1.863

Values are means ± SD for groups of 10 rats; *p < 0.05; **p < 0.01.

4. Discussion

In the current study, 2′-FL tested negative in both mutagenicity assays; thus, 2′-FL can be considered as non-genotoxic. With the negative outcomes in both genotoxicity tests, the genotoxic potential of the tested 2′-FL is sufficiently evaluated from a regulatory perspective (e.g. to comply with REACH regulations) and no further (in vivo) experiments are required. The results from the current genotoxicity

studies are corroborated by data available from three earlier GLP-compliant genotoxicity tests performed with synthetic 2′-FL: in an Ames test, an in vitro mammalian cell gene mutation test and an in vitro micronucleus assay, the synthetic 2′-FL tested negatively for genotoxicity (EFSA NDA Panel, 2015).

In the sub-chronic oral toxicity study, the safety of 2′-FL was examined in Wistar rats. 2′-FL was administered at constant concentrations in the diet at levels of 0% (control), 3%, 6% and 10% to groups of 10 rats/sex, during 13 weeks. The exposure to 2′-FL was well tolerated at all dose levels, and did not induce any relevant changes in general condition, growth, water intake, neurobehavioral observations, ophthalmoscopy, hematology, clinical chemistry, urinalysis, organ weights or in macroscopy and microscopy of organs and tissues. The no-observed-adverse effect level (NOAEL) is placed at the highest level tested: ≥7.25 g/kg body weight/day for males and ≥7.76 g/kg body weight/day for females.

Only a few observed changes were attributed to the administration of 2′-FL. In female rats of the high-dose group overall food consumption was slightly decreased. In males there was no statistically significant effect, but there was a similar trend in the high-dose group. Since the relative difference with controls was small (less than 10%), and no clear corroborative changes were observed in any of the other parameters investigated (especially growth), this finding – although likely treatment related – was considered to be of little, if any, toxicological significance.

Cecal enlargement was noted in mid- and high-dose males and females and in low-dose males. This finding is ascribed to the fact that the test substance is a non-digestible carbohydrate. It is well established that cecal enlargement in rats may arise from the feeding of large amounts of a heterogeneous family of products, referred to as 'dietary fiber' or 'poorly digestible carbohydrates (Levrat et al., 1991; Campbell, 1997; Lu et al., 2000; Kim, 2002). These substances are incompletely absorbed, yet fermented in the gastrointestinal tract. The fermentation results in the production of short chain fatty acids (SCFA), which raises the osmotic value of the cecal content and may promote the growth of the mucosal layer (Jin et al., 1994; Frankel et al., 1994; Knapp et al., 2013). Another cause of cecal enlargement can be the feeding of large amounts of substances with water binding properties. The increased cecal weights in the present study were not accompanied by hypertrophy or other histopathological changes. In the absence of such histopathological correlates, cecal enlargement is interpreted as a physiological response rather than a toxic effect (WHO, 1987).

The relative weight of the liver was increased by 8.25% in males in the high-dose group. This increase was not accompanied by changes in clinical chemistry (ALT and ASAT in particular, which are indicators for liver damage) and microscopic examination of the liver did not reveal any histopathological changes. The toxicological interpretation of liver hypertrophy has been discussed in detail on the 3rd international workshop of the European Society of Toxicologic Pathology (ESTP). One of the main conclusions was that liver hypertrophy without histopathological evidence of alterations indicative of liver toxicity can usually be considered as non-adverse (Hall et al., 2012). Therefore, in accordance with this recommendation, the increase in liver weight observed in the current study is not considered adverse.

The set-up of the sub-chronic oral toxicity study slightly differs from the recommendations made in OECD guideline 408 to accommodate the use of juvenile animals; in contrast to what is described in the test guideline, detailed clinical observations and ophthalmoscopy were not performed prior to the exposure. This was not possible due to the use of very young animals, that were weaned only shortly before the start of the exposure. This deliberate and planned omission is not considered to affect the validity of the study results; in fact, because of the intended application of 2'-FL as an additive to infant formula, the use of juvenile animals is of added value.

In the current study, animals were exposed via their feed. The alternative would be to expose animals by oral gavage, providing the significant advantage that exposure can be initiated at a very young age (e.g. PND 7, as in Coulet et al., 2014) corresponding to the human neonatal/infant stage. Although this would indeed be relevant, oral gavage has a number of setbacks. When oral exposure occurs via gavage, the test substance is not allowed to interact with surfaces in the oral cavity, including the buccal, sublingual, gingival, palatal and labial mucosa (Vandenberg et al., 2014). Thus, potential toxicity to the upper digestive system cannot be investigated when delivering the test chemical via oral gavage. Importantly, it is also well known that dosing of rats and mice via oral gavage is a stressful procedure (e.g. Brown et al., 2000; Bonnichsen et al., 2005). Performing oral gavage in animals can induce rapid and pronounced effects on stress-related responses (Balcombe et al., 2004). For instance, an effect on cardiovascular endpoints such as blood pressure and heart rate has been shown (Balcombe et al., 2004; Okva et al., 2006). Higher levels of the stress-response hormone corticosterone were measured in feces of mice upon oral gavage, indicative of activation of the hypothalamic-pituitary-adrenal axis (Walker et al., 2012). Besides stress, oral gavage can induce a number of toxicological phenomena, such as passive reflux if the stomach is overfilled, aspiration pneumonia, pharyngeal, esophageal, and gastric irritation or injury with stricture formation, esophageal and gastric rupture, asphyxia, inflammation, weight loss and hemorrhage (Turner et al., 2011; Bonnichsen et al., 2005; Damsch et al., 2011). Because of these considerations, in the current sub-chronic oral toxicity study animals were exposed to 2′-FL via their feed.

Similar outcomes have been described in two earlier repeated-dose oral toxicity studies with various 2′-FL samples produced using biotechnology. In a 13-week sub-chronic oral toxicity study in which juvenile rats were administered 10% (w/w) biotechnologically produced 2′-FL in their diet, corresponding to a dose of 7.7 or 8.7 g/kg b.w./day in males and females respectively, no toxicity was observed. Thus, the dose level tested was considered the NOAEL (described in GRAS notice GRN 571, 2015). In another 13-week oral toxicity study, Wistar rats were exposed to 2, 4 or 5 g/kg b.w./day biotechnologically produced 2-FL via their diet. In this study, no 2′-FL exposure-related toxicity was observed, and thus the NOAEL was placed at the high dose level of 5 g/kg b.w./day (described in GRAS notice GRN No. 650, 2016).

Furthermore, a sub-acute (3-week) oral toxicity study was performed in neonatal piglets (Hanlon and Thorsrud, 2014); from day 2 of lactation, crossbred farm piglets received a liquid diet containing 0, 200, 500 or 2000 mg/L 2′-FL produced using biotechnological methods, which corresponded to doses of 29, 72 and 292 mg/kg/day in males and 29, 74 and 299 mg/kg/day in females. After 3 weeks of exposure, no exposure-related toxicity was observed; thus, the NOAEL was placed at the high concentration level (2000 mg/L; 292 mg/kg/day for males and 299 mg/kg/day for females) (Hanlon and Thorsrud, 2014).

Somewhat deviating findings were observed in a third 13-week oral toxicity study (GLP-compliant) with chemically synthesized 2′-FL; in the high-dose group (6 g/kg b.w./day), mortality (one male and one female animal died), reduced kidney weight (females) and minimal renal pathology (females) were observed (Coulet et al., 2014). The NOAEL was placed at the mid-dose level by the authors, which was 5 g/kg b.w./day. However, it should be noted that in this study, animals were exposed via oral gavage, and thus it is possible that stress or non-specific toxicity attributable to the daily gavage procedure contributed to the observed effects. Also, in this study chemically synthesized 2′-FL was used, which is slightly different from the biotechnologically produced samples in the other studies.

Thus, the NOAEL for 2′-FL in the current sub-chronic oral toxicity study (≥7.25 and ≥ 7.76 g/kg b.w./day for males and females, respectively) is comparable to the NOAELs obtained from the other sub-chronic oral toxicity studies with biotechnologically-produced 2′-FL: ≥7.7 and ≥ 8.7 g/kg b.w./day for males and females, respectively (GRAS notice GRN 571, 2015) and ≥5 g/kg b.w./day (GRAS notice GRN No. 650, 2016).

5. Conclusions

2′-Fucosyllactose tested negative in both the bacterial reverse mutation test and the in vitro micronucleus test and should thus be considered as non-genotoxic. In the 90-day oral toxicity study, 2′-Fucosyllactose did not induce adverse changes in any test group. Thus, the no-observed-adverse effect level (NOAEL) is placed at the highest concentration tested, corresponding to ≥7.25 g/kg body weight/day for males and ≥7.76 g/kg body weight/day for females. The findings from the current study are in good agreement with available data generated using other biotechnologically produced 2′-FL samples and support its safe use as a food additive.

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