Safety Assessment of Lacto-*N*-neotetraose (LNnT)

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Novel Food Classification: 1.2.

Introduction

An application for the authorisation of Lacto-*N*-neotetraose (LNnT) was submitted to the Food Safety Authority of Ireland (FSAI) by Glycom A/S from Denmark in accordance with *Article 4* of the novel food Regulation (EC) No. 258/97. The application was accepted by the FSAI on January 13th, 2014.

The novel ingredient is a synthetic oligosaccharide produced using D-Lactose as a starting raw material. The novel ingredient is a linear tetrasaccharide made up of two D-Galactose molecules, one D-Glucose molecule and one N-Acetyl-D-glucosamine molecule and is chemically identical to an oligosaccharide present in human breast milk. While definitive information on the nutritional value of this oligosaccharide is limited, the applicant provides data to suggest that this ingredient, like other similar oligosaccharides is poorly absorbed in humans. The major proportion of ingested LNnT reaches the large intestine where it is primarily metabolised by intestinal microflora and is therefore considered to have prebiotic characteristics. Only a small proportion of ingested LNnT is absorbed and then excreted in urine.

The applicant intends to market the novel ingredient in a number of general foodstuffs, food supplements and some foods for particular nutritional uses including infant formula. This ingredient has not been used in food production previously in the EU and is classed by the applicant as novel in accordance with *Article 1.2(c)* of the novel food Regulation (EC) No 259/97; "Foods and food ingredients with a new or intentionally modified primary molecular structure".

The application dossier was prepared pursuant to Commission Recommendation 97/618/EC and in order to assess wholesomeness, LNnT was considered in Class 1 "pure chemicals or simple mixtures from non-genetically modified sources" and sub-class (2) "the source of the novel food has no history of food use in the Community".

I. Specification of the novel food

The applicant provides comprehensive information about the chemical and structural identity of the novel ingredient which demonstrates that it is the same as the naturally occurring LNnT which is present in human breast milk. The novel ingredient is a white to off-white powder with a minimum specified purity of 95.0% (HPLC analysis), though batch analysis puts the actual purity at ≥99% on average. Protein content is specified at a maximum level of 0.1%, but batch analysis indicates that

protein content is generally below the limit of quantification. Solvent residues as well as heavy metal and microbiological contaminants are controlled by specifications. The stability of crystalline (bulk) LNnT for six months under accelerated conditions has been demonstrated. The novel ingredient is hygroscopic and for bulk stability testing was packaged in polyethylene bags which were then placed in polyethylene/aluminium/polyester triple layer foil bags as secondary packaging. No appreciable changes were observed in water content, microbial load or known breakdown products after six months of accelerated storage (40°C and 75% RH). The stability of bulk LNnT at ambient temperature and controlled humidity (25°C and 60% RH) has been established for 36 months, with an ongoing study to determine stability over a five year period. The stability of LNnT in infant formula powder has been demonstrated for 18 months in an ongoing three year study. In addition, the stability of LNnT in yoghurt, flavoured milk and fruit juice under normal conditions of preparation and storage has also been demonstrated.

II. Effect of the production process applied to the novel food

LNnT is produced through a series of physical and chemical reactions in compliance with good manufacturing practices and in line with HACCP principles. The applicant designates Benzyl-LNnT as the primary raw material which in turn is derived from D-Lactose as starting raw material in Stage 1 of the production process. The novel ingredient (LNnT) is then produced in Stage 2 of the process, with quality control checks in place throughout the process and on the final product.

IX. Anticipated intake/extent of use of the novel food

The novel ingredient is intended for use in PARNUTS, particularly infant and follow-on formula, among other children's foods. It will also be added to a range of other foods (dairy products and analogues, bakery wares as well as syrups, sweeteners and infusions). The applicant included a comprehensive analysis of intake estimates using data from four sources: (a) EFSA estimates of infant formula intake; (b) UK Diet and Nutrition Survey of Infants and Young Children (DNSIYC) for estimates of infant formulae and infant specific foods only, (c) UK National Diet and Nutrition Survey programme to estimate total dietary intake; (d) EFSA 'Food Additives Intake Model' (FAIM) for estimates of total dietary intakes in 17 EU countries using data from 26 dietary surveys.

The proposed maximum levels of LNnT in all food categories are based on the intake by infants from breast milk. This is estimated by the applicant at 20-100mg/kg body weight/day, up to a maximum of 385mg/kg body weight/day for a 6.5Kg infant drinking approximately 1L of breast milk per day. Intake estimates are based on the "worst case" scenario where the novel food replaces all existing similar foods and safety was assessed by comparison with concentrations of LNnT naturally found in human milk, along with a NOAEL of 5,000mg/kg body weight/day. EFSA has estimated the average daily intake of liquid infant formula at 1,060 mL/day for infants aged 0-6 months, based on a 3 month old infant weighing 6.1Kg consuming 174 mL/kg body weight (bw)/day at the 95th percentile of intake.

Using this conservative estimate and a proposed addition of LNnT at 0.6g/L, the applicant estimates that consumption of LNnT at the 95th percentile of intake would be 636.8 mg/day (104.4 mg/kg body weight/day) in infants aged 0-6 months. The proposed intakes by all users at the 95th percentile of all population subgroups (or the maximum use level for high level users within the EFSA FAIM tool) are presented below.

Summary table of proposed intakes of LNnT for different population groups

Population Group	Estimated 95th percentile intake (maximum use level) (mg/kg bw/d)
Infants (0-6 months formula only)	104.4
DNSIYC (all users of infant formulae & infant specific foods)	
Infants (4-6 months)	329
Infants (7-12 months)	295
Young children (13-17 months)	159
UK NDNS (proposed food uses for all users)	
Toddlers (1-3y)	132
Children (4-10y)	72
Teenagers (11-18y)	35
Women of child bearing age (19-40y)	53
Female adults (19-64y)	40
Male adults(19-64y)	26
Elderly adults (≥65y)	28
EFSA FAIM tool (proposed food uses; heavy level intakes maximum use level)	
Toddlers (12-35 months)	370-528
Children (3-9y)	86-338
Adolescents (10-17y)	34-118
Adults (18-64y)	45-152
Elderly (≥65y)	45-143

The impact of supplement use was determined by adding the proposed LNnT supplement dose (1.5g/d) to mean daily intakes of the population groups in the UK NDNS. In all cases, the subsequent combined intake was below the 95th percentile of intake.

X. Information from previous human exposure to the novel food or its source

LNnT is a naturally occurring linear tetrasaccharide found in the milk of some mammalian species, though it is present at highest concentrations in human milk. The level in human breast milk varies considerably between individuals and is generally at its highest in colostrum. LNnT is an important part of a complex mixture of oligosaccharides found in human milk and which the applicant claims may have a

prebiotic role. However, though the addition of other oligosaccharides such as fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS) to infant formula is permitted in the EU, LNnT is not currently added specifically as an ingredient to any foodstuffs in the EU and so the history of human exposure is limited primarily to that of breast fed infants.

XI. Nutritional information on the novel food

Consumption of LNnT at the levels proposed and in the food categories intended should not have a significant nutritional impact. Ingested LNnT is not expected to undergo significant metabolism in the upper gastrointestinal tract or to be absorbed intact to any significant extent, but is likely to be metabolised through fermentation by bacteria residing in the large intestine, with some amount excreted unaltered in the faeces. Additionally, a small proportion of any absorbed LNnT may be excreted unchanged in urine. Oligosaccharides such as LNnT have been shown to provide a growth advantage to certain bacterial strains residing in the human intestines and as such may act as prebiotics. Therefore, the novel ingredient is not likely to be of any direct nutritional or calorific value to humans, and is intended to be a selective nutritional support for certain microorganisms in the gut microflora.

XII. Microbiological information on the novel food

The microbiological status of the novel ingredient, including endotoxin content is controlled via product specifications and supported by batch test results.

XIII. Toxicological information on the novel food

The applicant noted there were no acute studies on LNnT evident in the scientific literature. However, information was provided on rat feeding studies of LNnT where FOS was used as a reference ingredient since it is permitted internationally for use in infant and follow-on formulae.

Repeated Exposure Studies

The potential for toxicity related to the ingestion of the novel ingredient was assessed by a combination of standard rat feeding studies (14-day, 28-day and 90-day) that were adapted with the use of juvenile and standard weanling rats to allow for the risk assessment for all the proposed consumers, including infants. A dose range finding study was carried out by gavage feeding of the novel ingredient at different dose levels. An upper feeding limit of 5,000 mg/kg bw/day was set as any higher levels could result in a nutritional imbalance. A reference control group was fed 5,000 mg/kg bw/day of the plant-derived fructo-oligosaccharides (FOS) over the same period. LNnT intake was not associated with changes in mortality, clinical signs, body weight or histopathology in any dose group. A similarly designed 28-day oral gavage study was conducted to OECD principles of good laboratory practice and testing Guideline 407 and also included a 14 day recovery period. The reference control group was again administered at 5,000 mg/kg bw/day of FOS. Histopathological examination of all organs and tissues concluded that mortalities in the low dose and control groups were not treatment-related and no treatment-related clinical

abnormalities were observed. The testing laboratory concluded that statistically significant increases in total white blood cell (30%) and absolute monocyte (very slight) counts evident in the high-dose female group were incidental in nature and therefore of no toxicological significance. Moreover, changes in total white blood cell and absolute monocyte counts were not observed in females in the 90-day study. Certain differences in mean corpuscular volume, reticulocyte count and mean corpuscular haemoglobin concentration were observed in some male LNnT-treated animals when compared to controls. However, the differences were not dose-related and/or were very slight in magnitude compared to controls and none were corroborated by clinical or histopathological results.

Statistically significant changes in a number of clinical chemistry parameters were either sex-specific and/or not dose-related and/or not observed in the 90-day study. Where a dose-response trend was evident, statistical significance was reached at one or more doses, and the findings in the 90-day study corroborated those of the 28-day study. However, these changes were not mirrored by changes in other electrolytes, albumin/globulin ratio, other associated enzyme levels, urinalysis parameters, organ weights, macroscopic findings or histopathological results. The alanine aminotransferase levels in females and aspartate aminotransferase levels in males and females decreased with dose, while the increase trend in alkaline phposphatase in males may have been associated with food intake (although statistically significant changes were not recorded for this parameter). A few other clinical chemistry changes were observed but were sporadic and considered by the applicant to be unrelated to treatment. Furthermore, no treatment-related macroscopic or microscopic lesions were recorded. The NOAEL was determined to be 5,000 mg/kg bw/day, the highest dose tested.

A number of statistically significant changes in haematology parameters were recorded in the 90-day study which used a similar testing protocol carried out to GLP. There were no treatment-related mortalities and no effects on body weight, body weight gain or food intake, with ophthalmological examinations being unremarkable. Most significant was an observed reduction in the total white blood cell and lymphocyte counts in high-dose males which remained significantly lower (in all dose groups) after the recovery period. However, the reduction in all dose groups was not observed at the very end of the treatment period so the toxicological significance is debatable. The applicant considered these changes to be incidental or possibly the result of physiological adaptation to the administration of LNnT (and FOS) compared to the water control, with no apparent correlation to histopathological findings. Changes in clinical chemistry parameters were recorded in the high-dose group in this study, as they were in the 28-day study. Decreases in serum enzyme activities were sex-specific, whereas the decrease in sodium levels was observed in both sexes, but none of the changes were corroborated by histopathology or organ weight changes. Urinalysis was unremarkable and no treatment-related macroscopic or microscopic lesions were recorded. The NOAEL was set at 5,000 mg/kg bw/day, the highest dose tested.

Mutagenicity

The mutagenic potential of LNnT was investigated using the Ames test and the novel ingredient was deemed to be non-mutagenic at concentrations up to 5000 µg/plate. In addition, a mammalian cell gene mutation assay using L5178Y tk+/- mouse lymphoma cells did not reveal statistically or biologically significant mutagenic effects of LNnT at concentrations up to 4,250 µg/ml.

Human Studies

A study from the scientific literature on 228 healthy infants aged 6-24 months demonstrated that feeding of LNnT (approximately 500mL formula/day – LNnT of unknown purity produced by fermentation) for four weeks was well tolerated and without adverse health effects at concentrations up to 220 mg/L.

Allergenicity

Allergenicity is not considered an issue as protein is at or below detection limits in the novel ingredient.

Conclusions

Lacto-*N*-neotetraose (LNnT) is a natural constituent in mammalian milk, with human breast milk having the highest levels among animal species, in particular colostrum. The novel ingredient in this application is structurally and chemically the same as the corresponding constituent in human breast milk, but is derived from lactose through a series of chemical and physical interactions.

The applicant has identified the food types in which they intend to market the food. Based on food intake data from a number of sources, potential average intakes were estimated and no nutritional concerns were identified. The toxicological data provided is sufficient to demonstrate the safety of this ingredient when consumed as intended, even by vulnerable groups such as young children.

Recommendation

The Food Safety Authority of Ireland has not identified any safety concerns associated with the consumption of Lacto-*N*-neotetraose (LNnT) at the proposed use levels in foods or food supplements containing the novel ingredient and therefore considers that it meets the criteria for novel food set out in *Article 3.1.* of the novel food Regulation (EC) No 258/97.