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Safety assessment of the biotechnologically produced human-identical milk oligosaccharide 3-Fucosyllactose (3-FL)

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ABSTRACT

3-Fucosyllactose (3-FL), a highly abundant complex carbohydrate in human breast milk, functions as a prebiotic promoting early microbial colonization of the gut, increasing pathogen resistance and modulating immune responses. To investigate potential health benefits, 3-FL was produced by fermentation using a genetically modified *E. coli* K12 strain. The safety assessment of 3-FL included acute oral toxicity, *in vitro* and *in vivo* assessment of genetic toxicity, and a subchronic rodent feeding study. 3-FL was not acutely toxic at 5000 mg/kg bw, and there was no evidence of genetic toxicity in the bacterial reverse mutation test and chromosomal aberration assay. There was a repeatable statistically-significant trend in the 4-h S9-activated test conditions in the *in vitro* micronucleus assay; the confirmatory *in vivo* mouse micronucleus study was negative at all doses. Dietary subchronic exposure of rats to 3-FL (5% and 10%) did not produce any statistical or biologically-relevant differences in growth, food intake or efficiency, clinical observations, or clinical or anatomic pathology changes at average daily intakes of 5.98 and 7.27 g/kg bw/day for males and females, respectively. The weight of evidence from these studies support the safe use of 3-FL produced using biotechnology as a nutritional ingredient in foods.

1. Introduction

3-Fucosyllactose (3-FL), an oligosaccharide found in human breast milk, is composed of a lactose core of D-glucose and D-galactose units, linked via an α1-3 bond to L-Fucose (Smilowitz et al., 2014). First isolated from human breast milk in the mid-1950s (Kunz, 2012), 3-FL belongs to a group of complex carbohydrates described as human milk oligosaccharides (HMOs) that are highly abundant in breast milk (Bode, 2012; Thurl et al., 2017). 3-FL concentrations in breast milk of healthy women vary across geographically regions, ethnicity and lactation stage. In a survey involving breast milk samples collected from 435 women residing in 10 different countries, Erney et al. (2000) observed a significant difference in mean 3-FL concentrations in samples collected during the same postpartum timeframe (11–30 days) from Asian women (1.76 g/L) compared to those from Latin American women (0.75 g/L). Mean concentrations of 3-FL in breast milk samples from women in Asia and in the US were found to be significantly lower (1.38 g/L and 1.03 g/L, respectively) during the first 3–10 days postpartum compared to after the first month postpartum (2.15 g/L and 2.57 g/L respectively). Biosynthesis and secretion of 3-FL and other fucosylated neutral oligosaccharides has long been associated with the Lewis blood group (Blank et al., 2012). A study by Thurl et al. (2010) reported significantly higher average 3-FL concentrations (1.79 g/L) in breast milk from women with Lewis blood group who lack the gene to secrete 2′-Fucosyllactose (2′-FL). The biological reason for this variability in HMO concentrations is currently unknown although the variability is correlated with multiple factors, e.g., genetics (secretor vs. non-secretor), geography, ethnicity, parity, maternal age, etc. (Azad et al., 2018; McGuire et al., 2017).

HMO research in general, and studies on fucosylated neutral

Abbreviations: HMO, Human Milk Oligosaccharides; 3-FL, 3-fucosyllactose; FOS, fructo-oligosaccharides

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oligosaccharides, most often 2′-FL focused, point to the contribution of HMOs to infant health and the role they have in reducing the susceptibility of infants to diseases. These studies include observational studies on breastfeeding infants, correlating HMOs in breastmilk with gut microbiota profiles and infection and immune function as well as in-vitro cell culture and animal studies. Collectively, these studies report that HMOs and fucosylated oligosaccharides act as prebiotics, facilitating early colonization of the gut by *Bifidobacteria* (Yu et al., 2013; Lewis et al., 2015; German et al., 2008), act as decoy receptors, inhibiting adhesion of potential pathogens to epithelial surfaces in the intestine (Morrow et al., 2004; Coppa et al., 2006; Newburg et al., 2005) and have a role in modulating immune responses (Plaza-Díaz et al., 2018; Triantis et al., 2018).

Current knowledge on 3-FL is more limited. However, the following prospective studies suggest that 3-FL may exert biological functionality that is complementary to, if not unique from 2′-FL: (A) in a study on fucosylated HMOs in breast milk found over the course of lactation, 3-FL concentrations in breast milk gradually increased as 2′-FL levels dropped (Chaturvedi et al., 2001); (B) in an in-vitro study, 3-FL inhibited the adhesion to Caco-2 cells of enteropathogenic bacteria more strongly than 2′-FL (Coppa et al., 2006), and (C) 3-FL has been shown to be significantly more effective in reducing colon motor contractions compared to 2′-FL and other HMOs in a standardized *ex vivo* model (Bienenstock et al., 2013). Supplementary findings suggest an association between colon muscle contractility and neural activity. Together, these data provide preliminary indications of the potential benefits for gut motility disorders and raise speculations on effects on cognition and memory (Bienenstock et al., 2013).

The differential distribution of 3-FL across individual healthy women and emerging data reporting its potential benefits have raised interest for further clinical research and the prospect for dietary supplementation of infant formula and other foods to extend these potential benefits to a wider population. To accomplish this, it is first necessary to produce 3-FL in sufficiently large volumes for use in clinical studies and to establish the safety of the 3-FL produced.

DuPont's 3-FL is produced by fermentation using a modified *E. coli* K12 host production strain. Carefully controlled downstream processing yields a highly purified 3-FL product (> 90% 3-FL assay expressed as area-%, Table 1) with no detectable residual rDNA.[1] The remainder of the product is made up of other carbohydrates including other mono and disaccharides present in foods and infant formula such as lactose, fucose, glucose and galactose. DuPont's 3-FL molecule was internally evaluated by mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR) and confirmed as structurally identical to 3-FL molecules isolated from human breast milk.

The safety assessment of fermentation-produced 3-FL included an evaluation of acute oral toxicity in rats and a genotoxicity evaluation comprising: a bacterial reverse mutation (Ames) test, *in vitro* micronucleus and chromosomal aberration tests, and an *in vivo* micronucleus test. Additionally, the potential for adverse effects of repeated exposure was evaluated in a subchronic (90-day) rodent feeding study including pharmacokinetic evaluation.

2. Materials and methods

The characterization of the 3-FL test substance was performed by trained personnel using established procedures and a validated method, and was subjected to Quality Control review. With exception of the 1.4% described as “other carbohydrates”, 98.6% of the composition is defined (Table 1). Heavy metals, endotoxins and aflatoxins were all below required limits for nutritional ingredients. All studies described herein were conducted in accordance with Good Laboratory Practice (GLP) regulations (US FDA, 1978; OECD, 1998). All studies involving live animals were conducted in test facilities accredited by AAALAC International, and all protocols were reviewed and approved by the appropriate test facility animal welfare authorities prior to study initiation.

Table 1
Composition of 3-fucosyllactose.

Component	Value
3-Fucosyllactose	94.6%
Lactose	1.5%
Glucose/galactose	1.3%
Fucose	1.2%
Other carbohydrates[a]	1.4%
Protein	≤100 μg/g
Ash	≤0.5%
Moisture	1.9%

Note: Carbohydrate values expressed as area-% i.e. not considering moisture.
[a] Excluding 3-FL, lactose, fucose and glucose/galactose.

2.1. Test and comparative control substance information

The compositional characteristics of the 3-FL used for all studies is presented in Table 1. 3-Fucosyllactose (3-FL; Chemical Formula: $C_{18}H_{32}O_{15}$; MW: 488.44 g/mol; CASRN: 41312-47-4) was provided by DuPont Nutrition & Health in powder form. The test substance was produced through fermentation by a genetically engineered *Escherichia coli* K-12 strain followed by purification. The host strain *E. coli* K-12 MG1655 is not considered a human or animal pathogen, is not toxicogenic and is classified as Biosafety Level 1 (US EPA, 1997a; US EPA, 1997b; ATCC, 2018). The purity of 3-FL was 94.6%, expressed as area-%, determined using high performance liquid chromatography (HPLC, Table 1).

Fructooligosaccharide (FOS), an ingredient currently approved as safe for use in infant formula and follow-on formulae in the European Union (EC, 2006) was selected as a reference control in the subchronic rodent feeding study. FOS was obtained from a commercial source and contained 95.6% FOS on a dry weight basis.

2.2. Acute oral toxicity

In accordance with appropriate testing guidelines (US EPA, 2002; US FDA, 2007; OECD, 2008), a limit dose of 5000 mg/kg bw (body weight) was selected based on the history of consumption and lack of toxicity historically observed with HMOs (Phipps et al., 2018; Coulet et al., 2014). Five female Crl:CD®(SD) rats (Charles River Laboratories, Inc., Raleigh, NC) received a single bolus dose of 3-FL in a deionized water vehicle by gastric intubation at a dose volume of 20 mL/kg bw. All animals were fasted for 17.5 h prior to dose administration and food was returned 3.5 h following dosing. Observations for clinical signs of toxicity and body weights were recorded periodically over a 14-day observation period after which all animals were humanely euthanized and examined macroscopically.

2.3. Genetic toxicity

The genetic toxicity potential of 3-FL was evaluated *in vitro* using the bacterial reverse mutation (AMES) test, the mammalian cell micronucleus test in Chinese Hamster Ovary cells and chromosomal Aberration test in human lymphocytes. Additionally, 3-FL was evaluated in mice using the mammalian erythrocyte micronucleus test. All studies were considered valid based on prespecified acceptance criteria stated in the applicable test guidelines, and positive and negative

[1] EFSA 2018 *Guidance on the characterization of microorganisms used as feed additives or as production organisms (*EFSA Journal 2018; 16(3):5206 https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2018.5206).

control results for all studies were within test facility historical ranges for acceptability.

2.3.1. Bacterial reverse mutation (AMES) test

3-FL was evaluated for mutagenicity in the AMES test in accordance with methods reported previously (Karaman et al., 2009). The study was conducted in accordance with guidelines promulgated by the United States Food and Drug Administration (US FDA, 2000a), the Organization for Economic Cooperation and Development (OECD, 1997), and the European Commission (EC, 2008). The test was conducted with *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 and *Escherichia coli* strain WP2*uvr*A in the absence and presence of an exogenous metabolic activation system (Aroclor-induced rat liver S9) using sterile water as the solvent. Based on the results of a preliminary cytotoxicity test, conducted at 3-FL concentrations ranging from 33.3 to 5000 μg/plate, concentrations selected for the mutagenicity test were 333, 667, 1000, 3333, and 5000 μg/plate. All 3-FL concentrations were tested in duplicate for the cytotoxicity test, and in triplicate for the mutagenicity test. Concurrent vehicle and positive control groups were included for each tester strain and test condition.

2.3.2. Mammalian cell micronucleus test in Chinese Hamster Ovary (CHO) cells

3-FL was evaluated *in vitro* for its ability to induce micronuclei in Chinese Hamster Ovary (CHO–K1) cells in accordance with applicable testing guidelines (US FDA, 2000b; OECD, 2016c; EC, 2017b). The test was conducted in the absence and presence of an exogenous metabolic activation system (Aroclor-induced rat liver S9) using sterile water as the solvent. Concentrations of 500, 1000, 2500, 3500, and 5000 μg/mL were selected for the definitive micronucleus assay based on the results of a preliminary toxicity assay at 3-FL concentrations of 1250, 2500 and 5000 μg/mL, and in consideration of the guideline limit concentration (OECD, 2016c); concurrent vehicle and positive control groups were included in each test condition. All concentrations were evaluated in triplicate. Cells were exposed to 3-FL for 4 h in the presence and absence metabolic activation and for 24 h in the absence of metabolic activation, and harvested 24 h after treatment initiation. Cell harvest and staining was conducted according to specifications provided by the kit manufacturer (Litron Laboratories Inc., 2016), with modifications to accommodate the use of 48 well plates. At least 20,000 cells per replicate sample were analyzed by flow cytometry for micronuclei induction and toxicity. Cell proliferation calculations were conducted to ensure an adequate population doubling was achieved.

2.3.3. Mammalian cell chromosomal aberration test in human lymphocytes

The potential for 3-FL to produce structural chromosomal aberrations was evaluated in cultured human lymphocytes obtained from a healthy, non-smoking volunteer, in accordance with OECD testing guidelines (OECD, 2016a). Concentrations of 1250, 2500, and 5000 μg/mL were tested in a definitive assay wherein 3-FL, dissolved in sterile water, was administered to cultures for 4 and 24 h in the absence, and 24 h in the presence of exogenous metabolic activation (Phenobarbitol/β-Naphthaflavone-induced rat liver S9). Test condition-specific positive controls were included; all concentrations and conditions were tested in duplicate and all cultures were harvested 24 h after initial treatment. Cells were incubated with demecolcine (0.1 μg/mL) to induce mitotic arrest prior to expansion in 0.075M hypotonic potassium chloride and fixation with fresh methanol/glacial acetic acid (3:1 v/v). Fixed cells were adhered to slides, air-dried and stained with 5% Giemsa solution prior to evaluation. Mitotic index was calculated by counting 1000 lymphocyte nuclei per slide and expressed as a percentage of the vehicle control value. For each test condition and concentration, at least 300 well-spread metaphases were evaluated; when at least 15 cells with aberrations (excluding gaps) were observed, the evaluation was terminated. Metaphase cells with 44–48 chromosomes were evaluated for gaps, breaks or rearrangements in accordance with the method of Savage (1976). Data are presented as the percentage frequency of aberrant cells excluding (−) and including (+) gaps.

2.3.4. Mammalian erythrocyte micronucleus test

3-FL was evaluated *in vivo* for its ability to induce micronuclei in rodent bone marrow by analyzing peripheral blood reticulocytes (RETs) from 8-week old male and female Crl:CD1(ICR) mice (Charles River Laboratories, Inc., Raleigh, North Carolina, U.S.A.). The study was conducted according to published procedures (Hayashi et al., 1994; Mavournin et al., 1990), and in accordance with applicable regulatory guidelines (OECD, 2016b; US FDA, 2000b). Groups of male and female mice (at least 5/sex) received a single oral (gavage) dose of 3-FL at 500, 1000 or 2000 mg/kg bw. Concurrent negative and positive control groups were similarly administered deionized water or 30 mg/kg bw of cyclophosphamide, respectively. Peripheral blood samples were collected at 48- and 72 h post-dosing for the 3-FL and vehicle control groups, and at 48 h post-dosing for the positive control group. A micronucleus evaluation was conducted on 5 animals per sex per group. A minimum of 20,000 RETs per sample were analyzed using flow cytometry (Dertinger et al., 2006; MacGregor et al., 2006) for the induction of micronuclei and toxicity as indicated by the frequency of immature erythrocytes (RETs) among total erythrocytes. To confirm systemic exposure, blood samples were collected at 4 h post-dosing from one vehicle control animal per sex, and four animals/sex administered 500 mg 3-FL/kg bw. Whole blood was processed to plasma, pooled for each sex of the treated animals, and analyzed for 3-FL concentration using ultra-high-performance liquid chromatography (UHPLC) with tandem mass spectrometry detection.

2.4. 90-day subchronic rodent feeding study

2.4.1. Test system and animal husbandry

Male and female Crl:CD®(SD) rats (Charles River Laboratories, Inc., Raleigh, NC, USA) were maintained in accordance with the Guide for the Care and Use of Laboratory Animals (NRC, 2011) at DuPont Haskell Global Centers for Health and Environmental Sciences (Newark, DE, USA). Animal husbandry conditions were as described previously (Mukerji et al., 2016), except that the animals were pair-housed by gender. During the pretest period, animals were fed Certified Rodent LabDiet® 5002 (PMI® Nutrition International, LLC) *ad libitum*. Animals were acclimated to laboratory conditions for approximately 1 week and released from quarantine based on acceptable health status.

2.4.2. Study design

The study was conducted in accordance with the OECD 408 test guideline (OECD, 1998)[2] and the FDA guidance (US FDA, 2007). The current study was conducted essentially as a limit-test. When there is no expectation of adverse effects, even at very high mg/kg bw doses or intakes, this approach allows the use of only one test group at a

[2] This study was initiated on March 20, 2018, prior to publication of the updated OECD 408 guideline on June 25, 2018, and the in-life portion was completed on June 22, 2018. The updated guideline includes: novel reproductive endpoints (estrus staging by vaginal lavage, possible differential staining and evaluation of testes and epididymides), a different organization of reproductive tissue weights, and thyroid gland weights and serum thyroid hormone concentrations (T3, T4 and TSH) at necropsy. In the current study, stage of estrus was determined microscopically by evaluation of the vaginal cytology in H&E-stained, formalin-fixed 5 μm tissue sections, and all required reproductive tissues were weighed, although not together as specified in updated guidance. Similar to the other data generated in the 90-day study, and based on evaluations of other HMO substances, it is highly unlikely that thyroid response variables would have been different, and there was no *a priori* reason to suspect an endocrine or reproductive/developmental effect of a substance with a history of safe consumption by human infants. Thus, this study is considered valid for all registration purposes.

Table 2
aBacterial reverse mutation test without metabolic activation (mean revertants ± SD).

Conc. µg/plate	TA98		TA100		TA1535		TA1537		WP2 *uvr*A	
	Trial 1[a]	Trial 2[b]	Trial 1[a]	Trial 2[b]	Trial 1[a]	Trial 2[b]	Trial 1[a]	Trial 2[b]	Trial 1[a]	Trial 2[b]
0[c]	30 ± 1	26 ± 3	139 ± 6	146 ± 14	15 ± 1	18 ± 3	10 ± 3	8 ± 2	34 ± 6	45 ± 7
33.3	26 ± 9	NA	134 ± 3	NA	19 ± 1	NA	12 ± 4	NA	45 ± 2	NA
66.7	21 ± 1	NA	138 ± 18	NA	16 ± 2	NA	7 ± 0	NA	35 ± 1	NA
100	18 ± 1	NA	127 ± 8	NA	17 ± 1	NA	6 ± 1	NA	37 ± 6	NA
333	24 ± 4	27 ± 6	117 ± 4	136 ± 3	10 ± 0	21 ± 3	9 ± 1	11 ± 3	42 ± 4	48 ± 3
667	21 ± 1	27 ± 6	136 ± 7	141 ± 11	17 ± 2	19 ± 1	6 ± 4	12 ± 2	43 ± 11	48 ± 7
1000	30 ± 1	28 ± 9	145 ± 9	137 ± 10	15 ± 1	18 ± 1	8 ± 1	12 ± 3	45 ± 4	47 ± 6
3333	23 ± 0	26 ± 10	108 ± 15	149 ± 13	15 ± 4	18 ± 3	9 ± 4	11 ± 1	36 ± 13	46 ± 3
5000	17 ± 4	32 ± 11	132 ± 16	160 ± 14	14 ± 4	17 ± 6	6 ± 4	11 ± 4	38 ± 11	48 ± 6
Positive control	182 ± 8[d]	184 ± 19[d]	815 ± 49[e]	836 ± 12[e]	804 ± 15[e]	873 ± 71[e]	1297 ± 153[f]	1348 ± 121[f]	785 ± 49[g]	669 ± 39[g]

NA – not applicable.
[a] Average of two replicates per trial.
[b] Average of three replicates per trial.
[c] Control (sterile water). Positive controls.
[d] 2-Nitrofluorene.
[e] sodium azide.
[f] ICR-191 acridine.
[g] 4-Nitroquinoline-oxide.

Table 2b
Bacterial reverse mutation test with metabolic activation (mean revertants ± SD).

Conc. µg/plate	TA98		TA100		TA1535		TA1537		WP2 *uvr*A	
	Trial 1[a]	Trial 2[b]	Trial 1[a]	Trial 2[b]	Trial 1[a]	Trial 2[b]	Trial 1[a]	Trial 2[b]	Trial 1[a]	Trial 2[b]
0[c]	32 ± 6	33 ± 3	146 ± 8	163 ± 4	13 ± 3	13 ± 9	9 ± 1	16 ± 2	43 ± 14	58 ± 7
33.3	29 ± 1	NA	154 ± 17	NA	12 ± 5	NA	7 ± 1	NA	47 ± 12	NA
66.7	28 ± 14	NA	153 ± 8	NA	11 ± 1	NA	9 ± 1	NA	43 ± 3	NA
100	20 ± 1	NA	138 ± 5	NA	12 ± 4	NA	11 ± 1	NA	43 ± 6	NA
333	27 ± 5	33 ± 8	159 ± 1	152 ± 10	18 ± 4	11 ± 1	14 ± 4	15 ± 5	51 ± 6	54 ± 5
667	27 ± 1	37 ± 8	142 ± 14	171 ± 5	17 ± 1	18 ± 2	12 ± 0	14 ± 5	41 ± 3	60 ± 3
1000	30 ± 1	39 ± 6	168 ± 9	156 ± 12	11 ± 5	10 ± 1	9 ± 3	13 ± 2	53 ± 9	57 ± 4
3333	20 ± 6	40 ± 5	129 ± 8	154 ± 11	12 ± 4	16 ± 2	8 ± 3	19 ± 4	38 ± 5	61 ± 5
5000	21 ± 4	34 ± 10	130 ± 1	145 ± 4	16 ± 1	17 ± 2	5 ± 1	12 ± 6	39 ± 8	63 ± 2
Positive control	284 ± 28[d]	289 ± 25[d]	2201 ± 86[e]	2320 ± 294[e]	173 ± 10[e]	283 ± 15[e]	238 ± 3[e]	226 ± 21[e]	268 ± 17[e]	258 ± 14[e]

NA – not applicable.
[a] Average of two replicates per trial.
[b] Average of three replicates per trial.
[c] Control (sterile water). Positive controls.
[d] benzo-[a]-pyrene.
[e] 2-amino-anthracene.

prespecified limit. Existing FDA and OECD guidances currently recommend a maximum test substance concentration of 5% for dietary studies or equivalent to ≥1000 mg/kg bw/day, respectively, to avoid the potential introduction of artifacts such as caloric restriction or nutritional imbalance that could imitate or mask test-substance-related adverse effects. However, because of the nature of the use profile of this substance and based on historical information available in the published literature, which demonstrated tolerability by rodents of dietary HMO concentrations as high as 10%, a decision was made to choose a dietary concentration above the FDA-recommended limit of 5% and include a test group fed 3-FL at a high-concentration of 10% in the diet. To mitigate the low-risk potential for nutritional, or other adverse effects at the 10% dietary 3-FL concentration, a decision was made to include a low concentration test group fed diets containing 5% 3-FL, which still met the FDA recommended limit concentration for a subchronic rodent dietary study. The determination to include 2 groups rather than 1 group for a limit-dose test, or 3 groups for a traditional guideline-based toxicity test, was considered to represent an appropriate balance between animal welfare, regulatory expectations, and business requirements for product stewardship.

Animals were computationally stratified into four groups of 10 males and 10 females, with body weights within 20% of the mean for each sex. At approximately 7 weeks of age, groups of animals were fed either the basal diet or diets containing 5% 3-FL, 10% 3-FL, or 10% FOS (w/w), *ad libitum* for at least 90 consecutive days. Fresh diets were prepared and administered weekly. The concentrations and homogenous distribution of 3-FL and FOS in the prepared diets were verified at the beginning and near the middle and end of the study. The stability of 3-FL and FOS in the prepared diets was verified under the conditions of use. Analyses were conducted by LC-MS/MS at Knoell USA, LLC (Garnet Valley, PA, USA).

2.4.3. Experimental variables

Animal observations: Ophthalmology examinations, daily observations for general clinical condition, and detailed physical examinations were conducted as previously described (Mukerji et al., 2016). Additionally, a cage-site clinical examination was performed daily and neurobehavioral assessments [functional observational battery (FOB) and motor activity] were evaluated prior to study initiation and near the end of the study.

Body weight: All animals were weighed periodically during the pretest period, at the initiation of experimental diet administration and

Table 3
In vitro micronucleus evaluation.

Conc. µg/mL	Average relative survival (%)				Average MN frequency (%)				Fold Increase in MN			
	-S9 mix		+S9 mix		-S9 mix		+S9 mix		-S9 mix		+S9 mix	
	4 h	24 h	4 h	4 h[a]	4 h	24 h	4 h	4 h[a]	4 h	24 h	4 h	4 h[a]
0[b]	100	100	100	100	0.69	0.87	1.31	1.94	NA	NA	NA	NA
500	93.60	86.08	112.44	227.42	0.59	1.01	1.59	2.02	0.86	1.17	1.21	1.04
1000	102.12	94.62	111.22	46.66	0.68	0.79	1.55	2.83[c]	0.98	0.91	1.18	1.46
2500	78.44	87.67	88.02	63.76	0.60	0.75	1.71[c]	2.27[c]	0.86	0.86	1.30	1.17
3500	114.35	98.84	108.57	70.76	0.81	0.71	2.15[c]	2.47[c]	1.16	0.82	1.63	1.27
5000	94.01	78.88	94.96	45.88	0.89	1.18	2.09[c]	2.24[c]	1.28	1.36	1.59	1.16
0.4 MMC[e]	65.27	45.64	NA	NA	3.33[d]	5.79[d]	NA	NA	4.79	6.28	NA	NA
0.8 MMC[e]	54.54	47.14	NA	NA	7.91[d]	8.61[d]	NA	NA	11.39	9.92	NA	NA
2 CP[f]	NA	NA	75.22	36.31	NA	NA	5.74[d]	5.75[d]	NA	NA	4.37	2.96
4 CP[f]	NA	NA	95.67	32.14	NA	NA	8.16[d]	10.90[d]	NA	NA	6.21	5.62

NA – not applicable.
[a] 4-h S9 activated confirmatory assay.
[b] Control (sterile water).
[c] Statistically significant concentration-related trend ($p \leq 0.05$).
[d] Statistically significant increase in micronuclei relative to the vehicle control ($p \leq 0.05$). Positive Controls.
[e] MMC – mitocycin C.
[f] CP – cyclophosphamide.

Table 4
In vitro chromosomal aberration evaluation.

Conc.	Frequency of Aberrant Cells (%)								
	4 h[a] -S9 mix			4 h[a] + S9 mix			24 h -S9 mix		
µg/mL	MI[b] (%)	- Gaps[c]	+ Gaps	MI (%)	- Gaps	+ Gaps	MI (%)	- Gaps	+ Gaps
0[d]	100	0.3	1.3	100	0.5	1.0	100	0.7	1.3
1250	91	1.0	1.0	101	0.3	0.3	80	1.3	1.7
2500	68	0.3	0.7	72	0.7	1.3	85	1.0	2.0
5000	85	0.0	0.7	86	1.0	1.0	58	0.0	0.3
0.2 MMC[f]	NA	NA	NA	NA	NA	NA	41	50.8[e]	50.8
0.4 MMC[f]	75	22.3[e]	22.3	NA	NA	NA	NA	NA	NA
2 CP[g]	NA	NA	NA	68	24.3[e]	26.4	NA	NA	NA

NA – not applicable.
[a] 4-h exposure followed by 20-h culture in treatment-free media.
[b] MI – mitotic index.
[c] Combination of chromatid and chromosome breaks and exchanges, and other aberrations, excluding gaps.
[d] Control (sterile water).
[e] Statistically significant increase in aberration frequency relative to the vehicle control ($p \leq 0.05$). Positive Controls.
[f] MMC – mitocycin C.
[g] CP – cyclophosphamide.

weekly thereafter. A fasted body weight was recorded for all animals on the day of scheduled necropsy for calculation of organ to body weight ratios.

Food consumption and test material intake: Food consumption was measured for each pair of animals within a cage by weighing the feeders at the beginning and end of each study week. Feed efficiency was calculated as a ratio of body weight gain to food consumption. The consumed 3-FL and FOS doses (mg/kg bw/day) were calculated weekly using the theoretical diet concentrations, and weekly body weight and food consumption values.

Pharmacokinetic Evaluation for 3-FL: Blood and urine samples were collected during week 12 for analysis of 3-FL concentration and kinetic evaluation. Animals were placed individually into metabolism cages for 16 h with access to their experimental diets and water. Urine was collected on dry ice, weighed, and stored at −80 °C until analysis. Following urine collection, blood samples were collected at three time points from 3 animals/sex/group at 6 a.m. and 10 a.m. and 4 animals/sex/group at 2 p.m. Whole blood was processed to serum and stored at −80 °C until analysis. Urine and serum samples were analyzed for 3-FL concentration using ultra-high-performance liquid chromatography (UHPLC) with tandem mass spectrometry detection. The systemic exposure of 3-FL was determined by area-under-the-curve (AUC_{24}) analysis of serum concentrations at four time points: The times were 6 a.m., 10 a.m., 2 p.m. and 6 a.m. starting on test day 80 for male rats or test day 81 for female rats. Test day 81 or 82 (6:00 a.m.) serum concentrations were set equal to the measured values on test day 80 or 81 (6:00 a.m.) assuming steady-state kinetics associated with diurnal dietary intake. The linear trapezoidal rule was used to calculate the AUC_{24} as follows:

$$AUC_{24} = \left[\frac{C_{6\,a.m.} + C_{10\,a.m.}}{2}\right] \times 4 + \left[\frac{C_{10\,a.m.} + C_{2\,p.m.}}{2}\right] \times 4 + \left[\frac{C_{2\,p.m.} + C_{6\,a.m.}}{2}\right] \times 16,$$

where C is the serum concentration at the designated time (Jochemsen et al., 1993).

Urine volume and analyte concentration values were used to calculate the recovery of 3-FL in urine as the mol percentage of daily dietary intake measured on test days 78–85.

Table 5
In vivo micronucleus evaluation (mean ± SD).

MALES	Control (0 mg/kg bw)	500 mg/kg bw	1000 mg/kg bw	2000 mg/kg bw	CP[a]
	N = 5	N = 5	N = 5	N = 5	
RET %					
48-Hour	2.26 ± 0.39	2.15 ± 0.50	2.05 ± 0.46	1.63 ± 0.28[b,c]	0.78 ± 0.28[b]
72-Hour	2.30 ± 0.35	2.27 ± 0.39	2.19 ± 0.54	1.76 ± 0.30[c]	NA
MN-RET %					
48-Hour	0.18 ± 0.03	0.18 ± 0.03	0.17 ± 0.04	0.18 ± 0.02	0.83 ± 0.18[b]
72-Hour	0.17 ± 0.07	0.21 ± 0.05	0.16 ± 0.03	0.24 ± 0.09	NA
FEMALES	N = 5	N = 5	N = 5	N = 5	
RET %					
48-Hour	1.51 ± 0.47	1.47 ± 0.78	1.44 ± 0.40	1.33 ± 0.44	0.76 ± 0.50[b]
72-Hour	1.68 ± 0.54	1.78 ± 0.69	1.62 ± 0.46	1.49 ± 0.30	NA
MN-RET %					
48-Hour	0.17 ± 0.06	0.19 ± 0.07	0.20 ± 0.08	0.16 ± 0.05	0.59 ± 0.07[b]
72-Hour	0.17 ± 0.05	0.23 ± 0.09	0.19 ± 0.04	0.24 ± 0.14	NA

NA – not applicable.
[a] Positive control: CP – cyclophosphamide (30 mg/kg bw).
[b] Statistically significant difference relative to the control group ($p < 0.05$).
[c] Statistically significant decreasing trend ($p < 0.05$). RET – reticulocyte, MN-RET – micronucleated reticulocyte.

Table 6
90-Day feeding study - body weight and dietary intake (mean ± SD).

Parameter	Units	Basal Diet Control	5% 3-FL	10% 3-FL	FOS Control
MALES		N = 10	N = 10	N = 10	N = 10
Final body weight[a]	g	575.9 ± 60.2	592.5 ± 66.7	573.4 ± 70.6	570.1 ± 59.3
Body weight gain[b]	g	380.0 ± 53.7	395.3 ± 58.6	378.4 ± 62.7	373.4 ± 55.4
Food consumption	g/day	26.5 ± 2.3	27.6 ± 2.4	26.2 ± 2.9	27.2 ± 2.2
Food efficiency	g/kg bw/day	0.157 ± 0.016	0.157 ± 0.014	0.158 ± 0.011	0.150 ± 0.013
Test Material Intake	g/kg bw/day	n/a	3.04 ± 0.16	5.98 ± 0.23	6.22 ± 0.26
FEMALES		N = 10	N = 10	N = 10	N = 10
Final body weight	g	314.1 ± 24.2	305.6 ± 14.5	311.5 ± 27.2	303.5 ± 36.6
Body weight gain	g	160.4 ± 21.3	153.5 ± 15.5	158.3 ± 24.9	150.6 ± 33.3
Food consumption	g/day	19.1 ± 1.0	19.9 ± 1.3	19.1 ± 0.7	19.0 ± 1.1
Food efficiency	g/kg bw/day	0.092 ± 0.011	0.085 ± 0.009	0.091 ± 0.015	0.087 ± 0.016
Test Material Intake	g/kg bw/day	n/a	3.87 ± 0.30	7.27 ± 0.59	7.41 ± 0.46

[b] Overall (test days 1–92). There were no statistically significant differences relative to the basal diet control group at $p < 0.05$.
[a] Final, non-fasted (test day 92).

Clinical Pathology: On the day prior to the scheduled sacrifice, animals were fasted overnight in metabolism cages for urine collection. On the day of sacrifice, blood samples for hematology, clinical chemistry and coagulation parameters were collected. Samples were collected into tubes containing EDTA (hematology) and citrate (coagulation) as anticoagulants. Sample for clinical chemistry were collected without anticoagulants. Animals were anesthetized by inhalation of isoflurane followed by exsanguination which completed euthanasia. Hematology, coagulation, serum chemistry and urinalysis variables were analyzed as described previously (Mukerji et al., 2016).

Anatomic Pathology: Following euthanasia, all animals received a complete gross necropsy. Absolute and relative organ weights were measured for brain, adrenals, heart, kidneys, liver, spleen, thymus, and for male accessory sex organs, epididymides, testes, prostate seminal vesicles, or for female ovaries and uterus. Tissues from the digestive, urinary, respiratory, cardiovascular, hematopoietic, endocrine, nervous, musculoskeletal and reproductive systems, along with skin and eyes, were preserved in fixative, processed to slides, and stained with hematoxylin and eosin (Mukerji et al., 2016). Slides prepared from animals in 10% 3-FL, 10% FOS, and basal diet control groups were evaluated microscopically by a veterinary pathologist and peer reviewed by a second veterinary pathologist.

2.5. Statistical analyses

2.5.1. Bacterial reverse mutation test

For all replicates, results are presented as the mean revertants per plate with the standard deviation. No further statistical evaluation was conducted.

2.5.2. Mammalian cell micronucleus test in Chinese Hamster Ovary cells

Statistical analyses of *in vitro* micronucleus data were conducted one-tailed and at a significance level of 5%. Data was transformed prior to analysis using an arcsine square root transformation (Freeman and Tukey, 1950), as the distribution of the transformed data more closely approximated a normal distribution compared with the non-transformed proportion of the data (Snedecor and Cochran, 1967). An ANOVA and Dunnett's test were performed on the transformed MN frequencies (Snedecor and Cochran, 1967; Dunnett, 1964). The frequency of MNs were evaluated only for an increasing response in relation to the concurrent vehicle control. A Williams test for dose responsiveness was conducted (Williams, 1971, 1972).

2.5.3. Mammalian cell chromosomal aberration test in human lymphocytes

The frequency of cells with aberrations excluding gaps was

Table 7
90-Day feeding study - hematology and coagulation (mean ± SD).

	Basal Diet Control	5% 3-FL	10% 3-FL	FOS Control
MALES Hematology	N = 10	N = 10	N = 10	N = 10
RBC (× 10^6/μL)	8.50 ± 0.54	8.20 ± 0.34	8.44 ± 0.54	8.22 ± 0.22
HGB (g/dL)	14.6 ± 0.6	14.8 ± 0.7	14.9 ± 0.7	14.3 ± 0.4
HCT (%)	44.7 ± 2.5	45.3 ± 2.3	45.3 ± 2.5	43.8 ± 1.3
MCV (fL)	52.6 ± 1.5	55.2* ± 2.3	53.8 ± 1.2	53.3 ± 1.2
MCH (pg)	17.2 ± 0.5	18.1* ± 0.7	17.6 ± 0.5	17.5 ± 0.3
MCHC (g/dL)	32.7 ± 0.6	32.8 ± 0.6	32.8 ± 0.6	32.7 ± 0.5
RDW (%)	12.7 ± 0.5	12.3 ± 0.7	12.2 ± 0.3	12.4 ± 0.5
PLT (× 10^3/μL)	978 ± 103	962 ± 84	971 ± 80	1001 ± 152
WBC (× 10^3/μL)	9.41 ± 1.74	9.06 ± 2.18	8.94 ± 1.34	8.49 ± 1.40
ANEU (× 10^3/μL)	1.33 ± 0.29	1.23 ± 0.38	1.35 ± 0.34	1.39 ± 0.64
ALYM (× 10^3/μL)	7.59 ± 1.40	7.38 ± 1.85	7.15 ± 1.39	6.71 ± 1.40
AMON (× 10^3/μL)	0.27 ± 0.09	0.26 ± 0.06	0.28 ± 0.13	0.24 ± 0.08
AEOS (× 10^3/μL)	0.14 ± 0.05	0.12 ± 0.05	0.11 ± 0.04	0.10 ± 0.05
ABAS (× 10^3/μL)	0.03 ± 0.02	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
ALUC (× 10^3/μL)	0.04 ± 0.02	0.04 ± 0.02	0.03 ± 0.01	0.03 ± 0.02
ARET (× 10^3/μL)	172.8 ± 24.3	187.6 ± 42.9	181.8 ± 22.6	174.9 ± 25.1
Coagulation	N = 10	N = 10	N = 10	N = 10
PT (sec)	17.1 ± 0.9	17.2 ± 1.0	17.0 ± 0.6	17.0 ± 0.7
APTT (sec)	16.2 ± 0.9	15.6 ± 0.8	15.7 ± 0.8	15.5 ± 1.4
FEMALES Hematology	N = 10	N = 10	N = 10	N = 10
RBC (× 10^6/μL)	8.02 ± 0.32	7.90 ± 0.44	8.05 ± 0.26	7.97 ± 0.31
HGB (g/dL)	14.8 ± 0.5	14.8 ± 0.8	15.0 ± 0.4	14.8 ± 0.6
HCT (%)	44.5 ± 1.9	44.6 ± 2.6	45.2 ± 1.3	44.5 ± 2.2
MCV (fL)	55.6 ± 1.6	56.5 ± 1.3	56.2 ± 1.6	55.9 ± 2.1
MCH (pg)	18.5 ± 0.7	18.8 ± 0.4	18.7 ± 0.6	18.6 ± 0.4
MCHC (g/dL)	33.2 ± 0.6	33.2 ± 0.5	33.3 ± 0.5	33.3 ± 0.6
RDW (%)	11.3 ± 0.4	11.2 ± 0.3	11.2 ± 0.3	10.9* ± 0.3
PLT (× 10^3/μL)	984 ± 103	995 ± 161	1048 ± 318	931 ± 71
WBC (× 10^3/μL)	6.39 ± 1.63	5.47 ± 1.38	6.39 ± 1.70	6.16 ± 1.68
ANEU (× 10^3/μL)	0.81 ± 0.49	0.67 ± 0.29	0.75 ± 0.28	0.68 ± 0.39
ALYM (× 10^3/μL)	5.29 ± 1.31	4.49 ± 1.32	5.32 ± 1.47	5.20 ± 1.33
AMON (× 10^3/μL)	0.18 ± 0.08	0.19 ± 0.08	0.19 ± 0.09	0.15 ± 0.06
AEOS (× 10^3/μL)	0.07 ± 0.02	0.07 ± 0.02	0.08 ± 0.04	0.07 ± 0.02
ABAS (× 10^3/μL)	0.01 ± 0.01	0.01 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
ALUC (× 10^3/μL)	0.03 ± 0.01	0.02 ± 0.01	0.04 ± 0.03	0.04 ± 0.02
ARET (× 10^3/μL)	166.7 ± 31.2	174.7 ± 30.2	167.0 ± 27.5	157.5 ± 40.9
Coagulation	N = 10	N = 10	N = 9	N = 10
PT (sec)	16.5 ± 0.5	16.3 ± 0.4	16.4 ± 0.3	16.5 ± 0.6
APTT (sec)	13.9 ± 1.3	14.5 ± 1.0	14.0 ± 1.0	14.0 ± 1.6

RBC – red blood cells, HGB – hemoglobin, HCT – hematocrit, MCV – mean corpuscular volume, MCH – mean corpuscular hemoglobin concentration, RDW – red blood cell distribution width, PLT – platelets, WBC – white blood cells, ANEU – absolute neutrophils, ALYM – absolute lymphocytes, AMON – absolute monocytes, AEOS – absolute eosinophils, ABAS – absolute basophils, ALUC – absolute large unstained cells, ARET – absolute reticulocytes.
*Statistically significant difference relative to the basal diet control group at $p < 0.05$.

compared with the concurrent vehicle control value using Fisher's exact test (Richardson et al., 1989). Polyploid cells were not observed.

2.5.4. Mammalian erythrocyte micronucleus test

In vivo micronucleus data analyses were one-tailed and conducted at a significance level of 5%. Statistical analysis of the positive control group as compared to the negative control group was conducted separately from the treatment groups. For each treatment group, the mean and standard deviation of % RETs and % micronucleated reticulocytes (MN RETs) were calculated. As with the *in vitro* micronucleus assay, data were transformed prior to analysis using an arcsine square root transformation (Freeman and Tukey, 1950; Sokal and Rohlf, 1969). Data was evaluated separately by gender for a decreasing frequency of RETs and an increasing frequency of MN RETs. For each sex and timepoint, an analysis of variance (ANOVA) and Dunnett's test were performed on both the RET and MN RET frequencies. (Snedecor and Cochran, 1967; Dunnett, 1964). Where the data were found not consistent with the requirements for a Dunnett's test, a Dunn's non-parametric test was used. A *t*-test was used to compare positive and negative controls. Statistical significance was further evaluated for a dose response using the Williams's test (Williams, 1971, 1972) or the non-parametric Jonckheere Terpstra test, where appropriate (Jonckheere, 1954).

2.5.5. 90-Day subchronic rodent feeding study

Body weight and food consumption parameters, clinical pathology parameters, and organ weights were analyzed by one-way analysis of variance (Snedecor and Cochran, 1967) followed by Dunnett's test (Dunnett, 1964). Transformations were applied (log, square root, or rank order) when appropriate, based on preliminary tests for homogeneity of variance (Levene, 1960) and normality (Shapiro and Wilk, 1965). Significance was judged at $P < 0.05$. Motor activity and grip

Table 8
90-Day feeding study - clinical chemistry (mean ± SD).

MALES	Basal Diet Control	5% 3-FL	10% 3-FL	FOS Control
	N = 10	N = 10	N = 10	N = 10
AST (U/L)	106[a] ± 54	70[c] ± 10	77[a] ± 12	76[b] ± 13
ALT (U/L)	37 ± 20	26 ± 6	27 ± 5	27 ± 5
SDH (U/L)	9.3[a] ± 5.7	5.7[c] ± 3.0	5.9[b] ± 2.2	6.5[b] ± 2.4
ALKP (U/L)	80 ± 18	71 ± 13	83 ± 19	83 ± 20
BILI (mg/dL)	0.12 ± 0.02	0.13 ± 0.02	0.11 ± 0.01	0.12 ± 0.02
BUN (mg/dL)	12 ± 2	12 ± 1	12 ± 1	12 ± 1
CREA (mg/dL)	0.32 ± 0.05	0.33 ± 0.03	0.32 ± 0.04	0.32 ± 0.05
CHOL (mg/dL)	58 ± 17	61 ± 9	57 ± 12	59 ± 13
TRIG (mg/dL)	51 ± 29	46 ± 17	41 ± 10	46 ± 19
GLUC (mg/dL)	207 ± 34	220 ± 33	199 ± 37	188 ± 44
TP (g/dL)	6.0 ± 0.3	6.1 ± 0.3	6.2 ± 0.2	5.9 ± 0.2
ALB (g/dL)	3.1 ± 0.1	3.1 ± 0.1	3.0 ± 0.1	3.0 ± 0.1
GLOB (g/dL)	2.9 ± 0.2	3.0 ± 0.2	3.1 ± 0.2	2.9 ± 0.2
CALC (mg/dL)	10.6 ± 0.4	10.7 ± 0.5	10.5 ± 0.5	10.3 ± 0.3
IPHS (mg/dL)	8.1[a] ± 0.6	8.1[c] ± 0.5	7.9[a] ± 0.9	7.5[b] ± 0.8
NA (mmol/L)	141.5 ± 0.8	141.6 ± 1.7	141.6 ± 1.6	142.3 ± 1.5
K (mmol/L)	6.70 ± 0.90	6.55 ± 0.85	6.47 ± 0.98	5.89 ± 0.83
CL (mmol/L)	102.8 ± 1.9	102.7 ± 1.5	103.1 ± 1.5	104.0 ± 1.5
TBA (μmol/L)	12.1 ± 15.5	5.2 ± 2.2	5.8 ± 2.2	7.5 ± 3.7
FEMALES	N = 10	N = 10	N = 10	N = 10
AST (U/L)	81 ± 19	117 ± 108	104[a] ± 54	90[a] ± 41
ALT (U/L)	25 ± 11	39 ± 40	41 ± 20	30 ± 19
SDH (U/L)	6.2 ± 3.6	15.0 ± 25.0	10.3[a] ± 6.3	9.2[a] ± 5.6
ALKP (U/L)	38 ± 9	40 ± 8	43 ± 14	41 ± 9
BILI (mg/dL)	0.14 ± 0.03	0.15 ± 0.03	0.15 ± 0.03	0.15 ± 0.02
BUN (mg/dL)	14 ± 1	15 ± 2	15 ± 2	15 ± 2
CREA (mg/dL)	0.37 ± 0.04	0.40 ± 0.04	0.38 ± 0.05	0.39 ± 0.03
CHOL (mg/dL)	71 ± 8	73 ± 12	64 ± 16	57* ± 10
TRIG (mg/dL)	34 ± 8	34 ± 7	32 ± 11	37 ± 9
GLUC (mg/dL)	161 ± 30	161 ± 37	164 ± 25	158 ± 19
TP (g/dL)	6.7 ± 0.4	7.0 ± 0.6	6.7 ± 0.4	6.6 ± 0.4
ALB (g/dL)	3.6 ± 0.2	3.8 ± 0.4	3.6 ± 0.3	3.5 ± 0.2
GLOB (g/dL)	3.1 ± 0.2	3.2 ± 0.3	3.1 ± 0.2	3.1 ± 0.3
CALC (mg/dL)	10.7 ± 0.3	10.9 ± 0.6	10.7 ± 0.5	10.8 ± 0.4
IPHS (mg/dL)	7.0 ± 0.7	7.1 ± 1.3	6.9[a] ± 0.8	7.3[a] ± 1.1
NA (mmol/L)	141.9 ± 0.8	141.5 ± 1.5	142.5 ± 1.2	141.6 ± 1.1
K (mmol/L)	5.39 ± 0.68	5.51 ± 1.02	5.31 ± 0.74	5.63 ± 0.95
CL (mmol/L)	104.4 ± 0.9	104.0 ± 1.1	104.8 ± 1.1	103.7 ± 1.2
TBA (μmol/L)	9.5 ± 5.4	10.7 ± 5.5	15.0 ± 8.8	14.2 ± 7.1

[a] N = 9. [b] N = 8. [C] N = 6 AST – alanine aminotransferase, ALT – alanine aminotransferase, SDH -sorbitol dehydrogenase, ALKP – alkaline phosphatase, BILI – bilirubin, BUN – urea nitrogen, CREA – creatinine, CHOL – cholesterol (total), TRIG – triglycerides, ALB – albumin, GLOB – globulin, CALC – calcium, IPHS phosphorus (inorganic), NA – sodium, K – potassium, CL – chloride, TBA – bile acids (total).
* Statistically significant difference relative to the basal diet control group at $P < 0.05$.

strength data were transformed (log, square root, or normalized rank order) when appropriate, based on the preliminary tests, and then analyzed using a repeated measures ANOVA followed by linear contrasts (Milliken and Johnson, 1984; Hocking, 1985). Descriptive FOB data were analyzed using Cochran-Armitage test for trend (Snedecor and Cochran, 1967). If the incidence was not significant, but a significant lack of fit occurred, then Fisher's Exact test with a Bonferroni-Holm correction was used (Fisher, 1985; Holm, 1979). Significance was judged at $P < 0.05$. Separate analyses were performed for each sex.

3. Results

3.1. Acute oral toxicity

Oral administration of a single, bolus dose of 5000 mg 3-FL/kg bw was well tolerated. There were no deaths or clinical signs of toxicity, and all animals gained weight during the 14-day post-exposure observation period. There were no macroscopic observations at necropsy.

3.2. Genetic toxicity

3.2.1. Bacterial reverse mutagenicity test

No positive mutagenic responses (≥2.0-fold with TA98, TA100 and WP2 *uvr*A or ≥3.0-fold with TA1535 and TA1537) or appreciable toxicity were observed with any tester strain in either the absence or presence of S9 metabolic activation (Tables 2a and 2b), and precipitation was not observed at any 3-FL concentration tested. All tester strain negative control values were within the laboratory historical control (HCD) range, and all positive control values demonstrated a > 3.0-fold increase compared to the concurrent negative control.

3.2.2. Mammalian cell micronucleus test in Chinese Hamster Ovary cells

In the *in vitro* micronucleus assay, there were no significant increases in the percentage of micronuclei ($p > 0.05$, Dunnett's test) at any 3-FL concentration in the presence or absence of S9 metabolic activation (Table 3), and all values were within the 95% confidence interval (CI) of the laboratory HCD ranges (4-h -S9 = 0.12%–3.62%; 24-h -S9 = 0.64%–4.413%; 4-h + S9 = 0%–5.38%). However, an increasing, statistically significant trend ($p \leq 0.05$, William's test) was observed in the 4-h S9-activated test condition at 3-FL concentrations ≥ 2500 μg/mL. To further characterize the observed statistical trend, a confirmatory assay was conducted using identical 3-FL concentrations and the 4-h S9-activated test condition (Table 3). Consistent with the first analysis, there were no significant ($p > 0.05$, Dunnett's test) differences in the percentage of micronuclei, and values were within the 95% CI of the laboratory HCD range. However, a statistically significant ($p \leq 0.05$, William's test), concentration-related trend was again observed at 3-FL concentrations ≥ 1000 μg/mL. The cell cycle length during exposure was within acceptable limits (1.5–2.0 normal cell cycles), positive control substances demonstrated statistically significant ($p \leq 0.05$, Dunnett's test) increases in MN frequency compared with the concurrent negative control (Table 3), and all controls were within the laboratory HCD range; therefore, the study was considered valid. Induction of micronuclei in CHO cells was not observed at any 3-FL concentration tested, as evidenced by the lack of statistical significance compared to concurrent controls, and the comparability to laboratory HCD ranges. However, based on the reproducible statistical trend test in the S9-activated test system, the study was concluded to be equivocal, and neither positive or negative (OECD, 2016c).

3.2.3. Mammalian cell chromosomal aberration test in human lymphocytes

No statistically significant differences or evidence of increased frequency of chromosomal aberrations, as assessed by evaluation of chromosome and chromatid breaks and exchanges, and gaps, was observed in metaphase cells of cultured human lymphocytes treated with 3-FL under any treatment condition at concentrations ≤5000 μg/mL (Table 4). Both positive control substances demonstrated statistically significant ($p \leq 0.05$, Fisher's Exact test) increases in the frequency of aberrant cells compared to the sterile water control, and positive and vehicle control responses were within the laboratory HCD ranges.

3.2.4. Mammalian erythrocyte micronucleus test

There were no statistically significant or biologically relevant increases in the frequency of MN RET in peripheral blood samples from mice administered 3-FL at any dose level or evaluation time point. Analyzed 3-FL plasma concentrations were 572 and 681 ng/mL, respectively, in pooled samples from male and female mice administered 500 mg 3-FL/kg bw; 3-FL was not detected in control plasma samples (data not shown). The frequency of RETs was significantly decreased ($p < 0.05$, Dunnett's test) at 48 h post-dosing, and a significantly-decreasing trend ($p < 0.05$, William's test) in RET frequency was observed in samples from male mice administered 2000 mg 3-FL/kg bw at

Table 9
90-Day feeding study - urinalysis (mean ± SD).

MALES	Basal Diet Control	5% 3-FL	10% 3-FL	FOS Control
	N = 10	N = 10	N = 10	N = 10
UVOL (mL)	14.3 ± 6.7	15.7 ± 9.5	14.4 ± 7.2	11.2 ± 6.0
pH	6.7 ± 0.4	7.0 ± 0.4	7.0 ± 0.6	6.8 ± 0.5
SG	1.031 ± 0.014	1.030 ± 0.012	1.029 ± 0.013	1.034 ± 0.014
URO (EU/dL)	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
UMTP (mg/dL)	92 ± 43	99 ± 55	110 ± 57	112 ± 55
FEMALES	N = 10	N = 10	N = 10	N = 10
UVOL (mL)	10.5 ± 8.6	9.5 ± 5.4	11.2 ± 7.8	11.5 ± 6.2
pH	6.7 ± 0.2	6.8 ± 0.4	6.7 ± 0.4	6.7 ± 0.6
SG	1.031 ± 0.017	1.029 ± 0.014	1.024 ± 0.010	1.025 ± 0.017
URO (EU/dL)	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
UMTP (mg/dL)	30 ± 17	32 ± 25	26 ± 14	29 ± 27

UVOL – urine volume, SG – specific gravity, URO – urobilinogen, UMTP – urine protein (total).
There were no statistically significant differences relative to the basal diet control group at $p < 0.05$.

Table 10
90-Day feeding study - organ weights as percentage of terminal body weight (mean ± SD).

MALES	Basal Diet Control	5% 3-FL	10% 3-FL	FOS Control
	N = 10	N = 10	N = 10	N = 10
Fasted body weight (g)	549.5 ± 60.1	565.1 ± 66.0	539.5 ± 66.8	539.0 ± 54.7
Brain	0.403 ± 0.048	0.385 ± 0.043	0.418 ± 0.049	0.412 ± 0.040
Adrenals	0.010 ± 0.002	0.010 ± 0.001	0.011 ± 0.002	0.011 ± 0.003
Heart	0.289 ± 0.032	0.283 ± 0.016	0.299 ± 0.033	0.292 ± 0.015
Kidneys	0.636 ± 0.047	0.640 ± 0.050	0.645 ± 0.062	0.638 ± 0.049
Liver	2.399 ± 0.209	2.448 ± 0.195	2.534 ± 0.269	2.422 ± 0.136
Spleen	0.151 ± 0.017	0.147 ± 0.024	0.153 ± 0.025	0.147* ± 0.023
Thymus	0.051 ± 0.014	0.056 ± 0.019	0.055 ± 0.016	0.055 ± 0.021
Accessory Sex	0.644 ± 0.107	0.633 ± 0.102	0.634 ± 0.108	0.617 ± 0.101
Epididymides	0.260 ± 0.033	0.255 ± 0.035	0.271 ± 0.031	0.270 ± 0.040
Testes	0.647 ± 0.051	0.650 ± 0.068	0.672 ± 0.088	0.662 ± 0.091
Prostate	0.137 ± 0.052	0.123 ± 0.042	0.151 ± 0.055	0.124 ± 0.049
Seminal Vesicles	0.507 ± 0.094	0.510 ± 0.074	0.483 ± 0.086	0.493 ± 0.067
FEMALES	N = 10	N = 10	N = 10	N = 10
Fasted body weight (g)	290.5 ± 23.5	281.9 ± 14.7	285.9 ± 23.3	276.6 ± 31.5
Brain	0.697 ± 0.061	0.719 ± 0.040	0.704 ± 0.054	0.737 ± 0.084
Adrenals	0.026 ± 0.005	0.026 ± 0.005	0.027 ± 0.005	0.026 ± 0.003
Heart	0.334 ± 0.021	0.338 ± 0.025	0.343 ± 0.016	0.352 ± 0.021
Kidneys	0.679 ± 0.049	0.688 ± 0.051	0.684 ± 0.065	0.710 ± 0.051
Liver	2.575 ± 0.128	2.679 ± 0.233	2.714 ± 0.274	2.764 ± 0.206
Spleen	0.187 ± 0.025	0.167 ± 0.027	0.195 ± 0.029	0.192 ± 0.032
Thymus	0.084 ± 0.019	0.086 ± 0.017	0.091 ± 0.017	0.089[a] ± 0.024
Ovaries	0.051 ± 0.008	0.048 ± 0.009	0.044 ± 0.007	0.056 ± 0.010
Uterus	0.216 ± 0.057	0.266 ± 0.108	0.223 ± 0.057	0.268 ± 0.146

[a] N = 9. There were no statistically significant differences relative to the basal diet control group at $p < 0.05$.

48- and 72 h post-dosing. Together with the plasma concentration data, these reductions are an indication of target cell exposure. No significant differences in RET frequency were observed in females administered 3-FL at any dose level or evaluation time point. The positive control group demonstrated a significant ($p < 0.05$, Dunnett's test) increase in MN RET frequency, and the positive and negative control values were within the laboratory HCD ranges.

3.3. 90-day subchronic rodent feeding study

Periodic analyses of diet samples confirmed the appropriate concentrations, stability and homogeneous distribution of 3-FL and FOS within the diet matrix. As expected when using a fixed-concentration feeding protocol and based upon age-related changes in body weight and feed intake, intakes of 3-FL and FOS declined gradually during the feeding period. The overall mean consumption of 3-FL at dietary concentrations of 5% and 10% was 3,038 and 5,975 mg/kg bw/day, respectively, for males, and 3,870 and 7,270 mg/kg bw/day, respectively, for females. In the 10% FOS control group, the overall mean consumption of FOS was 6,224 and 7,412 mg/kg bw/day for males and females, respectively (Table 6).

All animals survived to scheduled sacrifice. There were no ophthalmological findings, clinical or detailed physical observations, or effects on neurobehavioral parameters attributable to consumption of diets containing 3-FL or FOS (data not shown). No statistically significant or biologically-relevant differences in final body weight, or overall (test days 1–92) body weight gain, food consumption, or food efficiency were observed (Table 6).

No differences in hematology, coagulation, clinical chemistry, or urinalysis parameters were associated with consumption of diets containing 3-FL or FOS (Tables 7–9). A significantly higher mean cell volume and mean cell hemoglobin in the 5% 3-FL male group did not

Table 11
90-Day feeding study – selected microscopic observations[a].

Tissue	Observation	*MALES*			*FEMALES*		
		Basal Diet Control	10% 3-FL	FOS Control	Basal Diet Control	10% 3-FL	FOS Control
		N = 10	N = 10	N = 10	N = 10	N = 10	N = 10
Cecum	Inflammation, minimal	2	1	0	1	0	0
Epididymides	Inflammation, minimal	3	1	1	–	–	–
Eyes	Fold/rosette, minimal	0	0	2	0	0	0
Heart	Cardiomyopathy, minimal	1	2	1	0	0	0
	Cardiomyopathy, mononuclear, minimal	3	0	3	0	0	1
Kidneys	Chronic progressive nephropathy, minimal	5	4	3	0	0	0
	Dilatation, tubules, minimal	0	1	1	0	0	2
	Hydronephrosis, mild	0	0	2	0	0	0
	Infiltrate, lymphocyte, minimal	0	0	3	2	1	0
	Mineralization, minimal	1	0	0	0	0	2
Liver	Focal inflammation, minimal	3	3	0	0	1	0
	Vacuolation, median cleft, minimal	1	0	2	2	0	1
Lungs	Alveolar histiocytic infiltrate, minimal	3	4	1	1	2	0
	Inflammation, active, minimal	2	3	3	1	2	2
Nose	Septum degeneration, minimal	0	0	0	2	1	1
Pancreas	Fibrosis, islets, minimal	0	0	2	0	0	0
	Hyperplasia, acinar, minimal	0	2	0	1	0	0
	Inflammation, minimal	3	5	4	3	2	0
Prostate	Inflammation	4	3	4	–	–	–
	Minimal	[1]	[3]	[3]	[-]	[-]	[-]
	Mild	[2]	[0]	[1]	[-]	[-]	[-]
	Moderate	[1]	[0]	[0]	[-]	[-]	[-]
Salivary gland	Inflammation	0	0	2	1	2	2
	Minimal	[0]	[0]	[2]	[1]	[2]	[1]
	Mild	[0]	[0]	[0]	[0]	[0]	[1]
Spleen	Lymphoma, primary	0	0	1	0	0	0
Thyroid	Ultimobranchial cyst, minimal	0	1	0	2	1	1
Urinary bladder	Inflammation, minimal	2	0	0	0	0	0

[a] Except for spleen, table presents observations that occurred at an incidence of > 1 in any treatment group evaluated. A total of 40 tissues were examined (n = 10/sex/group).

demonstrate a dose-related response and was not associated with changes in any other hematology parameters. Similarly, a significantly lower red cell distribution width in females fed diets containing the FOS control substance was not associated with changes in any other hematology parameters. Cholesterol was significantly lower in females fed the FOS control diet. However, there were no correlative changes in triglycerides, nor were there changes in cholesterol or triglycerides in male rats, and individual cholesterol values for females were within the 95% historical control range (39–108 mg/dL). In addition, mild decreases in cholesterol have no known biologically significant effects. There were no statistically significant differences in coagulation or quantitative urinalysis parameters.

There were no statistically significant, biologically- or toxicologically-relevant differences in organ weights (Table 10), and no macroscopic or microscopic observations attributed to consumption of diets containing either 3-FL or FOS (Table 11). One male rat from the FOS control group exhibited an increased spleen weight, which correlated with multi-centric lymphoma observed microscopically. There were no correlating macroscopic observations or clinical pathology changes in this animal. Although uncommon, lymphoma has been described as a spontaneously-occurring lesion in rats (Son and Gopinath, 2004), and was therefore considered unlikely to be related to consumption of the FOS control diet. Other microscopic observations in this study were consistent with normal background lesions in rats of this age and strain.

Mean serum concentrations in male rats ranged from 984 (± 543) to 1520 (± 1200) ng/mL at 5% 3-FL and 2080 (± 592) to 2950 (± 669) ng/mL at 10% 3-FL dietary feeding concentrations, respectively. The serum concentrations in female rats were approximately 2-fold higher than male rats (Table 12). AUC_{24} estimates increased nearly 2-fold in both sexes and indicated dose proportional uptake with slight saturation of absorption based on minor decline in dose-normalized AUC_{24} at 10% 3-FL relative to 5% 3-FL feeding concentrations (Fig. 1).

Overnight urine collection for 16 h was selected as sufficient time to estimate 24-h excretion since rats are considered nocturnal feeders. Mean urine volumes (measured by weight, 1 g ≈ 1 mL) ranged from 10.3 to 13.6 mL across sex and dose level. The amounts recovered in urine (as the mol percentage of daily dietary intake) for male and female rats were 0.39 and 0.41% at 5% 3-FL and, 0.35 and 0.36% at 10% 3-FL, respectively. Overall, the percent of the daily intake dose recovered in urine was similar across dose and sex.

4. Discussion and conclusions

The present study examines the safety of 3-Fucosyllactose (3-FL) for use as nutritional ingredient in infant formula, dietary supplements and foods in general. HMOs promote health in nursing infants by reducing susceptibility to disease and supporting immune system development (Bode, 2012; Doherty et al., 2018) and acting as a principal growth factor for Bifidobacteria in the infant gut.

Preliminary studies suggest that 3-FL may also have important beneficial effects in breast milk (Bienenstock et al., 2013). Though HMOs are non-digestible to humans, intestinal bacteria ferment the oligosaccharides to generate organic acids (lactic acid) and short chain fatty acids, such as acetic, propionic and butyric acids which improve gut health (Smilowitz et al., 2014).

HMOs ingested during nursing are found in feces undigested with low systemic absorption into serum and excretion in urine (Goehring et al., 2014). Concentrations of 3-FL measured in urine and serum of breast-fed infants correlated to milk consumption and to its concentration in breast milk. Goehring et al. (2014) also reported the relative fractions of HMOs, including 3-FL, as 0.1% and 4% of breast milk

Table 12
90-Day feeding study – Pharmacokinetic parameters (mean ± SD).

Parameter	Units	5% 3-FL	10% 3-FL
MALES		N = 10[a]	N = 10[a]
Test Material Intake[b]	mg/kg bw/day	2340 ± 181	4650 ± 251
Test Material Intake[b]	mmol/rat/day	2.75 ± 0.192	5.28 ± 0.573
Serum 6 a.m. (N = 3), Day 80	ng/mL	1490 ± 177[c]	2540 ± 144[c]
10 a.m. (N = 3), Day 80	ng/mL	1520 ± 1200	2950 ± 669
2 p.m. (N = 4), Day 80	ng/mL	984 ± 543	2080 ± 592
6 a.m. (+24 h), Day 81		[c]	[c]
AUC_{24}[d]	h*ng/mL	30,800	58,000
Urine concentration	ng/mL	416,000 ± 177,000	931,000 ± 385,000
Urine mass (0–16 h)	g	13.6 ± 5.82	10.3 ± 2.90
Urine amount	mmol	0.0105 ± 0.00308	0.0187 ± 0.00724
Urine % of dose	mol %	0.385 ± 0.121	0.352 ± 0.123
FEMALES		N = 10[a]	N = 10[a]
Test Material Intake[b]	mg/kg bw/day	3230 ± 419	5880 ± 554
Test Material Intake[b]	mmol/rat/day	2.01 ± 0.245	3.70 ± 0.108
Serum 6 a.m. (N = 3), Day 81	ng/mL	2220 ± 660[c]	3190 ± 727[c]
10 a.m. (N = 3), Day 81	ng/mL	1700 ± 796	3690 ± 505
2 p.m. (N = 4), Day 81	ng/mL	4830 ± 6050	8450 ± 3980
6 a.m. (+24 h), Day 82		[c]	[c]
AUC_{24}[d]	h*ng/mL	77,300	131,000
Urine concentration	ng/mL	405,000 ± 152,000	633,000 ± 175,000
Urine mass (0–16 h)	g	10.8 ± 3.1	10.3 ± 3.8
Urine amount	mmol	0.00824 ± 0.00215	0.0132 ± 0.00564
Urine % of dose	mol %	0.414 ± 0.115	0.357 ± 0.156

Note: 3-FL concentrations in serum and urine from the Basal Diet Control samples were all < LOQ (200 ng/mL).
[a] Except for serum sample which used N = 3–4/time/sex/dose.
[b] Intake values for days 78–85 used for pharmacokinetic interpretation of serum and urine data.
[c] Test day 81 or 82 (6:00 a.m.) serum concentrations were set equal to the measured values on test day 80 or 81 (6:00 a.m.) as described in the methods.
[d] Composite estimate of systemic exposure.

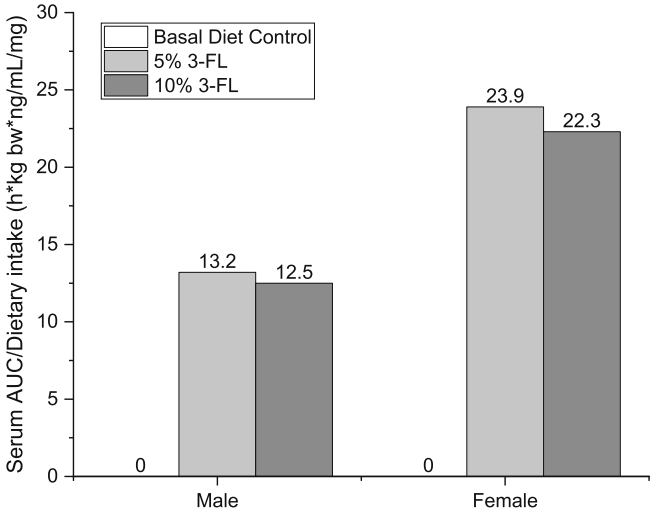

Fig. 1. Normalized daily systemic exposure (AUC_{24}/Dietary intake) of 3-FL.

concentrations present in plasma and urine, respectively. Although absorption from breast milk is low systemic exposure does occur.

The present studies were conducted to determine safety of 3-FL as a nutritional ingredient for general use. The battery of tests includes studies considered necessary to qualify a new material for safe use in food (EFSA Scientific Committee, 2017; US FDA, 2007). Future studies are planned to evaluate the safety of 3-FL for use in infant and follow-on formulas.

Like other naturally occurring HMOs, 3-FL was found to be non-toxic in an acute oral toxicity study in rats at a maximal dose level of 5000 mg/kg bw. There were no deaths or indications of toxicity at 5000 mg 3-FL/kg bw.

A battery of genotoxicity studies was conducted on 3-FL. All genotoxicity tests were conducted under GLPs in accordance with current OECD, FDA and EC guidelines and satisfied the criteria for adequate testing. 3-FL was not mutagenic in the bacterial reverse mutation (Ames) test with or without metabolic activation by a rat liver microsomal fraction (S9) (Tables 2a and 2b). 3-FL did not cause chromosome aberrations in human lymphocytes *in vitro* with or without metabolic activation (Table 4).

In an *in vitro* micronucleus test, 3-FL did not induce any statistically significant increases in micronuclei in CHO–K1 cells, compared to study controls, at any dose with or without metabolic activation by S9 (Table 3). A William's Trend Test found a statistically significant trend of higher incidences of micronuclei for the 4-h activated condition at doses of ≥2500 μg/mL in the first assay and at ≥1000 μg/mL in a second assay; however, the actual incidences of the micronuclei in all treated groups were within the 95% CI of the laboratory historical control database (HCD). Due to the statistically significant trend test, 3-FL is considered to be equivocal in the *in vitro* micronucleus test, based on the test guideline guidance (OECD, 2016c). While OECD classifies these result-types as equivocal, Fowler et al. (2012) has shown that the micronucleus assay with CHO cells can produce false positive rate up to 53%, most likely due as a result of p53 deficiency in these cell lines.

Therefore, an *in vivo* mouse micronucleus test was conducted to further determine if 3-FL has the potential to induce micronuclei in whole animals. 3-FL did not increase the frequency of micronuclei in reticulocytes in blood from mice exposed to an oral dose ≤2000 mg/kg bw (Table 5) and is considered non-genotoxic in this test. In summary, since there were no statistically significant elevated incidences of micronuclei in any treated group, compared to the study control *in vitro*; since the actual incidences of micronuclei at all 3-FL dose levels *in vitro* were within the HCD, and since the *in vivo* mouse micronucleus test was negative for all doses, it is concluded that 3-FL does not induce micronuclei.

Genotoxicity study results of 3-FL can be compared to other similar naturally occurring neutral fucosylated HMOs as these materials are oligosaccharides composed of D-galactose, D-glucose and L-fucose, with the main difference being the fucose linkage. For example, a mixture of naturally-occurring HMOs (2′-Fucosyllactose (2′-FL) and difucosyllactose (DiFL) with a ratio of 8:1 ratio 2′-FL:DiFL)) was negative in two *in vitro* tests: the Ames test and the mammalian cell micronucleus test using human peripheral blood lymphocytes (Phipps et al., 2018). In addition, 2′-FL alone was negative in the Ames test and the *in vitro* mammalian cell gene mutation assay in mouse lymphoma L5178Y cells (Coulet et al., 2014). 2′-FL was also tested more recently by Van Berlo et al. (2018) who confirmed the lack of genotoxicity in the Ames test and an *in vitro* micronucleus test in cultured human lymphocytes. Other non-fucose containing oligosaccharides (some also considered HMOs) were also negative in genotoxity studies. Lacto-N-neotetraose (LNnT), galactooligosaccharide and Neosugar, (a fructooligosaccharide mixture) were negative in Ames, mouse lymphoma, unscheduled DNA synthesis, *in vitro* chromosomal aberration and/or *in vitro* micronucleus studies. (Coulet et al., 2013; Clevenger et al., 1988; Kobayashi et al., 2009). Based on the structural similarities of 3-FL to these HMOs, the genotoxicity results on 3-FL are consistent with other HMOs in supporting a lack of genotoxicity, based on a weight of evidence approach using reliable GLP studies (EFSA Scientific Committee, 2017). In addition, 3-FL has a high purity, and lacks significant levels of impurities generated during the manufacture which could affect the genotoxicity results of the manufactured product (Table 1). The weight of evidence strongly indicates that 3-FL is not genotoxic.

The safety of 3-FL was further determined in a 90-day dietary study under OECD 408 test guideline in adult rats given diets containing 0, 5% or 10% 3-FL, or 10% fructooligosaccharide (FOS), a permitted food ingredient (EC, 2006, US FDA GRAS GRN Notice 000044, 2000a,b). In addition, blood samples were collected over an 8-h period and urine samples were collected overnight for 16 h during week 12 to determine concentrations of 3-FL. There were no treatment-related effects of 3-FL on survival, ophthalmology, clinical observations, neurobehavioral parameters, body weight, body weight gain, food consumption, food efficiency, hematology, coagulation, clinical chemistry or urinalysis parameters. There were no treatment-related differences in organ weights, macroscopic or microscopic histopathological findings in any treated groups. Determination of concentrations of 3-FL levels in serum and urine verified exposure.

Pharmacokinetic evaluation of 3-FL in serum indicated low absorption and systemic exposure from dietary intake that was proportional to dose with slight saturation between the 5% and 10% dose levels (Table 12, Fig. 1). Approximately 2-fold higher serum concentrations and thus dose-normalized AUC_{24} values were observed in female rats compared with male rats. This observation can be explained by the higher body weight (Table 6) and consequently higher volume of distribution of male rats. Despite this sex difference in serum concentrations, recovery of 3-FL in urine was ~0.4% (as mol %) of the administered dose in both dose groups regardless of sex (Table 12). Quantitation of 3-FL in serum and urine confirms negligible systemic exposure with absorption well below 1.0% of daily dietary intake.

The pharmacokinetics of 3-FL can be compared with data available from the analog 2′-FL tested in a 90-day dietary study at a dose level of 10%, equivalent to intake of 7.66 and 8.72 g/kg bw/day for male and female rats, respectively (LPT, 2014). Rats fed 10% 2′-FL for 90 days had C_{max} levels of 2963 (± 401) and 3618 (± 565) ng/mL for male and female rats, respectively (LPT, 2014). In the current study, rats fed 10% 3-FL for the same time period had C_{max} levels of 2950 (± 669) and 8450 (± 3980) ng/mL for males and females, respectively. In general, 2′-FL and 3-FL appear to have similar serum concentrations during dietary feeding. Comparison of 2′-FL and 3-FL urine concentrations was also possible. The mean (± SD, n = 3) urine concentrations for 2′-FL were 48,228 (± 53,258) and 94,511 (± 69,373) ng/mL for the male and female rats, respectively (LPT, 2014). 2′-FL urine samples were collected for 16 h from “fasted” rats. 3-FL urine samples were collected for 16 h from “non-fasted” rats. The 3-FL mean (± SD, n = 10) urine concentrations relative to 2′-FL were 19- and 7-fold higher for the male (931,000 ± 385,000 ng/mL) and female (633,000 ± 175,000 ng/mL) rats, respectively. For 3-FL, the 16-h mol percent of dose recovered in urine ranged from 0.352 to 0.357% of the daily dietary intake, as mentioned above (Table 12).

Vasquez et al. (2017) examined the time course of three HMOs in serum and urine after a single oral gavage dose to female rats to several equimolar dose equivalents of 2′-FL (0.2, 2, 5 g/kg bw), 6'-sialyllactose (0.2, 1, 3.75 g/kg bw) and LNnT (0.2, 1 g/kg bw). All three test materials were absorbed into serum in a dose-dependent manner with peak levels at 1–2 h post-dosing; maintained levels in serum until 5 h post-dosing, the end of the experiment. The peak serum levels of 2′-FL in female adult rats were about 40 μg/mL (Fig. 2 of Vasquez et al., 2017).

The results of the 90-day dietary study support the safety of 3-FL. The dietary concentrations of 5% and 10% were selected to meet and exceed the highest recommended dietary concentration for a food additive (US FDA, 2007), and for consistency with and comparability to similar studies conducted using other HMO test substances (LPT, 2014; Van Berlo et al., 2018), and did not compromise the nutritional value of the diets.

No toxicologically significant or treatment-related effects on body weight, body weight gain or food consumption were observed at either the 5% or 10% dietary concentrations. The mean consumption of 3-FL was 3038 mg/kg bw/day in males and 3840 mg/kg bw/day in females at the dietary concentration of 5%. At the 10% dietary concentration, the mean consumption of 3-FL was 5975 mg/kg bw/day in males and 7270 mg/kg bw/day in females. Consumption of diets containing 10% FOS resulted in mean consumption of 6224 and 7412 mg/kg bw/day FOS in males and females, respectively. There were no treatment-related effects following consumption of 3-FL or FOS. These data indicate that 3-FL has the same safety profile of FOS when tested under the same conditions in the same study. FOS is currently permitted as a food ingredient for use in various foods, including infant formula and follow-formula (EC, 2006; US FDA GRAS Notices GRN 44, 2000a,b, and GRN 797, 2018).

2′-FL, another naturally-occurring and commercially produced HMOs, has been shown to have a similar safety profile to 3-FL when tested in rodent subchronic dietary studies. Van Berlo et al. (2018) conducted a 90-day dietary study with 2′-FL at dietary concentrations of 0, 3%, 6% and 10% in young rats commencing from postnatal day (PND) 25 to PND 115 to coincide with the development of the immune system through to maturation. In this study, there were no adverse treatment-related findings at all doses. The No Observed Adverse Effect Level (NOAEL) was considered to be the high dose, 10% in the diet, in which 2′-FL exposures were ≥7.7 g/kg bw/day in males and ≥8.7 g/kg bw/day in females (Van Berlo et al., 2018). 2′-FL is permitted for use in various foods, including infant formula (US FDA GRAS Notices GRN 571, 2015, and GRN 650, 2016). A 90-day dietary study was also conducted in adult rats administered 2′-FL at a concentration of 10% in diet (LPT, 2014). No treatment-related effects were reported at 10% 2′-FL, which can be considered a NOAEL. The pharmacokinetic analyses of 2′-FL from this study are discussed above.

Another naturally-occurring, chemically synthesized HMO, LNnT, was evaluated in a 90-day oral gavage in juvenile rats (PND 7) given doses of 0, 1000, 2500 or 5000 mg/kg bw/day LNnT or 5000 mg/kg bw/day FOS in water once daily (Coulet et al., 2013). The NOAEL for LNnT in this study was the high dose, 5000 mg/kg BW/day. Two other similar HMOs tested in subchronic 90-day oral gavage studies in juvenile rats (PND 7) showed no adverse, treatment-related effects at doses up to 5000 mg/kg bw/day: 2′-FL/DFL mixture (8:1) (Phipps et al., 2018) and 2′-FL (Coulet et al., 2014). Although the subchronic dietary studies are more relevant for comparison to the present study on 3-FL, the additional gavage studies further support the safety of this class of naturally-occurring, synthetically manufactured HMOs. LNnT is

permitted for use in various feeds including foods for infants and young children (US FDA GRAS Notice GRN 659, 2016; EC, 2017C).

Based on these results, biotechnologically produced 3-FL is safe for use as a nutritional ingredient for food use. Comparison to several HMOs currently approved as ingredients and infant formula/follow-on formula uses indicates that 3-FL has the same safety profile, based on the current acute toxicity, genotoxicity and subchronic dietary studies presented here. Further studies with neonatal piglets will be supportive in the safety evaluation of 3-FL for use in infant formula and follow-on formula.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2019.110818.

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