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Pre-clinical safety assessment of biotechnologically produced lacto-*N*-tetraose (LNT)

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ABSTRACT

Lacto-*N*-tetraose (LNT) is a human milk oligosaccharide with average concentrations ranging from 0.74 to 1.07 g/L in breastmilk, depending on the lactation stage. In this study, the preclinical safety of LNT produced by the *Escherichia coli* K-12 E2083 production strain was assessed. LNT was negative in both the bacterial reverse mutation assay and the *in vitro* micronucleus assay, demonstrating the absence of genotoxic potential for this substance. In the OECD 408 guideline compliant 90-day oral toxicity study rat, LNT did not induce any adverse effects in any treatment group up to and including the highest dose tested, and no LOAEL could be determined. Therefore, the no-observed-adverse effect level (NOAEL) is set at the highest dose level tested, i.e. a dietary level of 5 % (w/w), corresponding to ≥2856 mg/kg bw/day and ≥3253 mg/kg bw/day for males and females, respectively. This might be an underestimation of the NOAEL, caused by the range of dose levels tested. The results obtained in the current study are in good agreement with available data generated using other biotechnologically produced LNT batches and therefore support its safe use as a food ingredient.

1. Introduction

Human milk oligosaccharides (HMOs) are the third largest solid component in breast milk, after lactose and lipids. They are complex, non-digestible sugars and appear in breast milk at a concentration range of 10–15 g/L. The concentration of HMOs in breastmilk varies between mothers and depends on genetics of the mother, the stage of lactation and environmental factors (Bode, 2015). To date, more than 200 different HMOs have been found in breast milk (German et al., 2008). HMOs are composed of five mono-sugar building blocks, including glucose, galactose, fucose, N-acetyl-glucosamine and the sialic acid derivative N-acetyl-neuraminic acid (Bode, 2015). Based on their composition, HMOs can be divided into three categories: fucosylated neutral oligosaccharides, non-fucosylated neutral oligosaccharides, and acidic oligosaccharides (containing at least one sialic acid moiety). In term breastmilk, ~35–50% of HMO are fucosylated, 42–55% are non-fucosylated neutral HMO, and 12–14% are sialylated (Donovan and Comstock, 2017).

HMOs are non-digestible to humans: they resist the low pH in the stomach and degradation by gastro-intestinal digestive enzymes, and reach the colon relatively intact (Engfer et al., 2000). There, they serve as metabolic substrate for specific bacteria in the gut microbiota, like species of the genus Bifidobacterium (Asakuma et al., 2011). HMOs have been associated with a number of health-promoting effects. HMO content in human milk is considered a very important factor for the development of intestinal microbiota of infants (Newburg and Ruiz-Palacios, 2005; Kunz and Rudloff, 2006). Moreover, preclinical research has shown that HMOs can reduce pathogen adhesion to- and infectivity of the intestinal- or airway epithelium (Bode et al., 2004; Weichert et al., 2013, 2016; Laucirica et al., 2017). Furthermore, HMOs have been shown to modulate both innate and adaptive immunity, by affecting immune cell populations and cytokine secretion (Bode et al., 2004). Some HMOs are even absorbed into the blood stream at low levels (Goehring et al., 2014), where they may exert systemic effects on e.g. the immune system (Donovan and Comstock, 2017). In addition, HMOs have been suggested to support brain development (Bode, 2012). Overall, these functionalities of HMOs may be beneficial for the growth and development of infants.

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Table 1
Composition of lacto-N-tetraose (batch C256001B–W01).

Component	Content (%)
Lacto-*N*-tetraose (LNT)	83
Lacto-*N*-triose (LNT2)	1.0
D-Lactose	0.3
Isomaltose	1.5
Ribose	0.1
Glucose	<0.1
Galactose	0.1
Sum of other carbohydrates	9.7
Protein	<0.01
Moisture	4
Sulphated ash	0.2

Based on their potential beneficial health effects, the interest in the application of HMOs as ingredients of infant formula has developed over the years. Although breast milk is the best nutrition for every newborn, infant formula is the only safe alternative in cases when breast milk is not - or insufficiently available. Most infant formula on the market is based on cow's milk. However, because oligosaccharide levels in cow's milk are 10–100 times lower as compared to human milk (Robinson, 2019), infant formula traditionally lacks the oligosaccharides which are similar to those found in breast milk. Recent advances in chemical synthesis and biotechnology have enabled the commercial production of several HMOs, structurally identical to those found in breast milk, for application in infant nutrition and other foods (Zhu et al., 2022). The production of HMOs currently focuses on the ones with the highest reported concentrations in breast milk, and representing the most abundant candidates from the three HMO categories (fucosylated neutral oligosaccharides, non-fucosylated neutral oligosaccharides, and acidic oligosaccharides). The first HMO to go to market was 2′-fucosyllactose (2′-FL), which is the most abundant HMO in breast milk (20–30% of total HMO in most lactating women) and a representative of the neutral fucosylated oligosaccharide category (Soyyilmaz et al., 2021). Upon extensive ingredient characterization and (pre-)clinical safety testing, 2′-FL from different manufacturers received regulatory approval to enter several markets globally, including Europe and the USA (European Commission, 2017; GRAS Associates, 2017; Jennewein, 2017).

Lacto-*N*-tetraose (LNT) is a tetra-saccharide HMO (Gal-(β1-3)-GlcNAc-(β1-3)-Gal-(β1-4)-Glc), and the most abundant representative of the non-fucosylated neutral HMO category in breast milk. Its average concentration in breast milk ranges from 1.07 g/L in early lactation to 0.74 g/L in mature milk (Soyyilmaz et al., 2021), and has been reported to gradually decline to levels of around 0.35 g/L late in lactation, around 12 months (Lefebvre et al., 2020; Siziba et al., 2021). Preclinical research showed that LNT could be utilized as substrate by various *Bifidobacterium* strains, including *B. longum* subsp. *infantis, B. longum* subsp. *longum, B. bifidum* and *B. breve* (Asakuma et al., 2011; Ojima et al., 2022), all of which are bacterial strains typically present in the gut microbiota of infants. Furthermore, LNT was reported to have very specific antipathogenic potential. It can inhibit the growth of group B *Streptococcus* (Lin et al., 2017), which is a common cause of serious infections in newborns and can lead to meningitis, pneumonia, or sepsis. In addition, LNT has been shown to reduce both the attachment of the parasite *Entamoeba histolytica* to intestinal epithelial cells, as well as its cytotoxicity (Jantscher-Krenn et al., 2012). Finally, LNT has recently been found to enhance intestinal barrier function by reducing intestinal permeability induced by inflammatory cytokines (Natividad et al., 2022). Altogether, these preclinical findings suggest that the addition of LNT to infant formula may impact the health of infants by enhancing a healthy gut microbiota composition, by reducing risk of infection and by supporting intestinal development.

In the current study, the preclinical safety of LNT produced by the *Escherichia coli* K-12 E2083 production strain was assessed. A bacterial reverse mutation assay and an *in vitro* micronucleus assay were performed to evaluate its genotoxic potential, and its possible subchronic toxicity was investigated in a 90-day dietary toxicity study in rats. These studies are generally required by regulatory authorities (FDA, 2006; EFSA Panel on Dietetic Products Nutrition and Allergies, 2021; Government of Canada, 2022) for assessing the safety of novel ingredients such as HMOs for infant formula and other food applications.

2. Material and methods

The experiments described in this study were conducted in accordance with the OECD Principles of Good Laboratory Practice (OECD, 1998).

2.1. Test material

Lacto-N-tetraose (LNT; chemical formula: $C_{26}H_{45}NO_{21}$; molecular weight: 707.632 g/mol; CAS No: 14116-68-8) was provided by FrieslandCampina (Amersfoort, the Netherlands). The test material was produced through fermentation by the genetically modified *Escherichia coli* K-12 E2083 production strain, followed by purification. The production of LNT was performed according to the EFSA Guidance on the characterization of microorganisms used as feed additives or as production organisms (EFSA Panel on Additives and Products or Substances used in Animal Feed FEEDAP, 2018). During the purification step, proteins, DNA, and other large molecules that can originate from the production organism are removed. Analysis by a quantitative polymerase chain reaction (qPCR) method demonstrated that there was no detectable residual DNA (limit of detection of 1 ng/g) in the LNT material (*data not shown*). The purity of the test material was 83%, as determined by HPAEC-PAD (high performance anion exchange chromatography/pulsed amperometric detection). The specification is provided in Table 1. Of note: at the start of the genotoxicity studies, the batch of LNT used was determined to have a purity of 95%. However, upon validation of the analytical method, the purity of the LNT batch used was verified to be 83% LNT on dry matter basis. Retrospectively, the exposure levels in the genotoxicity assays were therefore 13% lower than intended,[1] and did not reach the limits prescribed by the OECD test guidelines. In the 90-day rat study, the diets were prepared taking into consideration the correct purity of 83%, in full compliance with the OECD test guideline (OECD, 2018).

2.2. Genotoxicity studies

The potential genotoxicity of LNT was assessed by two *in vitro* tests, including the bacterial reverse mutation (Ames) assay and the *in vitro* mammalian micronucleus test in cultured peripheral human lymphocytes. Both studies were conducted at Charles River Laboratories, 's-Hertogenbosch, The Netherlands, and according to the respective OECD guidelines for these assays (OECD, 2016, 2020).

2.2.1. Bacterial reverse mutation (Ames) assay

The bacterial reverse mutation assay was performed in accordance with OECD test guideline No. 471 (OECD, 2020). The standard plate incorporation method with the histidine-requiring *S. typhimurium* strains TA 1535, TA 1537, TA 98, TA 100 (master culture from Dr. Bruce Ames) and the tryptophan-requiring *E. coli* strain WP2 uvrA (master culture from The National Collections of Industrial and Marine Bacteria, Aberdeen, UK) was used. All bacterial strains and rat liver microsomal enzymes (S9 homogenate) were purchased from Trinova Biochem GmbH

[1] For the initially stated purity of 95%, no correction factor was applied, in line with the guidelines (OECD, 2016, 2020). A purity of 100% was assumed. The newly determined purity of 83% therefore led to 13% lower exposure levels.

Table 2
Reference mutagens serving as positive control in the bacterial reverse mutation (Ames) assay.

Strain	- S9 mix		+ S9 mix	5%	10%
	Reference	μg/plate	Reference	μg/plate	
TA 1535	SA	5	2AA	2.5	2.5
TA 1537	ICR-191	2.5	2AA	2.5	5
TA 98	NF	10	2AA	1	1
TA 100	MMS	650	2AA	1	2
WP2*uvrA*	4-NQO	10	2AA	15	15

SA: sodium azide; ICR-191: Acridine mutagen ICR-191; NF: 2-nitrofluorene; MMS: methylmethanesulfonate; 4-NQO: 4-nitroquinoline-N-oxide; 2AA: 2-aminoanthracene.
The vehicle for all reference mutagens was dimethyl sulfoxide, except for sodium azide, which was dissolved in physiological saline.

(Giessen, Germany). S9 homogenate was prepared from Sprague Dawley rats that had been induced with an intraperitoneal injection with 500 mg/kg bw Aroclor-1254. A detailed description of the experimental procedure is available in the Supplementary Material section.

Selection of an adequate dose range of the test substance was based on a dose-range finding test with the strains TA100 and WP$_2$*uvrA* in the presence and in the absence of 5% (v/v) S9-mix. Eight concentrations ranging from 1.4 to 4150 μg/plate were tested in triplicate. No cytotoxicity nor precipitation of test substance was observed, when assessed by visual inspection of the bacterial background lawn; therefore, the highest concentration of the test item used in the subsequent mutation assays was 4150 μg/plate. The test substance was subsequently tested at 5 concentrations (in triplicate) ranging from 43 to 4150 μg/plate, both in the absence and presence of 5% (v/v) S9-mix in the tester strains TA1535, TA1537 and TA98, to complete the panel of 5 bacterial strains under these standard conditions. In a confirmatory experiment, the test substance was tested at 5 concentrations (in triplicate) ranging from 408 to 4150 μg/plate both in the absence and presence of 10% (v/v) S9-mix (instead of 5% (v/v)) in all tester strains. In all experiments, the negative control (vehicle) and relevant positive controls (specified in Table 2) were concurrently tested with each strain in the presence and absence of S9-mix.

2.2.2. Micronucleus assay in cultured human lymphocytes

The ability of LNT to induce micronuclei *in vitro* was investigated in cultured human lymphocytes in accordance with OECD test guideline No. 487 (OECD, 2016). The metabolic activation system consisted of rat S9 homogenate (Trinova Biochem GmbH, Giessen, Germany) and was prepared from male Sprague-Dawley rats orally dosed with a suspension of 80 mg/kg bw phenobarbital and 100 mg/kg bw β-naphtoflavone. A detailed description of the experimental procedure is available in the Supplementary Material section.

The top concentration proved to give adequate viability in a dose-range finding test. This was based on the requirement that the maximum test should aim to achieve 55 ± 5% cytotoxicity (OECD, 2023), determined based on the Cytokinesis-Block Proliferation Index (CBPI). The level of cytotoxicity was calculated as:

$$\% \text{ cytostasis} = 100\text{-}100*\{(CBPI_{test} - 1)/(CBPI_{control} - 1)\}, \text{ with}$$

CBPI = {(No. mononucleate cells) + (2* No. binucleate cells) + (3* No. multinucleate cells)}/(Total No. of cells)

And: t = test culture, and c = vehicle control culture.

A vehicle control was included at each exposure time. Depending on the test conditions, mytomicin-C, colchicine or cyclophosphamide were used as positive controls. Cytochalasin B was used as actin polymerization inhibitor.

2.3. Ninety-day oral toxicity study in the rat

The general toxicity of LNT was investigated in a ninety-day oral toxicity study in the rat performed according to OECD test guideline 408 (OECD, 2018). A complete description of all experimental procedures is available in the Supplementary Material section.

2.3.1. Animals and maintenance

Six-week old male and female Wistar rats (Crl:WI) were obtained from a colony maintained under SPF conditions at Charles River Laboratories, Research Models and Services (Germany GmbH, Sulzfeld, Germany) Animals were group-housed, up to 2 animals of the same group and sex/cage in a controlled environment (temperature of 20–25 °C, relative humidity between 27 and 56%, 12-h light/dark cycle, 15–20 air changes/h). The welfare of the animals was maintained in accordance with the general principles governing the use of animals in experiments. The principles and criteria summarized in the OECD Humane Endpoints Guidance Document No. 19 were taken into consideration (OECD, 2000). The test facility (Charles River Laboratories Hungary Kft, Veszprém, Hungary) is AAALAC accredited.

2.3.2. Experimental design

The study included four experimental groups of 10 rats/sex. The experimental diets were prepared by adding LNT to the animals' feed (ssniff® S5677-E052 EF AIN93G purified diet (Ssniff Spezialdiäten GmbH, Soest, Germany)) at levels of 0% (control diet), 1.25%, 2.5% and 5% (w/w), and were administered *ad libitum* for 90 consecutive days. The prepared diets were stored refrigerated (2–8 °C) under dry conditions in sewed bags until use, and confirmed to be stable for 6 months at room temperature. The high dose of this study was selected based on the absence of test substance-related adverse findings in a 14-day pilot study in which daily dietary concentrations of up to 5% were administered to Wistar rats and 5% is the maximum concentration for dietary studies according to OECD guideline 408 (OECD, 2018). Evaluated parameters in this pilot study were body weight and body weight gain, food consumption, organ weight and necropsy observations (*data not shown*).

2.3.3. Diet analysis

Representative duplicate samples were collected from the diets of each dose group (before the start of treatment with the batch, and additionally near the end of the use of each batch of diets, to determine concentration and homogeneity, and to prove stability under use conditions. Samples were taken from five different places of the diet container from each dose group, whereas one sample was taken from the middle of the diet container of the control diet. Diet samples were kept at approximately 2–8 °C, under dry conditions, under which LNT in feed was demonstrated to be stable for up to 56 days (acceptance criteria: 100 ± 10%). The stability was demonstrated in a separate method validation study. The stability data are available in Table 10 in the Supplementary Material section. Analysis was performed using a validated High-Performance Liquid Chromatography with UV detection (HPLC-UV) method (detection at 245 nm), comprising a Zorbax Eclipse XDB C18 150 × 4.6 mm x 5 μm RP-HPLC column kept at a temperature of 35 °C, and a mobile phase system based on 0.1 M ammonium acetate in water (eluent A) and acetonitrile (eluent B). The Limit of Quantification (LOQ) was 0.2 mg/mL for the analytical samples, which equals to 0.2 (m/m%) diet concentration (counting with the sample preparation).

2.3.4. Observations

Observations and investigations of the animals during the in-life phase of the study are according to OECD test guideline 408 (OECD, 2018) and are described in detail in the Supplementary Material.

Necropsy and histopathology: On Day 91, euthanasia was performed under pentobarbital anesthesia by exsanguination. After sample collection for clinical pathology evaluation, necropsy and macroscopic examination were performed on all animals. After exsanguination, the

Table 3
Number of revertant colonies counted in the bacterial reverse mutation test (mean number of revertant colonies per 3 replicate plates (±S.D).

	WP_2uvrA						TA 100						TA 98						TA 1535						TA 1537					
	-S9			+S9			-S9			+S9			-S9			+S9			-S9			+S9			-S9			+S9		
	Dose-range finding experiment *Plate incorporation assay with 5% (v/v) S9*												*First experiment* *Plate incorporation assay with 5% (v/v) S9*																	
Dose[a] (µg/plate)																														
PC	1631	±	103	316	±	146	1011	±	17	1396	±	131	1480	±	44	1419	±	303	832	±	51	260	±	36	1018	±	52	214	±	24
0	20	±	3	22	±	8	104	±	10	88	±	13	14	±	4	14	±	4	11	±	7	7	±	2	3	±	2	3	±	3
1.4	24	±	9	18	±	5	104	±	10	87	±	17																		
4.5	20	±	9	22	±	6	94	±	8	84	±	12																		
14	19	±	5	22	±	3	98	±	7	78	±	12																		
43	23	±	7	18	±	4	116	±	14	102	±	10	14	±	3	19	±	5	7	±	3	13	±	12	4	±	4	2	±	1
136	18	±	5	21	±	4	107	±	6	99	±	13	16	±	2	10	±	5	8	±	4	9	±	2	2	±	3	2	±	1
425	19	±	10	21	±	2	115	±	16	89	±	6	10	±	6	13	±	2	6	±	2	7	±	4	1	±	2	2	±	1
1328	15	±	4	24	±	1	100	±	6	96	±	9	14	±	1	13	±	4	13	±	5	10	±	2	3	±	1	1	±	2
4150[b]	21	±	9	25	±	2	96	±	14	88	±	3	11	±	2	14	±	4	10	±	5	10	±	2	2	±	2	3	±	2
	Second experiment *Plate incorporation assay with 10% (v/v) S9*																													
PC	1417	±	84	359	±	18	922	±	29	424	±	52	1539	±	126	437	±	21	858	±	134	155	±	4	1037	±	201	84	±	8
0	16	±	4	17	±	5	88	±	10	33	±	6	12	±	3	17	±	2	9	±	3	11	±	1	3	±	2	1	±	0
408	22	±	4	20	±	2	112	±	7	39	±	15	13	±	9	20	±	1	7	±	4	8	±	4	4	±	3	5	±	2
729	17	±	6	26	±	8	93	±	5	44	±	2	11	±	2	17	±	2	9	±	2	10	±	2	4	±	1	4	±	1
1301	23	±	10	20	±	6	103	±	9	35	±	8	11	±	6	19	±	2	8	±	2	10	±	2	2	±	1	4	±	1
2324	17	±	3	17	±	4	106	±	4	40	±	12	15	±	3	19	±	4	8	±	6	7	±	4	1	±	2	2	±	2
4150[b]	18	±	3	21	±	8	93	±	15	50	±	13	11	±	4	17	±	3	9	±	4	10	±	0	3	±	2	4	±	3

[a] Actual dose levels, corrected for newly determined purity of 83% of the test substance.
[b] This concentration showed no precipitation and normal bacterial background lawn in any of the incubation types/strains.

Table 4
In vitro micronucleus test in cultured human lymphocytes.

Concentration (μg/mL)[c,d]	Without metabolic activation			With metabolic activation		
	Cytokinesis Block Proliferation Index (CPBI)		No. binucleated cells with micronuclei[a,b]	Cytokinesis Block Proliferation Index (CPBI)		No. binucleated cells with micronuclei[a,b]
	Mean CPBI	Cytostasis (%)		Mean CPBI	Cytostasis (%)	
Pulse treatment (3-h exposure – 27-h harvest time)						
0	2.00	0	4	2.02	0	4
415	1.99	2	2	2.02	−1	1
830	1.98	2	4	1.91	10	3
1660	2.05	−5	4	1.96	5	3
MMC-C 0.2	1.73	28	5	*n.d.*	*n.d.*	*n.d.*
MMC-C 0.25	1.68	33	*n.d.*	*n.d.*	*n.d.*	*n.d.*
Colch 0.05	1.58	42	30****	*n.d.*	*n.d.*	*n.d.*
Colch 0.1	1.09	91	*n.d.*	*n.d.*	*n.d.*	*n.d.*
CP 7.5	*n.d.*	*n.d.*	*n.d.*	1.64	37	41****
CP 10	*n.d.*	*n.d.*	*n.d.*	1.50	51	*n.d.*
Continuous treatment (24-h exposure – 24- hour harvest time)						
0	2.02	0	2			
415	2.00	3	1			
830	1.96	7	2			
1660	1.97	5	2			
MMC-C 0.125	1.63	38	55****			
MMC-C 0.15	1.50	51	*n.d.*			
Colch 0.01	1.37	64	16***			
Colch 0.05	1.02	98	*n.d.*			

n.d. no data.
[b] Significantly different from control group (Fisher's exact test), *P < 0.05, **P < 0.01, ***P < 0.001 or ****P < 0.0001.
[a] Sum of duplicate cultures (1000 binucleated cells/culture scored for the presence of micronuclei).
[c] Concentration of LNT or positive control substances MMC-C: mitomycin-C; Colch: colchicine or CP: cyclophosphamide.
[d] Actual concentrations, corrected for newly determined purity of 83% of the test substance.

Table 5
Food consumption, LNT intake and body weight; mean values calculated over the 90-day exposure period.

Parameter	LNT concentration in diet (%)											
	0			1.25			2.5			5.0		
	Males											
Food consumption[a] (g/rat/day)	25.26	±	0.78	24.44	±	0.81	24.28	±	1.25	26.55	±	1.71
Mean LNT intake[b] (mg/kg bw/day)	0.0			687			1385			2856		
Body weight gain[c] (g/rat)	353.6	±	51.7	338.7	±	30.6	329.2	±	37.7	379.3	±	75.3
	Females											
Food consumption[a] (g/rat/day)	17.61	±	1.10	16.30	±	0.75	17.44	±	1.73	18.00	±	1.46
Mean LNT intake[b] (mg/kg bw/day)	0.0			768			1623			3253		
Body weight gain[c] (g/rat)	151.2	±	31.3	151.4	±	19.0	158.8	±	39.5	169.7	±	32.9

[a] Values are means ± SD for groups of 5 rats.
[b] Calculated mean values based on individual animal daily food intake, the mean body weight from the start of the day to 24 h later, and the dietary concentration of test item. The mean of all animals/day/sex/group are shown.
[c] Values are means ± SD for groups of 10 rats.

external appearance was examined, all orifices, and the cranial, thoracic and abdominal cavities were opened, and the appearance of the tissues and organs were observed macroscopically. Any abnormality was recorded with details of the location, color, shape and size, as appropriate.

2.3.5. Statistical analysis

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

2.3.5.2. Micronucleus assay in cultured human lymphocytes. The frequencies of micronuclei in LNT-treated cultures and positive control cultures were compared with those of the concurrent solvent control cultures using Fischer's exact test (one-sided). The results were considered statistically significant when the *p*-value of Fischer's exact test was less than 0.05. A statistically significant increase was considered dose-related in at least one experimental condition when evaluated with a Cochran Armitage trend test.

2.3.5.3. Ninety-day oral toxicity study in the rat. The normality and heterogeneity of variance between groups was checked by Shapiro-Wilk and Levene tests, using the most appropriate data format (log-transformed when justified). In case both tests showed no significant heterogeneity, an ANOVA/ANCOVA (one-way analysis of variance) test was carried out. If the obtained result was positive, Dunnett's (Multiple Range) test was used to assess the significance of inter-group differences; identifying differences of <0.05 or <0.01, as appropriate. This parametric analysis was the better option when normality and heterogeneity assumptions were adequate. If either of the Shapiro-Wilk or Levene tests showed significance of data, then the ANOVA-type approach was not valid, and a non-parametric analysis was required. A Kruskal-Wallis analysis of variance was used after the Rank Transformation. If there

Table 6a
Hematology analysis in rats exposed for 90 days to LNT; red blood cell parameters.

% LNT in diet	RBC (10E12/L)			Hb (g/dL)			HCT (%)			MCV (fL)			MCH (pg)			MCHC (g/dL)			RCDW (%)			Reticulocytes (%)		
Males[a]																								
0.0	7.970	±	0.778	14.91	±	1.04	42.12	±	2.38	53.09	±	3.23	18.78	±	0.86	35.42	±	0.90	14.33	±	3.10	3.42	±	3.86
1.25	7.964	±	0.575	14.94	±	0.84	41.67	±	2.09	52.43	±	2.00	18.82	±	0.57	35.86	±	0.67	13.49	±	0.64	2.12	±	0.39
2.5	8.336	±	0.392	15.09	±	0.66	42.78	±	1.83	51.38	±	1.66	18.12	±	0.54	35.28	±	0.77	13.23	±	0.36	1.96	±	0.30
5.0	8.278	±	0.277	15.10	±	0.51	42.54	±	1.48	51.39	±	1.54	18.24	±	0.74	35.51	±	0.85	13.53	±	0.63	2.27	±	0.45
Females[b]																								
0.0	7.643	±	0.441	14.23	±	0.88	40.34	±	2.48	52.77	±	1.12	18.62	±	0.39	35.29	±	0.31	12.19	±	0.60	1.93	±	0.37
1.25	7.598	±	0.522	13.91	±	0.84	39.53	±	2.73	52.06	±	1.55	18.35	±	0.55	35.25	±	0.63	12.31	±	0.54	1.98	±	0.39
2.5	7.238	±	0.909	13.55	±	1.28	38.57	±	3.27	53.65	±	2.93	18.82	±	0.87	35.10	±	0.87	12.86	±	1.97	3.12	±	3.14
5.0	7.468	±	0.311	13.86	±	0.58	39.31	±	1.83	52.63	±	1.03	18.56	±	0.32	35.26	±	0.44	11.98	±	0.37	1.91	±	0.30

RBC, red blood cell count; Hb, hemoglobin concentration; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RCDW, red cell distribution width.
Values are means ± SD for groups of 10 rats. Statistical analysis showed no significant differences between the LNT exposed groups and controls.
[a] Calculated actual LNT intakes: 0.0, 687, 1385 and 2856 mg/kg bw/day for dietary levels of 0.0, 1.25, 2.5 and 5.0%, respectively.
[b] Calculated actual LNT intakes: 0.0, 768, 1623 and 3253 mg/kg bw/day for dietary levels of 0.0, 1.25, 2.5 and 5.0%, respectively.

was a positive result, the inter-group comparisons were performed using the Dunn test, identifying differences of <0.05 or <0.01, as appropriate.

For non-continuous data, the Cochran-Armitage test for trend was applied and the Chi-square test was used for statistical differences relative to control.

For pathology data (macroscopic and microscopic data), the Cochran-Armitage test for trend was applied and if appropriate, the Chi-squared homogeneity test. If significance was plausible based on a user-defined value (0.05), a pairwise test of each treatment group versus the control group was made. If the groups size was <5, the Fisher's Exact Test was used; if the group sizes were larger, the Chi-squared test was used, identifying differences of <0.05, <0.01, or <0.001, as appropriate.

Any statistically significant effect was evaluated for toxicological relevance based on expert review, where appropriate.

3. Results

3.1. Genotoxicity studies

3.1.1. Bacterial reverse mutation (Ames) assay

Table 3 shows the numbers of reverted colonies counted in the bacterial reverse mutation assay for each of the strains tested. LNT was not toxic to any of the bacterial strains up to and including the highest tested concentration of 4150 μg/plate, as demonstrated by the absence of a decrease in mean number of revertants and/or a clearing of the background lawn of bacterial growth. The number of revertants counted in the negative (vehicle) control and the positive control incubations were in line with historical control data for the respective bacterial strains in the Test Facility (historical data not shown), therefore, the test was considered valid. Both in the absence and in the presence of S9-mix, the test substance did not induce a more than 2-fold increase in revertant colonies in strains WP_2uvrA and TA 100, or a more than 3-fold increase in the other strains, nor was there a dose-related increase in the mean number of revertant colonies compared to the background spontaneous reversion rate observed in the negative control incubations for any of the strains tested. It can therefore be concluded that LNT did not cause any mutations in the bacterial reverse mutation assay under the conditions tested.

3.1.2. Micronucleus test in cultured human lymphocytes

The potential clastogenic and/or aneugenic effects of LNT were investigated in an *in vitro* micronucleus test in which human lymphocytes were treated with cytochalasin B prior to mitosis. As shown in Table 4, the maximum percentage cytostasis did not exceed 10% after pulse- and continuous treatment with LNT, respectively, providing evidence for the absence of cytotoxicity up to the highest tested concentration of 1660 μg/mL In addition, no statistically significant, dose-dependent increase in the number of binucleated cells containing micronuclei was observed, when compared with the concurrent vehicle control cultures, under both the pulse treatment (with and without S9) and the continuous treatment (without S9) conditions with LNT concentrations up to 1660 μg/mL. Moreover, the number of binucleated cells containing micronuclei were within the test facility's historical data range of all positive and respective negative control groups. Thus, all three criteria for a clearly negative outcome of the study were met. It can therefore be concluded that LNT is not aneugenic or clastogenic under the conditions tested.

3.2. Ninety-day oral toxicity study in the rat

Diet analysis: The measured LNT concentrations in the diet samples were 83.7–96.3% of the nominal LNT concentrations, and thus within the acceptance criteria of 100 ± 20% of the nominal concentrations. The Relative Standard Deviation (RSD) of replicates was $<20\%$ in all cases. Therefore all samples were considered to homogenous. A summary of the data is available in Table 11 of the Supplementary Material Section.

Table 6b
Hematology analysis in rats exposed for 90 days to LNT; coagulation.

% LNT in diet	PlC (10E9/L)			MPV (fL)			PT (s)			APTT (s)		
Males[a]												
0.0	937.6	±	130.6	6.99	±	0.42	10.08	±	0.18	11.87	±	0.71
1.25	905.5	±	61.2	6.83	±	0.33	10.11	±	0.20	11.72	±	0.51
2.5	850.8	±	78.2	7.65	±	1.15	10.00	±	0.22	12.01	±	0.92
5.0	861.1	±	108.5	7.55	±	0.96	9.96	±	0.16	11.95	±	0.54
Females[b]												
0.0	862.0	±	79.7	7.70	±	0.79	9.73	±	0.29	12.62	±	0.85
1.25	**754.2***	±	90.9	7.37	±	0.58	9.67	±	0.27	11.79	±	1.04
2.5	758.8	±	118.3[c]	7.54	±	0.59	9.72	±	0.36	12.12	±	0.73
5.0	**754.3***	±	98.2	7.35	±	0.45	9.44	±	0.37	11.66	±	1.14

PlC, platelet count; MPV. Mean platelet volume; PT, prothrombin time; APTT.
Values are means ± SD for groups of 10 rats.
*Dunnett 2-sided $p < 0.05$.
[a] Calculated actual LNT intakes: 0.0, 687, 1385 and 2856 mg/kg bw/day for dietary levels of 0.0, 1.25, 2.5 and 5.0%, respectively.
[b] Calculated actual LNT intakes: 0.0, 768, 1623 and 3253 mg/kg bw/day for dietary levels of 0.0, 1.25, 2.5 and 5.0%, respectively.
[c] Relatively high standard deviation was due to high PlC in 2 individuals.

Food consumption and intake of the test substance: Analysis of the prepared diets confirmed the stability, homogeneity and dose levels of the test substance in the diet throughout the study (data not shown). Table 5 shows the mean food consumption and the calculated daily intake of the test substance. There were no statistically significant differences in food consumption between treatment groups during the study. Calculated average daily LNT intake based on the food consumption data and the analyzed LNT levels were 687, 1385 and 2856 mg/kg bw/day in males and 768, 1623 and 3253 mg/kg bw/day in females of respectively the 1.25, 2.5 and 5% dietary exposure groups.

Body weight development: There was no test substance related effect on absolute body weights (*data not shown*) or body weight gains, as shown in Table 5. Throughout the duration of the study, body weights of the groups exposed to LNT remained comparable with the body weights recorded in the control group.

Clinical observations: There were no test substance-related mortality or clinical signs during the study. Pale feces was recorded in all animals, including from the control group from approximately Day 3–10 until the end of the study. This was not considered related to the test substance.

Ophthalmic evaluation: No treatment related changes as compared to pre-treatment were noted at ophthalmoscopy examination.

Neurological assessment: No treatment related effect was observed in the Irwin test (Irwin, 1968), the assessment of grip strength, foot splay and locomotor activity (LMA) (*data not shown*). LMA was considered to have shown a normal response in all dose groups: it was initially higher in the beginning and then reduced to a plateau at approximately 25–30 min in both sexes. All LMA data were considered as normal. Altogether, the neurological assessment did not provide any indications for a neurotoxic effect by the test substance under the test conditions.

Vaginal smears evaluation: There were no test substance-related observations in the animal estrus cycles evaluation prior to necropsy, and the animals showed a normal distribution of estrus phases.

Hematology and clinical chemistry: There were no test substance related changes in any of the measured hematology parameters, including red blood cell parameters (Table 6a), coagulation parameters (Table 6b) and white blood cell parameters (Table 6c). In addition, there were no test substance related changes in the clinical chemistry parameters, as shown in Table 7a–c. Some sporadic statistically significant changes were observed compared to the concurrent controls. These were however small in magnitude and/or did not show any dose-response relationship, and therefore were not considered relevant.

Thyroid hormones: There were no test substance related changes in the levels of the thyroid hormones T3, T4 or TSH in any of the male or female treatment groups (Table 8).

Organ weights: As shown in Table 9, there were no test substance-related effects on absolute organ weights in males and females. Sporadic statistically significant changes were considered to be incidental. This was also the case for the relative organ weights, when expressed both as a percentage of terminal body weight and of brain weight (*data not shown*).

Pathology: There were no test item-related macroscopic findings at necropsy or microscopic findings. All sporadic findings appeared in the control group as well, or were considered as common background, seen with similar incidence and severity in control and test substance-treated groups.

4. Discussion

The interest in HMOs such as LNT has developed over the past years, due the expanding knowledge on their health benefits (Asakuma et al., 2011; Bode, 2012; Jantscher-Krenn et al., 2012; Lin et al., 2017; Natividad et al., 2022; Ojima et al., 2022), as well as to the increasing possibilities for large-scale (biotechnological) manufacturing of these complex oligosaccharides, with the aim to apply them in infant formula and other foods. For regulatory approval of the application of newly produced HMOs in any food, including infant formula, its safety must be demonstrated in a required set of studies investigating the genotoxic potential and the general toxic effects of the novel ingredient. In the present study, the safety of biotechnologically produced LNT, the most abundant representative of the non-fucosylated neutral HMO category in breast milk (Soyyilmaz et al., 2021), was investigated. The average LNT intake of a 5 month-old breastfed infant can be estimated at 0.11 g/kg bw/day.[2]

LNT was negative in the bacterial reverse mutation assay and the *in vitro* micronucleus assay. These tests investigate the regulatory required aspects of genotoxicity of a substance, including its mutagenic, aneugenic and clastogenic potential. Of note, based on the respective OECD guidelines, LNT should have been tested at the limit dose of 5000 μg/plate in the bacterial reverse mutation assay (OECD, 2020) and 2000 μg/mL in the *in vitro* micronucleus assay (OECD, 2016). In the present study, however, these maximum levels were not reached, despite the intention to do so at the time the study was conducted (see Section 2.1). The maximum test concentration in the *in vitro* micronucleus assay was still in line with the ICH S2 (R1) guideline prescribed for pharmaceuticals for human use (ICH, 2011). The genotoxic potential of

[2] Mature milk (15–90 days lactation) contains approximately 0.74 g/L LNT (Soyyilmaz et al., 2021). Based on a maximum intake of 1 L/day (around 5 months of age) and a body weight of 7 kg this would correspond to an LNT intake of 0.11 g/kg BW/day.

Table 6c
Hematology analysis in rats exposed for 90 days to LNT; absolute white blood cell counts and relative sub-populations.

% LNT in diet	WBC (10E9/L)			LUC (%)			Lymphocytes (%)			Neutrophils (%)			Eosinophils (%)			Basophils (%)			Monocytes (%)		
Males[a]																					
0.0	5.266	±	2.335	0.42	±	0.15	53.30	±	14.31	40.38	±	14.54	1.81	±	0.95	0.11	±	0.09	4.02	±	0.91
1.25	5.506	±	2.378	0.48	±	0.33	59.84	±	10.19	33.57	±	9.60	2.09	±	0.52	0.15	±	0.13	3.88	±	1.20
2.5	6.983	±	2.414	0.31	±	0.18	**39.37***	±	10.87	**55.35***	±	10.78	1.46	±	0.59	0.08	±	0.08	3.41	±	0.86
5.0	5.296	±	2.877	0.41	±	0.17	62.04	±	8.50	31.53	±	8.29	2.02	±	0.38	0.13	±	0.09	3.86	±	1.07
Females[b]																					
0.0	3.327	±	1.539	0.57	±	0.29	49.47	±	14.16	45.17	±	13.62	1.73	±	0.67	0.22	±	0.25	2.80	±	1.05
1.25	2.817	±	1.175	0.59	±	0.24	54.09	±	13.25	40.93	±	13.34	1.65	±	0.59	0.11	±	0.09	2.58	±	1.03
2.5	3.344	±	1.584	0.43	±	0.27	48.42	±	8.79	47.09	±	8.30	1.62	±	1.24	0.17	±	0.14	2.27	±	0.75
5.0	3.004	±	1.385	0.76	±	0.48	54.81	±	11.24	39.21	±	10.74	2.29	±	1.66	0.20	±	0.21	2.73	±	1.02

WBC, white blood cell count; LUC, large unclassified cells.
Values are means ± SD for groups of 10 rats.
*Dunnett 2-sided $p < 0.05$.
[a] Calculated actual LNT intakes: 0.0, 687, 1385 and 2856 mg/kg bw/day for dietary levels of 0.0, 1.25, 2.5 and 5.0%, respectively.
[b] Calculated actual LNT intakes: 0.0, 768, 1623 and 3253 mg/kg bw/day for dietary levels of 0.0, 1.25, 2.5 and 5.0%, respectively.

Table 7a
Clinical chemistry analysis in plasma from rats exposed for 90 days to LNT.

% LNT in diet	ALP (U/L)			ALT (U/L)			AST (U/L)			GGT (U/L)			Total Bilirubin (μmol/L)			Total Protein (g/L)			Albumin (g/L)			A/G		
Males[a]																								
0.0	63.2	±	15.1	36.4	±	6.8	124.1	±	20.2	0.0	±	0.0	6.06	±	1.95	61.17	±	3.66	39.87	±	2.58	1.88	±	0.08
1.25	63.9	±	9.9	41.8	±	10.4	121.0	±	24.2	0.0	±	0.0	5.28	±	1.74	60.39	±	3.28	38.86	±	2.39	1.81	±	0.15
2.5	59.9	±	9.8	44.5	±	14.7	135.4	±	26.2	0.1	±	0.3	5.69	±	1.77	58.71	±	4.44	37.92	±	3.25	1.84	±	0.12
5.0	69.0	±	15.8	37.4	±	10.1	114.9	±	33.6	0.0	±	0.0	5.70	±	1.61	60.52	±	4.14	39.29	±	2.77	1.86	±	0.13
Females[b]																								
0.0	38.4	±	10.7	41.5	±	8.9	184.4	±	56.0	0.1	±	0.3	5.58	±	1.22	66.27	±	3.31	44.66	±	2.81	2.08	±	0.21
1.25	34.3	±	3.7	40.3	±	11.2	171.4	±	64.8	0.1	±	0.3	5.32	±	1.22	67.73	±	4.26	45.84	±	2.45	2.11	±	0.09
2.5	41.1	±	15.8	44.8	±	6.3	155.8	±	31.6	0.0	±	0.0	5.23	±	1.40	62.75	±	3.63	42.56	±	2.51	2.11	±	0.15
5.0	37.6	±	9.5	**31.7***	±	3.6	**116.6****	±	28.1	0.1	±	0.3	5.73	±	1.45	65.63	±	5.66	44.07	±	4.13	2.04	±	0.12

ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase; A/G, Albumin/Globulin ratio.
Values are means ± SD for groups of 10 rats.
*Dunn 2-sided $p < 0.05$; **Dunnett 2-sided $p < 0.01$.
[a] Calculated actual LNT intakes: 0.0, 687, 1385 and 2856 mg/kg bw/day for dietary levels of 0.0, 1.25, 2.5 and 5.0%, respectively.
[b] Calculated actual LNT intakes: 0.0, 768, 1623 and 3253 mg/kg bw/day for dietary levels of 0.0, 1.25, 2.5 and 5.0%, respectively.

Table 7b
Clinical chemistry analysis in plasma from rats exposed for 90 days to LNT.

% LNT in diet	Cholesterol (mmol/L)			HDL (mmol/L)			LDL (mmol/L)			Triglycerides (mmol/L)			Bile acids (μmol/L)		
Males[a]															
0.0	1.642	±	0.291	0.896	±	0.162	0.301	±	0.054	0.948	±	0.421	5.375	±	3.684
1.25	1.628	±	0.276	0.911	±	0.175	0.299	±	0.043	0.902	±	0.220	**12.026***	±	7.616
2.5	1.598	±	0.389	0.852	±	0.226	0.296	±	0.056	1.034	±	0.207	6.436	±	3.455
5.0	1.600	±	0.257	0.888	±	0.137	0.281	±	0.055	1.159	±	0.487	**10.592***	±	4.154
Females[b]															
0.0	1.509	±	0.185	0.814	±	0.120	0.297	±	0.049	0.580	±	0.136	21.989	±	8.589
1.25	1.413	±	0.298	0.775	±	0.185	0.270	±	0.043	0.538	±	0.168	17.603	±	9.488
2.5	1.278	±	0.191	0.698	±	0.098	0.249	±	0.039	0.591	±	0.343	23.244	±	9.407
5.0	1.504	±	0.267	0.838	±	0.166	0.292	±	0.048	0.691	±	0.225	19.632	±	5.846

Values are means ± SD for groups of 10 rats.
*Dunnett 2-sided $p < 0.05$.
[a] Calculated actual LNT intakes: 0.0, 687, 1385 and 2856 mg/kg bw/day for dietary levels of 0.0, 1.25, 2.5 and 5.0%, respectively.
[b] Calculated actual LNT intakes: 0.0, 768, 1623 and 3253 mg/kg bw/day for dietary levels of 0.0, 1.25, 2.5 and 5.0%, respectively.

biotechnologically produced LNT has been investigated previously. An LNT batch with a purity of ≥77.0% produced by fermentation with an *E. Coli* K-12 DH1 MDO-derived strain (MP813) was negative at exposure levels of up to and including 5000 μg/plate and 2000 μg/mL in the bacterial reverse mutation assay and *in vitro* micronucleus assay, respectively (Glycom A/S, 2018; Phipps et al., 2018; EFSA Panel on Nutrition, Novel Foods and Food Allergens, 2019; EFSA Panel on Nutrition, Novel Foods and Food Allergens, 2022). In line with this outcome, an LNT batch with a purity of ≥80.0% produced by fermentation with a different *E. Coli* K12-derived strain (MG1655) was negative when tested up to the limit dose levels in these assays (Inbiose N.V., 2023). Parschat et al. evaluated the genotoxic potential of an HMO mix containing 23.7% (w/w dry matter) LNT at equivalent maximum exposure levels of 142,000 μg/plate LNT in the bacterial reverse mutation assay and 14,200 μg/mL LNT in the *in vitro* micronucleus assay. In both assays, a clearly negative response was obtained (Parschat et al., 2020). The Parschat-study showed that LNT does not elicit a genotoxic response at exposure levels of up to ~28-fold and ~7-fold higher than the prescribed limit dose levels in the test guidelines. Therefore, even if the limit dose was not completely reached in the present study, LNT can be considered non-genotoxic, based on the total body of evidence, and the genotoxic potential of the LNT batch under investigation considered sufficiently evaluated from regulatory perspective (EFSA Scientific Committee, 2011; FDA, 2012; EFSA Scientific Committee, 2017). In this context, it is import to note that the *in vitro* micronucleus assay was performed according to the former version of OECD test guideline 487 (OECD, 2016). As compared to the update from July 2023 (OECD, 2023), there are no fundamental differences in the interpretation of the data. Therefore, the conclusions of the performed study are also valid under the updated version of the test guideline. Given the negative outcomes in these *in vitro* assays, no further *in vivo* genotoxicity experiments are required.

In the dietary 90-day toxicity study, the exposure to the test substance occurred via the feed, the same exposure route as intended for LNT as ingredient of infant formulae. A different exposure method often used for exposure in an oral study is via gavage, which ensures equal daily test substance intake for every animal. The advantage is that exposure to juvenile animals can start prior to weaning. However, the test substance is brought immediately into the stomach and does not interact with surfaces in the oral cavity, including the buccal, sublingual, gingival, palatal and labial mucosa (Vandenberg et al., 2014). This means that potential effects of the test substance in these areas could be missed. In addition, oral gavage is associated with stress-reactions (Brown et al., 2000; Bonnichsen et al., 2005; Walker et al., 2012), that may interfere with the outcome of these studies. 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 (Bonnichsen et al., 2005; Damsch et al., 2011; Turner et al., 2011). Lastly, via oral gavage, a test substance is administered in one or a few boluses per day, which may not adequately reflect human intake over the course of the day. Because of these considerations, animals were exposed to LNT via their feed in the current 90-day oral toxicity study.

LNT was well-tolerated and did not cause any adverse effects in any of the treatment groups up to and including the maximum tested dose level of 2856 and 3253 mg/kg bw/day in males and females, respectively, which is equivalent to the limit dietary dose level[3] of 5% recommended by OECD test guideline 408 (OECD, 2018). The No-Observed-Adverse-Effect-Level (NOAEL) of the present study was therefore at least 2856 and 3253 mg/kg bw/day in males and females, respectively. This more than covers the estimated daily intake of breastfed infants (Soyyilmaz et al., 2021).

The design of the 90-day study was in accordance with OECD Test Guideline 408, version 2018 (OECD, 2018), and included endpoints addressing endocrine disruptive properties, such as the analysis of thyroid hormone levels, weights of endocrine relevant organs such as testes, epididymides, adrenal glands, prostate and seminal vesicles with coagulating glands, uterus, ovaries, and pituitary and thyroid gland, and concurrent histopathology. Furthermore, cholesterol-related parameters, known to be influenced by thyroid mediated processes, were included. In addition, sperm parameters and vaginal smears were investigated. With the inclusion of these parameters, the study design should allow to detect the flags for any potential to cause neurotoxic, endocrine-, immunological- or reproductive organ effects (OECD, 2018). In the present study, none of these parameters were affected in rats exposed to up to and including the highest dose level of the test substance. Therefore, no indication for neurotoxic effects or adverse interactions with the endocrine, immunological or reproductive system by LNT was obtained.

Since infants and young children are an important part of the intended target population of LNT, juvenile animals were included in this 90-day study. Although exposure via oral gavage would allow direct pre-weaning exposure of rats, intake via diet is considered the most relevant exposure route. In order to reach adequate exposure to the test substance when administered via the diet, in the present study, rats were around 6 weeks old at the start of treatment, which is approximately 3 weeks after weaning. Given the considerations discussed earlier, and because besides infants, also the general population is part of the envisaged target population of this LNT, the choice for dietary exposure

[3] The guidance prescribes a maximum high dose level of 1000 mg/kg bw/day. Assuming a daily feed intake of 20 g (ECHA, 2010), this is equivalent to a dietary level of 5%.

Table 7c
Clinical chemistry analysis in plasma from rats exposed for 90 days to LNT.

% LNT in diet	Glucose (mmol/L)			P (mmol/L)			Ca (mmol/L)			K (mmol/L)			Na (mmol/L)			Cl (mmol/L)			Urea (mmol/L)			Creatinin (μmol/L)		
Males[a]																								
0.0	11.25	±	1.58	2.561	±	0.339	2.853	±	0.080	5.86	±	0.72	144.3	±	0.8	102.60	±	0.83	6.99	±	0.86	57.78	±	8.65
1.25	9.80	±	1.45	2.415	±	0.260	2.863	±	0.094	5.85	±	0.46	144.3	±	1.3	102.52	±	0.93	7.50	±	1.14	59.23	±	10.88
2.5	9.58	±	1.90	2.355	±	0.198	2.817	±	0.103	5.32	±	0.68	144.8	±	1.6	103.03	±	1.57	7.43	±	1.10	49.62	±	11.97
5.0	10.27	±	2.09	2.421	±	0.311	2.864	±	0.096	5.23	±	0.64	144.8	±	1.1	101.98	±	1.69	7.14	±	1.30	51.79	±	17.61
Females[b]																								
0.0	9.10	±	0.93	2.431	±	0.255	2.916	±	0.099	4.89	±	0.36	143.5	±	0.8	102.42	±	1.37	6.98	±	1.49	76.34	±	11.55
1.25	8.91	±	1.72	**2.075***	±	0.288	2.942	±	0.074	4.93	±	0.55	144.0	±	1.0	103.60	±	1.90	7.28	±	1.01	81.93	±	15.70
2.5	8.33	±	1.48	2.340	±	0.252	2.854	±	0.089	4.52	±	0.24	144.0	±	1.3	102.86	±	0.67	7.31	±	1.45	81.19	±	16.44
5.0	9.54	±	1.15	2.357	±	0.219	2.958	±	0.104	5.15	±	0.69	144.3	±	0.8	102.67	±	1.05	7.73	±	1.33	78.69	±	13.48

Values are means ± SD for groups of 10 rats.
*Dunnett 2-sided $p < 0.01$.
[a] Calculated actual LNT intakes: 0.0, 687, 1385 and 2856 mg/kg bw/day for dietary levels of 0.0, 1.25, 2.5 and 5.0%, respectively.
[b] Calculated actual LNT intakes: 0.0, 768, 1623 and 3253 mg/kg bw/day for dietary levels of 0.0, 1.25, 2.5 and 5.0%, respectively.

instead of oral gavage was made in the present 90-day study.

A number of publications addressing the sub-chronic toxicity of different biotechnologically produced LNT in juvenile rats are available. In these studies, exposure started at post-natal day 7, implying that gavage was the route of exposure, and lasted 90 days. In one study, neonatal Sprague-Dawley (Crl:CD*(SD)) rats were exposed to LNT (purity 77.0% (w/w)) produced via a fermentation process involving an *E. Coli* K-12 DH1 MDO-derived strain (MP813), up to a high dose level of 4000 mg/kg bw/day (Glycom A/S, 2018; Phipps et al., 2018; EFSA Panel on Nutrition, Novel Foods and Food Allergens, 2019; EFSA Panel on Nutrition, Novel Foods and Food Allergens, 2022). In the second study a biotechnologically obtained LNT (purity ≥80.0% (w/w), produced by a different *E. coli* K-12 strain (MG1655), was tested in juvenile Sprague-Dawley rats up to a high dose level of 5000 mg/kg bw/day (Inbiose N.V., 2023). In both studies, no test substance-related findings were reported, and the NOAELs were set at 4000 (Glycom A/S, 2018; Phipps et al., 2018; EFSA Panel on Nutrition, Novel Foods and Food Allergens, 2019; EFSA Panel on Nutrition, Novel Foods and Food Allergens, 2022) and 5000 mg/kg bw/day, respectively (Inbiose N.V., 2023). Like the present study, these gavage studies in juveniles were conducted according to the criteria listed in the OECD 408 test guideline. Despite the difference in the specifications between the LNT test materials, the NOAELs are of similar order of magnitude and all at least equivalent to the highest dose levels tested. In addition, there is no indication for a difference in hazard profile when LNT exposure occurs via gavage as compared to via the diet. This suggests that besides the main component LNT, the impurity fraction in each of the test materials can also be considered of low toxic potential. The combination of these three OECD guideline studies in the rat constitute robust evidence supporting the safety of this food ingredient for the target population, including infants.

LNT has also been tested in piglets. Two GRAS Notices filed for biotechnologically produced LNT batches (Spherix Consulting Group, 2020; Inbiose N.V., 2023) have included in their weight of evidence supporting the safety of LNT a 21-day neonatal piglet study (Hanlon, 2020), in which groups of 6 male and female LD-2 domestic Yorkshire cross-bred swines were exposed to a mixture of HMOs,[4] including LNT, from day 2 of lactation, as part of a liquid diet administered via a feeding bowl. Based on the food consumption, the total intake of HMO-mix (containing 23.7% LNT by dry weight) was calculated for males as 2556 and 3576 mg/kg bw/day in the low and high dosing groups, respectively, and in females as 2604 and 3660 mg/kg bw/day, respectively (Hanlon, 2020). The resulting LNT intake was calculated to be 606 and 848 mg/kg bw/day in males and 617 and 867 mg/kg bw/day in females of the low and high dose groups, respectively. In the absence of any adverse test substance related findings, the NOAEL for LNT based on this piglet study can be considered to be at least 848 and 867 mg/kg bw/day in males and females, respectively. Although the animals were exposed to the other components of the HMO mix as well, this study further supports the safety of LNT for juvenile animals. This same HMO mix was tested in a 90-day dietary toxicity study in 65-days old CD rats (Parschat et al., 2020; Spherix Consulting Group, 2020). The study was conducted as a limit test according to OECD test guideline 408 (OECD, 2018) and comprised only one treatment group, in which 10 males and females were exposed to 10% HMO mix (containing 23.7% LNT by dry weight) via the diet, versus a control diet group. This dose level of the HMO-mix was equivalent to an overall dietary exposure of 2.37% LNT, calculated as a mean LNT intake of 1340 mg/kg bw/day in males and 1650 mg/kg bw/day in females during the study (Spherix Consulting Group, 2020). In the absence of any adverse findings related to exposure to the test substance, this dose can be considered a NOAEL for LNT in

[4] blend of a fixed combination of 2′-fucosyllactose (47.1% DW), 3-fucosyllactose (16.0% DW), LNT (23.7% DW), 3′-sialyllactose (4.1% DW), 6′-sialyllactose (4.0% DW) and other carbohydrates (5.1% DW).

Table 8
Thyroid hormone analysis in serum from rats exposed for 90 days to LNT.

% LNT in diet	T3 (ng/mL)			T4 (ng/mL)			TSH (pg/mL)		
Males[a]									
0.0	1.3100	±	0.3245	44.50	±	8.07	702.0	±	162.6[c]
1.25	1.2770	±	0.2909	43.00	±	4.52	647.6	±	47.6
2.5	1.2690	±	0.3573	42.90	±	7.22	663.1	±	61.5
5.0	1.3180	±	0.3462	44.50	±	4.12	630.2	±	16.4
Females[b]									
0.0	1.1578	±	0.2588	37.44	±	9.21	921.3	±	316.2
1.25	1.2610	±	0.3796	34.10	±	8.80	937.5	±	329.4
2.5	1.1980	±	0.3941	33.90	±	6.14	882.5	±	317.1
5.0	1.1970	±	0.3294	34.40	±	7.81	937.5	±	329.4

Values are means ± SD for groups of 10 rats.
[a] Calculated actual LNT intakes: 0.0, 687, 1385 and 2856 mg/kg bw/day for dietary levels of 0.0, 1.25, 2.5 and 5.0%, respectively.
[b] Calculated actual LNT intakes: 0.0, 768, 1623 and 3253 mg/kg bw/day for dietary levels of 0.0, 1.25, 2.5 and 5.0%, respectively.
[c] Relatively large standard deviation, in line with historical control analyses, showing a mean of 517.6 pg/mL and an SD of 681.6 pg/mL (6 studies; 60 animals).

Table 9
Absolute organ weights (g) from rats exposed for 13 weeks to LNT.

Parameter	LNT concentration in diet (%)[a,b]											
	0			1.25			2.5			5.0		
Males												
Terminal body weight (g)	**577.8**	**±**	**57.2**	**568.9**	**±**	**34.3**	**558.2**	**±**	**43.7**	**607.0**	**±**	**83.1**
Adrenals	0.0650	±	0.0120	0.0731	±	0.0119	0.0693	±	0.0073	0.0684	±	0.0071
Brain	2.251	±	0.107	2.247	±	0.115	2.206	±	0.090	2.211	±	0.064
Epididymis	1.643	±	0.156	1.634	±	0.105	1.713	±	0.211	1.720	±	0.147
Heart	1.487	±	0.133	1.458	±	0.121	1.463	±	0.124	1.554	±	0.147
Kidneys	3.214	±	0.427	3.157	±	0.300	3.045	±	0.304	3.279	±	0.483
Liver	13.211	±	2.064	12.769	±	1.425	12.835	±	1.471	14.607	±	2.892
Pituitary gland	0.0152	±	0.0019	0.0146	±	0.0008	0.0142	±	0.0016	0.0148	±	0.0012
Prostate gland	1.531	±	0.366	1.470	±	0.344	1.542	±	0.199	1.655	±	0.341
Seminal vesicles	2.664	±	0.497	2.661	±	0.334	2.687	±	0.384	2.775	±	0.375
Spleen	0.967	±	0.207	0.946	±	0.143	0.845	±	0.135	1.025	±	0.196
Testis	4.087	±	0.429	4.169	±	0.200	4.134	±	0.487	4.210	±	0.222
Thymus	0.477	±	0.147	0.435	±	0.073	0.373	±	0.079	0.475	±	0.126
Thyroid/parathyroid	0.0305	±	0.0024	0.0310	±	0.0035	0.0311	±	0.0042	0.0331	±	0.0048
Females												
Terminal body weight (g)	313.1	**±**	28.4	307.2	**±**	25.4	315.8	**±**	46.0	325.7	**±**	36.7
Adrenal glands	0.0805	±	0.0103	**0.0651***	±	0.0082	0.0776	±	0.0130	0.0726	±	0.0144
Brain	1.924	±	0.127	2.011	±	0.059	1.999	±	0.089	2.030	±	0.065
Heart	0.933	±	0.060	0.894	±	0.036	0.947	±	0.087	0.948	±	0.091
Kidneys	1.761	±	0.140	1.740	±	0.121	1.722	±	0.236	1.792	±	0.280
Liver	7.431	±	0.631	7.327	±	0.412	7.326	±	1.305	7.766	±	1.235
Ovaries	0.1296	±	0.0243	0.1086	±	0.0117	0.1138	±	0.0294	0.1380	±	0.0342
Pituitary gland	0.0177	±	0.0028	0.0165	±	0.0021	0.0162	±	0.0019	0.0171	±	0.0031
Spleen	0.656	±	0.107	0.649	±	0.109	0.747	±	0.141	0.721	±	0.166
Thymus	0.294	±	0.046	0.298	±	0.069	0.336	±	0.103	0.364	±	0.076
Thyroid/parathyroid	0.0265	±	0.0035	0.0241	±	0.0038	0.0252	±	0.0041	0.0262	±	0.0046
Uterus incl. cervix	0.702	±	0.216	0.841	±	0.280	0.765	±	0.242	0.698	±	0.191

Values are means ± SD for groups of 10 rats.
*Dunnett 2-sided $p < 0.05$.
[a] Calculated actual LNT intakes in males: 0.0, 687, 1385 and 2856 mg/kg bw/day for dietary levels of 0.0, 1.25, 2.5 and 5.0%, respectively.
[b] Calculated actual LNT intakes in females: 0.0, 768, 1623 and 3253 mg/kg bw/day for dietary levels of 0.0, 1.25, 2.5 and 5.0%, respectively.

this 90-day study. Moreover, in addition to all preclinical data, recently, the safety and tolerability of HMO-mix enriched infant formula, containing relatively high levels of LNT (1.5 g/L), was confirmed in a randomized, controlled clinical study, in which healthy-term infants were supplemented with the HMO-mix as part of their infant formula for 15–16 weeks (Parschat et al., 2021; Lasekan et al., 2022). The safety data generated with the HMO-mix of which LNT constitutes 23.7% on dry weight basis can be considered supportive and extent the total weight of evidence for the safety of LNT as food ingredient.

In conclusion, the NOAEL of at least 2856 and 3253 mg/kg bw/day in male and female rats, respectively, in the present study, is of the same order of magnitude as the NOAELs set in sub-chronic rat toxicity studies with other biotechnologically produced LNT batches, ranging from 1340 to 5000 mg/kg bw/day in males and 1650–5000 mg/kg bw/day in females (Glycom A/S, 2018; Phipps et al., 2018; EFSA Panel on Nutrition, Novel Foods and Food Allergens, 2019; Parschat et al., 2020; Spherix Consulting Group, 2020; Inbiose N.V., 2023). The derived NOAEL-values appeared always to be equivalent to the highest dose level tested, even in the limit dose studies. Since no Lowest Observed Effect Level (LOAEL) could be determined in any of the *in vivo* studies with LNT, the NOAEL is probably higher than the tested range.

5. Conclusions

LNT, produced through fermentation by the genetically modified *E. coli* K-12 E2083 production strain, tested negative in both the bacterial reverse mutation assay and the *in vitro* micronucleus assay, demonstrating the absence of genotoxic potential for this substance. In

the OECD 408 guideline compliant 90-day oral toxicity study rat, LNT did not induce any adverse effects in any treatment group up to and including the highest dose tested, and no LOAEL was determined in the present study. Therefore, the no-observed-adverse effect level (NOAEL) is set at the highest dose level tested, i.e. a dietary level of 5 % (w/w), corresponding to ≥2856 mg/kg bw/day and ≥3253 mg/kg bw/day for males and females, respectively. This might be an underestimation of the NOAEL, caused by the range of dose levels tested. The results obtained in the current study are in good agreement with available data generated using other biotechnologically produced LNT batches and therefore support its safe use as a food ingredient.

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CRediT authorship contribution statement

Hester van der Woude: Writing – original draft, Writing – review & editing. **Sylvia M.J.G. Pelgrom:** Writing – review & editing. **Carin Buskens:** Investigation. **Roy Hoffmans:** Investigation. **Nora Krajcs:** Investigation. **Dianne J. Delsing:** Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Dianne J. Delsing reports a relationship with FrieslandCampina that includes: employment. Hester van der Woude reports a relationship with FrieslandCampina that includes: consulting or advisory. Sylvia M.J.G. Pelgrom reports a relationship with FrieslandCampina that includes: consulting or advisory. Carin Buskens reports a relationship with FrieslandCampina that includes: consulting or advisory. Roy Hoffmans reports a relationship with FrieslandCampina that includes: consulting or advisory. Nora Krajcs reports a relationship with FrieslandCampina that includes: consulting or advisory.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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