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7 August 2019

Dr Paulette Gaynor
Office of Food Additive Safety
HFS-200
5001 Campus Drive
College Park, MD 20740-3835

New submission
Ref: GRAS Notice for Trehalose

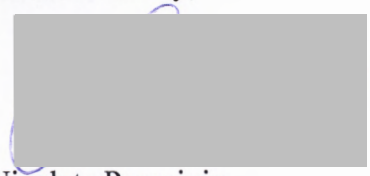
Dear Dr Gaynor,

Pursuant to 21 CFR § 170 Subpart E consisting of § 170.203 through 170.285, c-LEcta GmbH (Perlickstr. 5, 04103 Leipzig, Germany), through Pen & Tec Consulting S.L.U as its agent, hereby informs the United States Food and Drug Administration of the conclusion that Trehalose, manufactured by c-LEcta GmbH, is GRAS under the specifie conditions of use as a food ingredient in food excluding infant formulas, on the basis of scientific procedures, and therefore, is not subject to the premarket approval requirements of the Federal Food, Drug and Cosmetic Act.

I hereby certify that the enclosed electronic files have been checked and found to be virus free.

Should you have any questions regarding this GRAS Notice at any point during the review process, please contact Paula.Pescador@c-LEcta.com, as the contact person for all correspondence. C-LEcta GmbH, Perlickstraße 5, 04103 Leipzig, Germany. Phone +49 341 355 214-0/ Fax -33.

Yours sincerely,



Nicoleta Pascinic
Regulatory Affairs Manager
Pen & Tec Consulting S.L.U.

Enclosures:
1. one CD

GRAS NOTICE FOR TREHALOSE

PREPARED FOR:
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Campus Drive
College Park, MD 20740-3835

DATE:
7 August 2019

GRAS Notice for Trehalose

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GRAS Notice for Trehalose

Part 1 §170.225 Signed Statements and Certification

c-LEcta GmbH prepared and submitted this GRAS notice in accordance with 21 CFR Part 170, Subpart E.

1.1 Name and Address of Notifier

c-LEcta GmbH
Perlickstrasse 5
40103 Leipzig
Germany

1.2 Common Name of Notified Substance

The common name of the notified substance is trehalose or α,α-trehalose. The commercial product is the dihydrate.

1.3 Conditions of Use

c-LEcta intends to market trehalose produced from sucrose by enzymatic conversion in accordance with current Good Manufacturing Practice (cGMP) as a multipurpose food ingredient in the United States (U.S.) for use in food in general excluding infant formulas.
The ingredient is to be used in bakery products, beverages, dairy products and dairy analogs, grains and cereals, dressings and sauces, fruits and vegetables, meat, poultry, fish and egg products, nut products, gelatins and fillings, jams and jellies, snacks and sweets, seasonings and flavors, soups.
The ingredient is used as a nutritional sweetener, flavor enhancer, texturizer, humectant, and for other functional uses such as color preservation.

1.4 Basis for GRAS

Pursuant to Title 21, Section 170.30 (a) and (b) of the *Code of Federal Regulations* (CFR), trehalose has been determined by c-LEcta to be GRAS on the basis of scientific procedures. A comprehensive scientific literature search was conducted from 01 January 2000 until 12 November 2018 to evaluate any updates on the safety of trehalose based on in vivo and in vitro studies.

1.5 Exemption from Premarket Approval

In accordance with 21 CFR §170 Subpart E consisting of §170.203 through 170.285, c-LEcta GmbH hereby informs the U.S. Food and Drug Administration (FDA) of the view that its trehalose produced by enzymatic conversion from sucrose is not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on its conclusion that the notified substance is Generally Recognized as Safe (GRAS) under the conditions of its intended use described in Part 1.3 above.

1.6 Availability of information

The data and information that serve as the basis for this GRAS Notice can be disclosed by c-LEcta GmbH upon request either during or after U.S. FDA evaluation. Requested data and information will be sent to the U.S. FDA in an electronic format or on paper or made available for review and copying during business hours at c-LEcta offices, at the above address.

1.7 Freedom of Information Act, 5 U.S.C. 552

None of the data and information presented in Parts 2 through 7 of this GRAS Notice are exempt from disclosure under the Freedom of Information Act, 5 U.S.C. Section 552.

1.8 Certification

c-LEcta GmbH certifies that all data and information presented in this notice constitute a complete, representative, and balanced submission that includes all unfavorable as well as favorable information known to c-LEcta GmbH and pertinent to the evaluation of the safety and GRAS status of trehalose produced by enzymatic conversion from sucrose as a food ingredient.

Signed,

7 August 2019

Dr. Paula Pescador
Head of Regulatory Affairs
c-LEcta GmbH

Date

Part 2 §170.230 Identity, Method of Manufacture, Specifications, and Physical or Technical Effect

2.1 Identity

2.1.1 Common or Usual Name

Trehalose; α,α-trehalose; α-D-glucopyranosyl-α-D-glucopyranoside.

Empirical Formula: $C_{12}H_{22}O_{11} \cdot 2H_2O$

Molecular Weight: 378.33

CAS Number: 6138-23-4

2.1.2 Chemical and Physical Characteristics

Trehalose is a white or almost white crystalline or powdery solid, virtually odorless and with a sweet taste. Trehalose is freely soluble in water and very slightly soluble in ethanol. The chemical structure of trehalose is presented in Figure 2.1.2-1. The commercial product is the dihydrate.

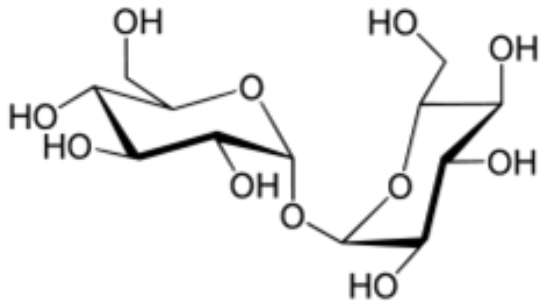

Figure 2.1.2-1 Chemical structure of trehalose

2.2 Method of Manufacturing

The starting material for c-LEcta trehalose produced by enzymatic conversion from sucrose is food-grade sucrose. The conversion of sucrose to trehalose takes place in a one-pot reaction by action of three different enzymes: a sucrose phosphorylase (SP), a trehalose phosphorylase (TP) and a glucose isomerase (GI).

Once the reaction is completed, the biotransformation mixture is clarified via solid/liquid separation and further ultrafiltered to remove the enzymes. The resulting solution is then continuously concentrated, leading to the crystallization of trehalose. Finally, the slurry is filtered (centrifuged), washed with water, and dried by evaporation. A flow diagram of the manufacturing process is presented in Figure 2.2.3-1.The final purified product contains ≥98% total trehalose, which is consistent with the purity criteria for trehalose as established by JECFA (2000).

2.2.1 Raw materials and Processing Aids

All raw materials and processing aids used in the manufacturing process are food-grade ingredients[1] permitted by U.S. regulation or have GRAS status for their respective uses. Production of c-LEcta trehalose produced by enzymatic conversion from sucrose is carried out in accordance with the principles of Hazard Analysis and Critical Control Points (HACCP) and current Good Manufacturing Practices for Food (cGMP), including quality control checks at every stage of the production process.

Table 2.2.1-1 Regulatory compliance of raw materials and processing aids used in the manufacture of trehalose

Sucrose	Reactant	GRAS
Enzymes (SP, TP, GI)	Processing aids	NA
Magnesium sulfate	Processing aid	Permitted for use in food as a direct food substance with no limitations apart from cGMP, 21 CFR §184.1443 (U.S. FDA, 2018)
Sodium hydroxide	pH control	Permitted for use in food as a direct food substance with no limitations apart from cGMP, 21 CFR §184.1763 (U.S. FDA, 2018)
Phosphoric acid	pH control/reactant	Permitted for use in food as a multiple purpose GRAS substance, 21 CFR §182.1073 (U.S. FDA, 2018)
Potassium phosphate buffer	pH control	NA
NA= not applicable		

2.2.2 Enzymes

The enzymes used in the manufacturing process to convert sucrose to trehalose are manufactured in accordance with cGMP for food and HACCP principles. The enzymes are produced by microbial fermentation of the production strain LE1B109, a derivative of Escherichia coli K-12, carrying an expression plasmid containing the corresponding enzyme gene.

The production strain LE1B109 is a genetically modified derivative strain of *E. coli* K-12 W3110, a non-pathogenic, non-toxigenic laboratory strain. The parental strain *E. coli* K-12 W3110 has been modified by site-directed recombination at different chromosomal loci to suit production purposes in terms of genetic stability, especially plasmid stability, and efficiency of expression and biotransformation. In particular, the genes encoding a number of proteases have been eliminated by deletion. Unwanted recombination events are also prevented through elimination of one specific gene, and one additional deletion has been carried out to allow for selection of target clones without the use of antibiotic resistance markers. Finally, to ensure a strong and regulated enzyme expression, chromosomal insertion of two further genes has been performed: one gene coding for the T7 RNA polymerase (from the *E. coli* T7 phage), and an extra copy of the lacI repressor naturally present in *E. coli*. Insertions and deletions of chromosomal DNA have been performed by integration of plasmid-based fragments carrying antibiotic resistance genes for selection purposes. After selection of the correct chromosomal mutants, resistance genes are excised and all plasmids are removed. No residual vector sequences or antibiotic resistance genes are left in the final LE1B109 strain, as confirmed by whole-genome sequencing.

The specific production strain used for manufacturing each enzyme is constructed from the LE1B109 recipient strain by introducing an expression vector carrying the gene for the corresponding enzyme (Table 2.2.2-1). The plasmids used to

[1] Compliant with the specifications set forth in the Food Chemicals Codex or equivalent international food standard [e.g. JECFA].

transform the *E. coli* recipient strain are based on the well-known vector pRSF-1b. All plasmids have been fully sequenced and do not carry antibiotic resistance genes or any other sequences of concern.

Table 2.2.2-1 Enzymes used in the biotransformation process

Enzyme	Function
Sucrose phosphorylase (SP)	Catalyzes the conversion of sucrose into fructose and glucose-1-phosphate
Trehalose phosphorylase (TP)	Catalyzes the formation of trehalose from glucose and glucose-1-phosphate
Glucose isomerase (GI)	Catalyzes the conversion of fructose into glucose

The *E. coli* LE1B109 production strain is a microorganism of biosafety level 1. Separate specifications for the 3 enzymes have been established and are presented in Table 2.2.2-2. The enzymes are of food-grade quality and conform to the recommended purity criteria established by the Food Chemicals Codex, 11th Edition (FCC, 2018a) and JECFA (2006). No antibiotics are used in the manufacturing process. The absence of the production microorganism is demonstrated for each enzyme batch.

Table 2.2.2-2 Product specifications for the enzymes used to manufacture c-LEcta Trehalose

Specification Parameter	Specification		
	SP	TP	GI
Activity	≥ 3000 U/mL	≥ 100 U/mL	≥ 90 U/mL
Total viable count	< 50,000 CFU/g	< 50,000 CFU/g	< 50,000 CFU/g
Salmonella spp.	Absent in 25 g	Absent in 25 g	Absent in 25 g
Escherichia coli	Absent in 25 g	Absent in 25 g	Absent in 25 g
Total coliforms	≤ 30 CFU/g	≤ 30 CFU/g	≤ 30 CFU/g
Staphylococcus aureus	Absent in 1 g	Absent in 1 g	Absent in 1 g
Anaerobic sulfite reducing bacteria	< 30 CFU/g	< 30 CFU/g	< 30 CFU/g
Antimicrobial activity	Negative	Negative	Negative
Lead	≤ 5 mg/kg	≤ 5 mg/kg	≤ 5 mg/kg
Cadmium	≤ 0.5 mg/kg	≤ 0.5 mg/kg	≤ 0.5 mg/kg
Mercury	≤ 0.5 mg/kg	≤ 0.5 mg/kg	≤ 0.5 mg/kg
Arsenic	≤ 3 mg/kg	≤ 3 mg/kg	≤ 3 mg/kg

2.2.3 Manufacturing Process

A schematic overview of the manufacturing process for c-LEcta Trehalose is illustrated below in Figure 2.2.3-1.

The processes utilized for the enzymatic conversion as well as for the subsequent product purification are very similar to those described in GRN No. 45, concerning the manufacture of trehalose from liquefied starch by a multistep enzymatic process. In the case of c-LEcta trehalose produced by enzymatic conversion from sucrose, food-grade sucrose is used as starting material. To formulate the reaction mixture, sucrose is dissolved in buffer and the solution is sanitized at 90 °C. After decreasing the temperature, the three enzymes, SP, TP and GI are added to the solution and the biotransformation reaction is allowed to proceed until the desired degree of conversion is reached. Once the biotransformation is completed, the solution is first clarified by liquid/solid separation to remove protein precipitates.

Next, ultrafiltration is carried out to separate and recover the enzymes. The trehalose-containing solution is then concentrated by evaporation. Optionally, a seed of trehalose crystals is added to the concentrated solution to initiate the crystallization process, which is carried out under controlled temperature conditions. The trehalose crystals are washed with water, separated from the mother liquor by centrifugation, and dried in an air drier. Finally, the trehalose crystals are packaged in food-grade bags.

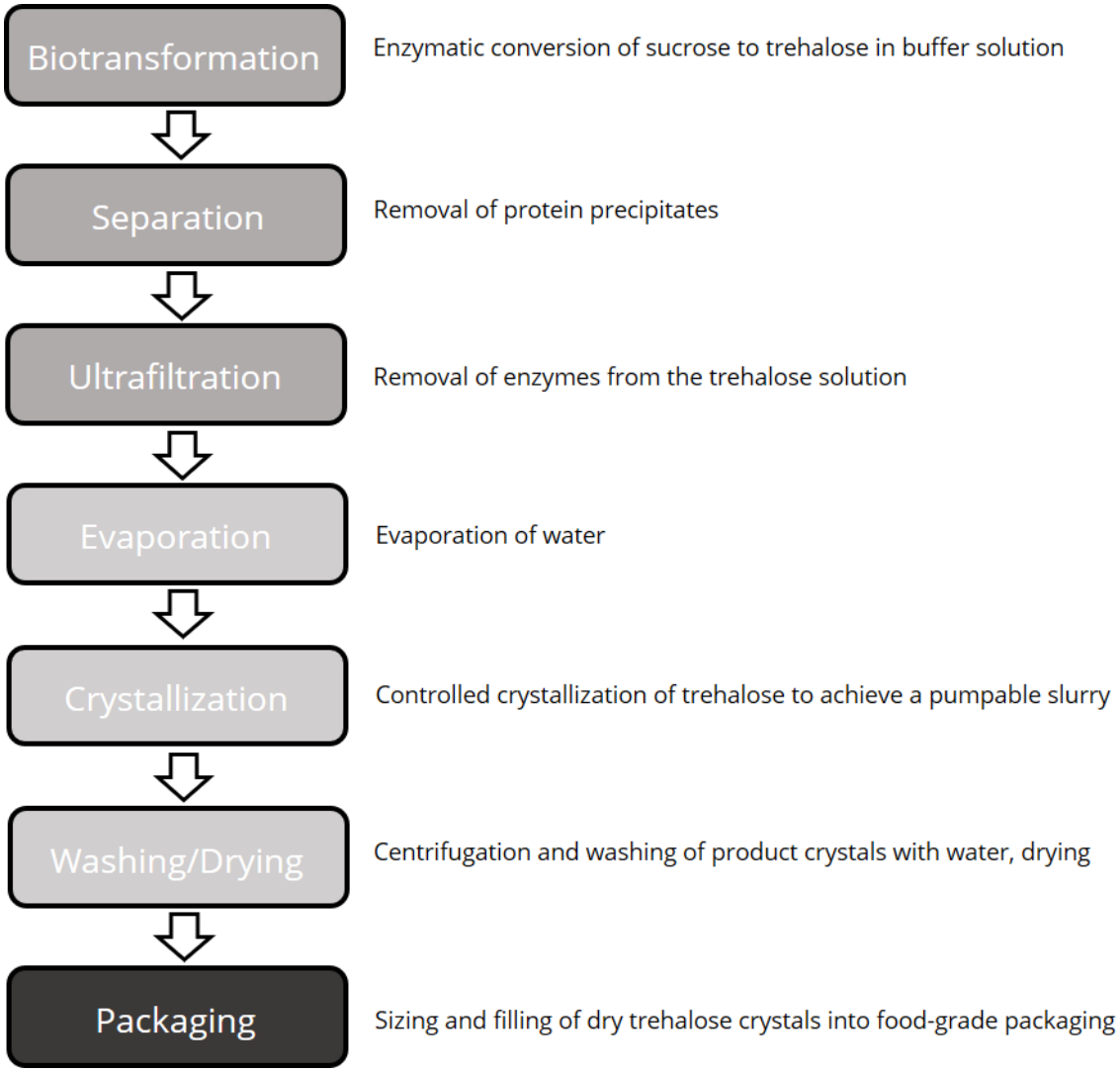

Figure 2.2.3-1 Schematic overview of the manufacturing process for c-LEcta Trehalose

2.3 Product Specification and Batch Analysis

2.3.1 Description and Identification

Trehalose occurs as a non-hygroscopic, white, crystalline powder. It is a stable, non-reducing disaccharide with two glucose molecules linked in an α,α-1,1 configuration. The powder is freely soluble or readily dispersible in water.

The identity of c-LEcta trehalose produced by enzymatic conversion from sucrose was confirmed by infrared absorption according to the current FCC method, by comparison with an USP Reference Standard (FCC, 2018b).

2.3.2 Physical and Chemical Specifications

The product specifications for trehalose produced by enzymatic conversion from sucrose are presented in Table 2.3.2-1.

Table 2.3.2-1 Physical and chemical specifications for c-LEcta Trehalose

Parameter	c-LEcta Trehalose	Current FCC specifications (FCC, 2018b)	Current JECFA specifications (JECFA, 2000)	Method of analysis
Appearance	White crystalline powder	White or almost white crystals	White or almost white crystals	Sensory Evaluation
Assay (anhydrous basis)	≥98%	≥98%	≥98%	HPLC (JECFA, 2000)
Loss on drying	≤1.5%	n.s.	≤1.5% (60°C, 5h)	JECFA (2006)
Turbidity	<0.05%	≤0.05%	≤0.05%	FCC (2018b)
Color in solution	<0.1%	≤0.1%	≤0.02%	FCC (2018b)
pH (30% solution)	4.5-6.5	4.5-6.5	n.s.	FCC (2018b)
Water	≤11.0 %	≤11.0 %	n.s.	
Total ash	<0.05%	≤0.05%	≤0.05%	FCC (2018b) (sulphated ash)
Lead (as Pb)	<0.1 ppm	≤0.1 ppm	≤1.0 ppm	FCC (2018b)
Arsenic (as As)	<1.0 ppm	n.s.	n.s.	AAS/ICP-AES/ICP/MS

AAS = Atomic absorption spectroscopy; FCC = Food Chemicals Codex; HPLC = High Performance Liquid Chromatography; ICP-AES = Inductively coupled plasma atomic emission spectroscopy; MS = mass spectrometry; n.s. = not specified.

2.3.3 Microbiological Specifications

The microbiological specifications for trehalose produced by enzymatic conversion from sucrose are presented in Table 2.3.3-1.

Table 2.3.3-1 Microbiological specifications for c-LEcta Trehalose

Specification Parameter	Specification	Method of Analysis
Salmonella	Absent in 25 g	DIN EN ISO 6579
Total aerobic count	≤ 300 cfu/g	DIN EN ISO 4833-2
Coliforms	Absent in 10 g	ISO 4832
Yeast and mold	≤100 cfu/g	ISO 21527-2
cfu = colony forming units		

2.3.4 Batch-to-batch variation

2.3.4.1 Physical and Chemical Analysis

The batch to batch variation of three commercial batches of trehalose is presented in Table 2.3.4-1. Data demonstrate the consistency of the manufacturing process and compliance with the physical and chemical specifications.

Table 2.3.4-1 Physical and chemical analysis of 3 lots of c-LEcta Trehalose

Specification Parameter	Limit	Manufacturing Lot		
		NW-U4D1	NW-U8D1-1	NW-U10D1-1
Appearance	White crystalline powder	conforms	conforms	conforms
Assay (anhydrous basis)	≥98%	99.6%	99.9%	99.1%
Loss on drying	≤1.5%	0,04	0,06	0,05
Turbidity	<0.05%	0.001	0.009	0.009
Color in solution	<0.1%	0.034	0.058	0.068
pH (30% solution)	4.5-6.5	5.8	6.4	6.4
Water	≤11.0 %	9.4%	9.46%	9. 28%
Total ash	<0.05%	0.007%	0.04%	0.01%
Lead (as Pb)	<0.1 ppm	<0.02 ppm	<0.02 ppm	<0.02 ppm
Arsenic (as As)	<1.0 ppm	<0.05 ppm	<0.05 ppm	<0.05 ppm
ppm= parts per million				

2.3.4.2 Microbiological Analysis

Data from the analysis of 3 lots of c-LEcta trehalose demonstrating the consistency of the manufacturing process and compliance with the microbiological specifications are presented in Table 2.3.4-2.

Table 2.3.4-2 Microbiological product analysis for 3 lots of c-LEcta Trehalose

Specification Parameter	Limit	Manufacturing Lot		
		NW-U4D1	NW-U8D1-1	NW-U10D1-1
Salmonella	Absent in 25 g	complies	complies	Complies
Total aerobic count	≤ 300 cfu/g	<100	<100	<100
Coliforms	Absent in 10 g	complies	complies	Complies
Yeast and mold	≤100 cfu/g	<10	<10	<10
cfu = colony forming units				

c-LEcta trehalose produced by enzymatic conversion from sucrose has a purity of ≥ 98%. On HPLC analyses according to the specified method, the only other detected saccharides were glucose, fructose and sucrose. As an example, in a representative lot with a purity of 99.6% (as determined by HPLC), these three by-products were detected at levels of 0.07, 0.18 and 0.12%, respectively. According to the data provided by Hayashibara (GRN 000045), the existing commercial product may contain the by-products glucose, glucosyltrehalose and α-D-isomaltosyl-α-D-glucoside in typical levels of 0.5, 0.3 and 0.1%, respectively (GRN 000045).

2.3.5 Residual Protein and DNA

To confirm the success of the purification techniques as well as the absence of protein in c-LEcta trehalose, three different product batches were analyzed. Using the Bradford method (Bradford, 1976), no protein was detected in a 50% trehalose solution down to a detection limit of 1.2 µg/ml (according to the instructions of the manufacturer; related to a BSA standard), corresponding to less than 2.5 ppm.

Moreover, a specific PCR-based test for the detection of recombinant DNA in the trehalose product was developed with primers designed to amplify the genes corresponding to the three enzymes used in the manufacturing process. The analysis of three different product batches showed the absence of residual recombinant DNA within a detection limit of 1 ng/g trehalose.

2.4 Stability Data

The stability of trehalose has been analyzed in several published studies (Higashiyama, 2002; Wolfenden, 2008). Trehalose is very resistant to acid hydrolysis and also remarkably heat-resistant, with hydrolysis rates much lower than those of sucrose (Ohtake and Wang, 2011). As a non-reducing sugar, trehalose does not undergo Maillard reactions (browning). In addition, GRN 000045 evaluated the stability of trehalose under various conditions mimicking its use in foods. Trehalose was stable in solution within a broad range of pH from 3.5 to 10.0. Furthermore, the color value of a 10% solution of trehalose was not affected by heating to 120°C. This was also true for trehalose solutions containing either 1% glycine or 5% polypeptone as a protein source.

2.4.1 Storage Stability

The storage stability of c-LEcta trehalose produced by enzymatic conversion from sucrose (Lot NW-U4D1) was assessed. c-LEcta trehalose was stored in polyethylene bags at 22± 5°C under dry conditions and without direct sunlight. After 92 weeks, samples were analyzed and the following parameters determined: trehalose content ("Assay"), pH, turbidity, color in solution, loss on drying, water content, and microbiological parameters. As reported in Table 2.4.1-1, after 92 weeks of storage c-LEcta trehalose produced by enzymatic conversion from sucrose was stable in all the examined parameters.

Table 2.4.1-1 Specifications of trehalose stored for 92 weeks

		Week	
Specification Parameter	**Limit**	**0**	**92**
Appearance	White crystalline powder	conforms	conforms
Assay (anhydrous basis)	≥98%	99.6	99.7
Loss on drying	≤1.5%	0.04	0.01
Turbidity	<0.05%	0.001	0.0003
Color in solution	<0.1%	0.034	0.06
pH (30% solution)	4.5-6.5	5.84	6.03
Water	≤11.0 %	9.4	9.75
Total viable count, cfu/g	<300	<100	<10
Salmonella	Absent in 25 g	Complies	complies
Yeasts and molds	<100 cfu/g	<10	<10
Coliforms	Absent in 10 g	Complies	complies

Part 3 §170.235 Dietary Exposure

3.1 Intended Use of Trehalose and Levels of Use in Foods

Trehalose has been determined to be GRAS as a multipurpose ingredient for use in food in general in accordance with current good manufacturing practice (GRN 000045; US FDA, 2000). Applications for trehalose include its use as a coloring adjuvant, flavor enhancer, humectant, nutritive sweetener, stabilizer, thickener, synergist, and texturizer in a broad range of food categories, including bakery products, frozen desserts, dairy-based foods and toppings, hard and soft confectionery, processed fruits and vegetables, and beverages, among others. Trehalose may be used as a binding and purge control agent up to a level of 2% in various meats and poultry products (GRN 000045) and as a flavoring agent in non-standardized ready-to-eat meat and poultry products (USDA FSIS 7120.1, 2018). The uses and use levels of c-LEcta trehalose produced by enzymatic conversion from sucrose are likely to reflect those currently found for trehalose in the U.S. Trehalose has also been proposed for use in sport rehydration beverages or low-caloric products as it has been shown to lower glucose and insulin response in comparison with glucose (Arai et al., 2013; van Can et al., 2009; Maki et al., 2009; Yoshizane et al., 2017).

Due to its lower sweetness, trehalose may be used to replace some or all of the sucrose in products where it is desirable to reduce the sweetness intensity for a more balanced flavor profile.

Trehalose has also been researched for its role as an autophagy modulator in the control of neurodegenerative diseases (Hosseinpour-Moghaddam et al., 2018) and suppressor of fatty acid oxidation (Higashiyama, 2002). Thus, trehalose may additionally be found in manufactured medications, food supplements and cosmetics (Ohtake and Wang, 2011; Fabbrocini et al., 2017).

3.2 Estimated Dietary Consumption of Trehalose based upon Intended Food Uses

3.2.1 History of Consumption

Trehalose is a disaccharide (sugar) consisting of two molecules of glucose. Trehalose can be found in bacteria, yeast, fungi and algae (Kemp and Lindley, 2007). It has been a regular component of the human's diet for centuries since this naturally occurring sugar is present in mushrooms, sea algae, honey, fermented drinks, bread and sunflower seeds (among others). Despite not being widely present in vegetables, it is commonly found in insects´ exoskeleton, and it can be synthesized by bacteria present in the human digestive tract. In countries where insects, fungi and algae are commonly consumed as part of the locals' diet, there is a higher exposure to trehalose than in those where these foods are not regularly consumed (Richards and Dexter, 2012).

Trehalose has been used for a long time in Japan before being introduced to U.S. in 2000 (GRN 000045, US FDA, 2000). In the 90's, when Hayashibara (GRN 000045) developed an enzymatic production system which sharply decreased the cost of manufacturing trehalose, this sugar started to be used widely in the Japanese food market. Despite its extended use in the Japanese market, no limits or restrictions have been set for trehalose. Good Manufacturing Practices are commonly used as guidelines to limit the use of this sugar in the wide range of products where it is intended to be used.

Trehalose is mainly used as a flavoring and a preservative (or binder) agent. Even though being considered a sweetener, its sweetness index is approximately half as that of sugar, although they both possess a similar glycemic index. As a binder, it helps food maintain its texture and prevents water content loss, especially in foods that undergo treatments

that tend to dry the product (e.g. freezing/thawing). This function is also related to its use as a flavoring agent since it bonds with volatile aroma compounds that are partially lost during food processing (Richards and Dexter, 2012).

3.2.2 Estimated Consumption of Trehalose from Proposed Food Uses

To estimate total intake of trehalose from different dietary sources in the general population, the maximum intake of added trehalose in different food categories, as well as the mean daily intake of food categories and naturally occurring levels of trehalose in foods were considered.

First, for estimating the use of trehalose in different foodstuffs, food categories from Title 21 CFR § 170.3 were considered. The Code of Federal Regulations (CFR) and the U.S. Department of Agriculture's Food Safety and Inspection Service (FSIS) have only established a maximum permitted value of 2% for trehalose in meat and poultry products when acting as a binder, purge-control and flavoring agent (USDA FSIS 7120.1, 2018). Therefore, those were considered the maximum expected inclusion rates in meat and poultry products. For the remaining food categories, FEMA's Average Maximum Use Levels values for trehalose with intended use as flavor were used as the maximum inclusion rate of trehalose (Smith et al., 2009).

Table 3.2.2-1 Average usual use & maximum inclusion rate of trehalose in different food categories

Food categories	Average usual use levels (mg/kg)	Maximum inclusion rate (as flavoring) (mg/kg)
Baked goods and baking mixes	20,000	50,000
Alcoholic beverages	1,000	10,000
Nonalcoholic beverages	20,000	35,000
Breakfast cereals	15,000	30,000
Cheeses	3,000	50,000
Chewing gum	20,000	35,000
Coffee and tea	10,000	30,000
Condiments and relishes	20,000	50,000
Confections and frostings	20,000	50,000
Dairy products analogs	20,000	50,000
Egg products (without shell)	15,000	40,000
Fats and oils (only fats)	15,000	40,000
Fish products	15,000	50,000
Frozen dairy desserts and mixes	15,000	50,000
Fruit and water ices	10,000	50,000
Gelatins, puddings and fillings	50,000	50,000
Grain products and pastas		-
Gravies and sauces	20,00	50,000
Hard candy and cough drops	50,000	50,000
Herbs, seeds, spices, seasonings, blends, extracts and flavorings	20,000	50,000
Jams and jellies (commercial)	50,000	50,000
Meat products	15,000	20,000
Milk (whole and skim)	-	-
Milk products	20,000	50,000
Nuts and nut products	20,000	50,000
Plant protein products	-	-
Poultry products	15,000	20,000

Table 3.2.2-1 Average usual use & maximum inclusion rate of trehalose in different food categories

Food categories	Average usual use levels (mg/kg)	Maximum inclusion rate (as flavoring) (mg/kg)
Processed fruits and fruit juices	10,000	30,000
Processed vegetables and vegetable juices	35,000	50,000
Snack foods	20,000	50,000
Soft candy	50,000	50,000
Soups	20,000	50,000
Sugar	-	-
Sugar substitutes	-	-
Sweet sauces	50,000	50,000

NOTE: where there is no value and instead there is a hyphen, it represents that trehalose is not permitted to be added to those food groups.

The estimated daily intakes of trehalose from the expected intended uses in the U.S. are represented in Table 3.2.2-1. Given the large number of food categories where trehalose is intended to be used, the vast majority of the population will be considered as potential consumers. Most people tolerate trehalose without any adverse effects.

Next, food patterns were obtained from: Food Patterns Equivalents Intakes by Americans NHANES 2003-2004 (Bowman et al., 2017); Food Intakes Converted to Retail Commodities 2007-2008 (Bowman et al., 2013); CDC's Morbidity and Mortality Weekly Report (Kumar et al., 2014); Beverage caffeine intakes in the U.S. (Mitchell et al., 2014), Alcoholic Beverage Consumption by Adults 21 Years and Over in the United States: NHANES 2003-2006 (Guenther et al., 2010); Food Commodity Intake Database (WWEIA-FCID 2005-2010) and Candy consumption patterns (Duyff et al., 2015). Given the current publicly available food pattern data, it is not possible to provide a more accurate daily intake of the different food categories included in Table 3.2.2-2. (e.g. processed vegetables, processed fruit). The estimated daily intake of trehalose added to food has been calculated by applying the maximum usage levels reported by the FEMA (Smith et al., 2009) and the estimated daily food category intakes.

The daily intake of certain categories had to be adjusted. The food pattern intake data that was identified above did not provide information on the amount of processed food consumed. The intake values for broad food categories such as fish were not given separately for raw and processed food products (i.e whole fish and processed fish finger products). Consequently, applying the maximum inclusion rate to unprocessed foods, such as fish, fruit and vegetables, would result in overestimating trehalose daily intake. U.S. food trends point out that processed food consumption has sharply increased in the past decades, accounting now for 60% of the total daily calories (Baraldi et al., 2018). Therefore, for added accuracy, 60% of the estimated daily intake for fish, meat, fruit & fruit juices, nut & nut products and vegetables was considered the more appropriate amount when calculating trehalose daily intake for those food categories. Lastly, to assess trehalose ingestion from natural sources, mushrooms (17% w/w) and honey (1.7% w/w) have been included in Table 3.2.2-2 as the two main products contributing to its exposure. Thus, the maximum trehalose concentration in those foods was considered when calculating the trehalose intake from honey and mushrooms.

Trehalose daily intake/food category =[(Adjusted) daily intake food category x (Maximum inclusion rate) or (Maximum trehalose concentration)]

The formula below describes calculation of the estimated daily trehalose consumption from all food sources.

Trehalose average daily intake = Daily intake of trehalose added to food + daily intake of trehalose naturally present in food

Table 3.2.2-2 Summary table of the estimated daily consumption of food and trehalose from all intended uses

Food categories	Estimated daily intake (gram/person/day)[1]	Trehalose daily intake (gram/person/day)[1]
Nonalcoholic beverages[2]	20	0.7
Alcoholic beverages[3]	50	0.5
Coffee[4]	190.8	5.7
Tea[4]	53	1.59
Cheeses	34	1.7
Refined grains[5]	124	6.2
Dairy products	22	1.1
Egg products (without shell)	25	1
Fats and oils (only fats)	31	1.24
Fish	16	0.48
Meat	89	1.068
Fruit & fruit juices	305	5.49
Poultry	65	1.3
Nuts and nut products	7	0.21
Vegetables	341	10.23
Mushrooms (natural source)[6]	10.96	3.16
Honey (natural source)[6]	1.41	0.2
Candies (with & without chocolate)[7]	10	0.5
	Average daily intake:	**42.37**

[1]Mean values for males and females; 2 years-old and over; [2]Kumar et al., 2014; [3]Guenther et al., 2010; [4]Mitchell et al., 2014 ; [5]USDA Food Patterns, 2015; [6]WWEIA-FCID 2005 – 2010; [7]Duyff et al., 2015

The average estimate of trehalose daily intake for consumers of all ages is 42.37 grams per person per day, and the 90th percentile intake would be approximately 84.74 grams per person per day (twice the average daily intake). Our estimate of the average total trehalose intake is close to Hayashibara's estimate of total intake made almost 2 decades ago (34.43 grams, GRN 00045), although more accurate as we have used current maximum inclusion rates for trehalose in different food categories.

It is to be noted that trehalose is also found in active dry yeast; the amount of trehalose in *Saccharomyces cerevisiae* can constitute up to 23% or more of the cells dry weight (Bolat, 2008). Thus, brewer's and baker's yeast consumed as food complements could be significant sources of trehalose in a subpopulation that regularly consumes those. Fermented beverages and bread could also contain traces of trehalose, however the data currently available do not allow to estimate the daily intake of such products, which constitutes a limitation of our total trehalose intake estimate.

Finally, given the scientific data supporting the safety of trehalose, U.S. FDA and JEFCA have not determined any ADI for this compound. Safety data exposed in this GRAS Notice suggests that trehalose will not cause adverse effects up to 5 g/kg/day. Considering that the average adult weight is around 60 kg, our estimated trehalose consumption would be 0.7 g/kg/day, with a 90th percentile of approximately 1.41 g/kg/day. Therefore, it is reasonable to assume that the average daily intake will not represent a health concern for the U.S. population. Moreover, this value is still far below trehalose daily intake in Japan (GRN 000045, US FDA, 2000), where the use of trehalose is more widely extended than in the U.S., especially considering that natural sources of trehalose are consumed as part of the daily diet. Also, no contaminants or degradation products are expected from its intended use.

Part 4 §170.240 Self-Limiting Levels of Use

The use of trehalose is largely limited by the amount that can technically be added to a particular food or beverage product without negatively affecting its quality and consumer acceptability. Therefore, the use of trehalose as a multipurpose ingredient in foods in general is self-limiting based on its organoleptic properties. FEMA's average maximum use for trehalose in different food categories (Smith et al., 2009) serves as a guide for levels of use which should not be exceeded, and which are generally recognized as safe. Levels are incorporated and shown in Table 3.2.2-1.

Part 5 §170.245 Experience Based on Common Use in Food Before 1958

Not applicable as trehalose is not an ingredient commonly used in food prior to January 1, 1958 and thus general recognition of safety for this GRAS notice is based upon scientific procedures in accordance with Title 21, Section 170.30 (a) and (b) of CFR.

Part 6 §170.250 Narrative and Safety Information

Over the last few decades, the safety of trehalose has been considered by several scientific bodies and regulatory agencies, including the U.S. FDA, JECFA, the UK Advisory Committee on Novel Foods and Processes (ACNFP), FSANZ, and Health Canada. A large safety database exists which includes a thorough examination of the metabolism and pharmacokinetics of trehalose in experimental animals and humans, acute toxicity studies, short- and long-term toxicity and carcinogenicity studies, reproductive and developmental toxicology studies, *in vitro* and *in vivo* mutagenicity/genotoxicity studies, and human studies.

The safety of trehalose can be established based on the conclusions of the safety reviews conducted by numerous scientific bodies and regulatory agencies, as well as the publicly available scientific literature related to the safety of trehalose. The following sections provide a detailed summary of: i) the metabolic fate of trehalose; ii) the data considered by the scientific bodies and regulatory agencies (i.e. JECFA, EFSA[2], FSANZ, Health Canada) to conclude on the safety of trehalose; and iii) the studies available in the scientific literature published since the U.S. FDA review of the related GRAS notice GRN 000045 for trehalose produced from liquefied starch by a multistep enzymatic process.

6.1 Absorption, Distribution, Metabolism, and Elimination of Trehalose

Apart from the fact that the specific enzyme trehalase is required for its digestion, it appears that ingestion, hydrolysis, absorption and metabolism of trehalose is identical to all other digestible disaccharides (Richards et al., 2002). The enzyme trehalase is present in humans and most animals and is found at the brush border of the intestinal mucosa, as well as in the kidney, liver and blood plasma (van Handel, 1970; Demelier et al., 1975).

A small minority of the population has trehalase deficiency, which may be hereditary or acquired. The incidence of trehalase deficiency is in general much lower than that of lactase deficiency (Murray et al., 2000). When trehalose is ingested by individuals with trehalase deficiency, it is either incompletely digested or undigested, and a small fraction (approximately 0.5%) may be absorbed by passive diffusion, as shown for other disaccharides (van Elburg et al., 1995). The absorbed trehalose may then be metabolized to glucose in the liver or kidney or be excreted unchanged in the urine (Demelier et al., 1975). Unabsorbed trehalose is likely to be fermented by the intestinal microflora to short-chain fatty acids such as acetate, propionate, and butyrate. After ingestion of excessive amounts of trehalose, individuals with trehalase deficiency may experience intestinal discomfort; however, smaller amounts of trehalose are tolerated without any symptoms (ACNFP, 2000).

Trehalose entering the circulating blood is converted to glucose by trehalase in serum, kidney, liver, and bile, depending on the species (van Handel, 1970; Arola et al., 1999). In mice, rabbits, dogs, pigs, and humans, trehalase in the brush border of the proximal tubular cells of the kidney is expected to cleave the excreted trehalose to glucose (Demelier et al., 1975).

[2] European Food Safety Authority

6.2 Summary of Safety Opinions on Trehalose by Scientific and Regulatory Authorities

6.2.1 United States

In the U.S., GRAS notice 000045 received no questions from the U.S. FDA regarding the GRAS status of Hayashibara trehalose (U.S. FDA, 2000). Similar to c-LEcta trehalose produced by enzymatic conversion from sucrose, the trehalose in GRN 000045 is produced from liquefied starch by a multistep enzymatic process.

6.2.2 The Joint FAO/WHO Expert Committee on Food Additives (JECFA)

The safety of trehalose was reviewed by JECFA at its 55th meeting in 2000. The Committee reviewed the available information regarding the estimated daily intake of trehalose, as well as its metabolic fate in humans. Furthermore, the data from toxicological studies in mice, dogs, rats, and humans were considered. On the basis of the available information, the Committee established an ADI "not specified" for trehalose (JECFA, 2000). At the same meeting, specifications for trehalose were prepared (FAO & WHO, 2001a).

6.2.3 UK Advisory Committee on Novel Foods and Processes (ACNFP)

Shortly after JECFA's 55th meeting, ACNFP conducted an evaluation of the safety of trehalose in the context of an application submitted by Hayashibara for its approval as a novel food ingredient under the European Novel Food Regulation (Commission Decision 2001/721/EC). In its assessment, ACNFP considered data provided in the application dossier regarding the estimated daily intake of trehalose, the previous human exposure to the food ingredient and its metabolism in animals and humans. In addition, a toxicological assessment was carried out on the basis of toxicological studies carried out *in vitro* in bacteria and in cultured mammalian cells and *in vivo* in mice, rats, and rabbits. Finally, human studies investigating trehalose tolerance after oral ingestion were considered. The Committee concluded that trehalose complying with the specification agreed at the 55th JECFA meeting was safe to be used in the range of foodstuffs detailed in the application dossier (ACNFP, 2000).

6.2.4 Food Standards Australia/New Zealand (FSANZ)

FSANZ conducted a safety assessment of trehalose in the context of a Novel Food application by Hayashibara (FSANZ, 2003). FSANZ reviewed the data used by JECFA to assess the safety of trehalose in humans and also considered new data submitted by Hayashibara on tolerance levels for trehalose in humans. FSANZ concluded that trehalose at doses up to 33 g from a single exposure in food would not be expected to cause gastro-intestinal symptoms in the most sensitive individuals (Asian populations) as identified in the scientific literature, while for western populations a threshold level of up to 50 g would apply. Taking into account the conservative assumptions used in the dietary exposure calculations, FSANZ concluded that the possibility to exceed these levels of 33-50 g over a 24-hour period was very unlikely. In summary, FSANZ concluded that minimal public health and safety concerns exist if trehalose is used according to Good Manufacturing Practice (GMP).

6.2.5 Health Canada

In the context of a Novel Food application by Hayashibara in 2005, Health Canada conducted an assessment of the safety of trehalose (Health Canada, 2005). Health Canada corroborated the conclusions of JECFA, ACNFP and FSANZ and determined that no safety or nutritional concerns are associated with the ingestion of trehalose.

6.3 New Data Related to the Safety of Trehalose

The safety of trehalose was evaluated in GRN 000045 for trehalose produced from liquefied starch by a multistep enzymatic process. GRN 000045 included a search of the scientific literature to capture relevant publications. The safety information presented in GRN 000045 is incorporated by reference. To identify new publications related to the safety of trehalose since the U.S. FDA review in 2000 of GRN 000045, a comprehensive search of the scientific literature was conducted in Pubmed (November, 2018). The search was restricted to articles published in English and spanning the years 2000-2018 (November). New studies in relation to the tolerance and toxicological effects of trehalose are summarized below. In conclusion, the results of these recent studies provide further support for the safety of trehalose.

6.3.1 Toxicity

No adverse effects have been reported since Hayashibara first registered trehalose in 2000 (GRN 000045). Toxicological studies conducted posterior to its registration in the U.S. demonstrate that there was no toxicity associated with trehalose consumption up to 5 g/kg/day (Liu et al., 2013). These results are aligned with those evidenced by Hayashibara (GRN 000045).

Richards and colleagues (2002) summarized the results of 10 animal toxicological safety studies using trehalose that were presented in Hayashibara's GRAS Notice 000045. The results demonstrated no mutagenicity or genotoxicity in standardized Ames or clastogenic assays, even at the highest concentrations tested (5,000 μg/plate). High concentrations of trehalose were fed to mice, rats and rabbits in four separate toxicity, embryotoxicity/teratogenicity, and reproductive studies. No adverse effects were observed in any of the studies. Therefore, the no-observed adverse effect level (NOAEL) is based on the highest concentrations consumed (g trehalose/kg body weight/day). For the 90-day subchronic study, the NOAEL was calculated at 50,000 ppm (5.0% of the diet), as 9.3 g/kg bw/day for females and 7.3 g/kg bw/day for males. In the rat and rabbit embryotoxicity/teratogenicity studies the NOAEL for rats was 6.94 g/kg bw/day, and the level for rabbits was 1.99 g/kg bw/day. In the two-generation study in rats the NOAEL for males during the premating period was 7.09 g/kg bw/day. The NOAEL for females fed trehalose during the pre-mating, gestation and lactation periods was 7.61, 6.16, and 14.09 g/kg bw/day, respectively. The data from the latter three studies demonstrated that trehalose had no toxic effects on maternal variables, the reproduction of the F0 and F1 parents, or on the development of the pups of either generation. Taken together, these data demonstrate that trehalose presents no risk of toxicity to humans. The maximum concentrations tested, 10% of the dietary intake (rat and rabbit embryotoxicity/teratogenicity, and rat two-generation reproduction study), suggest that the use of trehalose as a macro-ingredient in food is safe. The authors concluded that on the basis of these toxicity studies, together with human studies in which doses of trehalose were administered to various populations, and consumption of trehalose in commercial products in Japan, trehalose is safe for use as an ingredient in consumer products when used in accordance with current Good Manufacturing Practices.

In a study by Eroglu et al. (2003), mouse oocytes and zygotes were directly injected with trehalose and implanted to foster mothers to evaluate the effects of trehalose and the method of delivery on their development. Microinjection of trehalose up to 0.15 M resulted in development to blastocyst stage similar to controls (85 and 87%, respectively) while the blastocyst rate was significantly decreased (43%) in the presence of 0.20 M intracellular trehalose. When transferred to foster mothers, trehalose-injected zygotes (0.1 M) implanted and developed to day 16 fetuses similar to controls, and healthy pups were born. No toxicity or development issues were observed in the new-born pups hence implying that trehalose does not bear teratogenicity.

In their study, Liu et al., (2013) performed multiple toxicity studies of trehalose in mice by intragastric administration. Aberration, body weight, food consumption, haematology, organ coefficients, and both gross and microscopic appearance of histiocytes were compared between the test and control groups. A sperm abnormality test, bone marrow

cell micronucleus test, and a haematology study were conducted at levels of 1.25 g/kg, 2.5 g/kg, and 5 g/kg of trehalose. In both the sperm abnormality test and bone marrow cell micronucleus test, statistically significant differences were observed between the positive control and treatment groups ($P < 0.05$), while no statistical difference was observed among the negative control, high dose, moderate dose and low dose groups ($P > 0.05$). In the haematology study, there was no significant difference found from the controls at $P > 0.05$. The results obtained in the present study support the conclusion that consumption of trehalose has no adverse effects for humans.

In 2018, Collins and colleagues published an article linking the consumption of trehalose with a *Clostridium difficile* epidemic in the U.S. (2000-2003) (Collins et al., 2018). However, researchers have failed to establish a correlation between trehalose consumption and the outbreaks (Abbasi, 2018). In addition, the study design was flawed as two treatments were fed antibiotics for two weeks, hence altering the gut microbiota. Thus, those deviations have most likely interfered with the results, as healthy individuals are usually protected from *C. difficile* infection by the normal flora of the gut, whereas disruption of the normal microflora by antibiotics allows *C. difficile* to proliferate, produce toxins and eventually cause disease (Martinez et al., 2012). Moreover, *C. difficile* outbreaks were recorded before trehalose was introduced to the U.S. market which suggests that those were not induced by its consumption.

6.3.2 Tolerance

The GRAS Notice by Hayashibara (GRN 000045) presents information on the tolerance of trehalose and trehalase deficiency in different populations, as well as on the potential correlation of mushroom intolerance with trehalose intolerance and trehalase activity in people with metabolic or digestive disorders. Richards and Dexter (2012) reported that approximately 30,000 metric tons of trehalose added to food products were consumed in Japan during 2010 without any reports of intolerance. A recent literature search, including publications covering the period 2000-2018, has revealed two articles relevant to tolerance of trehalose, one of which was already presented in GRN 000045. The studies are summarized below.

The main findings of the Murray et al. (2000) study were presented in the GRAS Notification by Hayashibara (GRN 000045). In his study, 400 patients were investigated for suspected trehalose malabsorption. Endoscopic distal duodenal biopsies were taken for histological assessment and maltase, sucrase, lactase and trehalase estimation. Disaccharidase activities were determined by Dahlqvist's technique (Dahlqvist, 1968). The study e found that the normal range of trehalose activity (mean ± 2 SD) was 4.79-37.12 U/g protein. One patient had an isolated borderline trehalase deficiency and 31 patients with villous atrophy had significantly reduced disaccharidase activities. It was observed that with ingestion of a gluten-free diet, maltase, sucrase and trehalase activities recovered to normal in most patients, whereas lactase activity did not. Murray concluded that the normal range and very low incidence of isolated enzyme deficiency in the UK population was comparable with that described in populations from the USA and mainland Europe and that there should be no concern over the introduction of trehalose-containing dried foods.

The study of Buts et al. (2008) aimed to measure trehalase activity in 200 Belgian patients with lactase deficiency and symptomatic intolerance. 144 adults (mean age 43 ± 17 years) and 56 children (mean age 5 ± 2 years) participated, and findings reveal that 9 % of patients had total trehalase deficiency (0-2 g/protein) and 19.5 % had partial trehalase deficiency (2-12 U/g protein). Altogether, 28 % (30 adults and 27 children) had total or partial trehalase deficiency. The results of this study are in agreement with Reif- Breitwieser´s study (2018) in which 30 % of Austrian patients with carbohydrate malabsorption symptoms (32 ± 15.5 years) had trehalose malabsorption and/or intolerance.

In her doctoral thesis, Reif-Breitwieser (2018) investigated the prevalence of trehalose malabsorption in 30 Austrian patients (32 ± 15.2 years) from the outpatient clinic of Gastroenterology and Hepatology of the Medical University of Graz that had symptoms suggestive of carbohydrate malabsorption. After administration of 50 g of trehalose, breath samples were measured using a Gastrolyzer for analysis of hydrogen concentration. Symptoms of abdominal discomfort

were also recorded. As a result, 1/3 of patients were diagnosed with trehalose malabsorption (H2 ≥ 20 ppm over basal hydrogen levels) and further 1/3 were diagnosed with trehalose intolerance (≥ 2 symptoms).

Trehalase deficiency is very rare in general, only a small minority of Greenland natives (reported at 8% of the population, Gudmand-Hoyer et al., 1988), Finns (Arola et al., 1999), Japanese (Oku and Nakamura, 2000) and presumably other populations (Bergoz et al., 1982; Buts et al., 2008; Reif-Breitwieser, 2018) have lower or absent intestinal trehalase activity and cannot tolerate modest amounts of mushrooms (Kohlmeier, 2013). The reason for this low activity is most likely due to genetical variation. Since there is no cure for trehalase deficiency, individuals bearing the symptoms should reduce or eliminate foods containing trehalose from their diets (Stipanuk and Caudill, 2013).

6.3.3 Other Physiological Effects

Kaplon and colleagues (2016) studied the effects of oral trehalose consumption on vascular function in 32 healthy adults, aged 50-77 years. Adults consumed 100 g/day of trehalose or maltose dissolved in 350 ml water during 12 weeks. The authors concluded that trehalose supplementation improved resistance artery endothelial function, a major risk factor for cardiovascular diseases, a finding consistent with studies done in rodents (Echigo et al., 2012; Sarkar et al., 2014; LaRocca et al., 2012). Reported side effects included minor to moderate gastrointestinal discomfort (n=4) and changes in perceived energy levels (n=2), characteristic of disaccharide consumption in general (Richards et al., 2002).

Yoshizane and colleagues (2017) investigated the glycemic, insulinemic and incretin responses after 25g of trehalose ingestion in 20 healthy Japanese volunteers, aged 23-48 years. Blood glucose and insulin levels were found to be significantly lower following trehalose loading compared to glucose. Diarrhea symptoms were observed in 2 subjects after ingestion of 25 g trehalose dissolved in 100 ml water.

Van Can and colleagues (2009) performed a trehalose loading experiment, in which healthy participants ingested 75 g trehalose dissolved in 400 ml water. Authors did not report any gastrointestinal discomfort or any other clinical symptoms.

Trehalose causes diarrhea when a large amount is ingested at one time and it is not completely digested in the small intestine (Oku and Nakamura, 2000). The transitory laxative threshold of trehalose is 0.65 g/ kg body weight/ingestion. Small amounts of trehalose, coming from the daily average diet, might be completely digested and absorbed in the small intestine. Oku and Nakamura (2000) showed that trehalose, when ingested at a concentration of 30 g in 100 ml of water, did not cause diarrhea, whilst when ingested at doses above 40 g/100 ml water it did cause diarrhea (n=5/20), showing that trehalase activity differs from person to person. Mizote et al., (2016) concluded that no clinical signs were observed in healthy adults (n=32, 32-58 years) that consumed 10 g/day of trehalose for a 12-week period.

Diarrhea symptoms are possibly caused by the concentration of trehalose solution given to subjects but not by total quantity, as it seems from the above studies that trehalose solutions approximately above 25% (dry weight/volume %) cause diarrhea symptoms in about 10% of the population under study, whereas when given in lower concentrations in one sitting, no symptoms are recorded. Trehalose has been proven safe and as seen with other sugars, only a minority of the population has low trehalase activity which is responsible for the maldigestion of trehalose in the small intestine and the observed gastrointestinal discomfort. Restricting dietary intake of large amounts of trehalose in populations with low trehalase activity or in patients with intestinal malabsorption disorders is the usual method of treatment. It is also worth mentioning that gastrointestinal symptoms may be more apparent when trehalose is administered in solution after an overnight fast versus when trehalose is used in a food, as in the case of the later, it will result in a longer period of digestion thus allowing for a more complete hydrolysis.

6.4 Safety of the Enzymes and the Production Microorganisms

The enzyme production strain *E. coli* LE1B109 is a derivative of the parental strain *E. coli* K-12 W3110. The K-12 strain, and in particular the W3110 substrain, has been safely used as a laboratory organism for more than 50 years and is one of the most extensively characterized bacteria (Bachmann, 1972; Jensen, 1993).

E. coli K-12 is not considered a human or animal pathogen and has accordingly been classified as belonging to Risk Group 1 in the NIH Guidelines (NIH, 2016). Moreover, it is often used as a non-pathogenic reference when studying the virulence factors of pathogenic *E. coli* strains (Blanc-Potard et al., 2002; Kaper et al., 2004). *E. coli* K-12 and its derivatives are essentially unable to colonize the mammalian gastrointestinal tract, do not produce toxins that cause illness upon ingestion, including Shiga toxin, and are unable to persist in either water or soil (Bogosian et al., 1996; U.S. EPA, 1997). The parental laboratory strain W3110 does not carry any introduced antimicrobial resistance genes. The complete genomes of *E. coli* K-12 and specifically of the sub-strain W3110 have been sequenced, confirming the absence of toxigenic potential (Blattner et al., 1997; Hayashi et al., 2006).

E. coli K-12 has a long history of safe use in the industrial production of specialty chemicals and human drugs (U.S. EPA, 1997). Furthermore, a food enzyme preparation (chymosin) obtained from a genetically modified *E. coli* K-12 strain was affirmed as GRAS by the U.S. FDA in 1990 (Flamm, 1991; Olempska-Beer et al., 2006) and has been used safely for cheese production worldwide. In the European Union there are currently 3 food enzyme preparations derived from *E. coli* K-12 being assessed by EFSA as part of the requirements for authorization in accordance with Regulation (EC) 1331/2008 of the European Parliament and of the Council (European Commission, 2016). One of them, D-allulose 3-epimerase, has recently been the subject of a GRAS notification, receiving no questions from the U.S. FDA (U.S. FDA, 2016). The other two food enzyme preparations derived from *E. coli* K-12, two different cyclomaltodextrin glucotransferases, have been safely used for years in the production of the novel food ingredients alpha- and gamma-cyclodextrin, authorized by the European Commission in 2008 and 2012, respectively.

6.5 Allergenicity

As discussed in Section 2.3.5, the final product does not contain residual protein, as demonstrated in 3 batches of c-LEcta trehalose produced by enzymatic conversion from sucrose. However, in order to evaluate the possibility that the food enzymes may cross-react with known allergens and induce a reaction in already sensitized individuals, a sequence homology search was conducted according to the approach outlined by the FAO/WHO (FAO/WHO, 2001b) and the Codex Alimentarius (FAO/WHO, 2009) using the AllergenOnline Database version 18B (available at http://www.allergenonline.org; updated March 23, 2018) maintained by the Food Allergy Research and Resource Program of the University of Nebraska (FARRP, 2017). The database contains a comprehensive list with 2089 peer-reviewed allergen sequences for the purpose of evaluating food safety.

The main criterion for the assessment of potential allergenicity according to the recommended procedure is a threshold of 35% sequence identity between the problem protein and a known allergen using a sliding window of 80 amino acids. This analysis was performed using default settings (E value cut-off = 1 and maximum alignments of 20). No hits above this 35% threshold were found for any of the three sequences of interest encoding the food enzymes sucrose phosphorylase, trehalose phosphorylase and glucose isomerase.

Further, full FASTA alignments between the food enzymes and known allergens were performed in order to identify any sequences with a high degree of homology. According to AllergenOnline, expectation value scores (E-scores or E-values) equal or higher than 1 are unlikely to be related in either evolution or structure. On the other hand, E-values less than 0.02 might indicate that the sequences are related in evolutionary terms. However, when assessing the possibility of immunologic or allergic cross-reactivity, matches with E-values larger than 10^{-7} are not likely to be relevant (Hileman et

al., 2002). Moreover, the percent identity of the sequence between aligned proteins should also be considered, since cross-reactivity typically involves more than 50% sequence identity (Aalberse, 2000). Besides, the likelihood of cross-reactivity is also greater for closely related species.

The results of the FASTA alignment using the AllergenOnline tool showed several hits with E-values below $1x10^{-7}$ (ranging between 1.7×10^{-30} and 5.9×10^{-8}), corresponding to matches with known allergens from wheat (*Triticum sativum*) and buckwheat (*Fagopyrum esculentum*). However, in all cases the sequence identity was below 17%. Together with the very low degree of relatedness between the bacterial source organisms of the food enzymes and the allergen sources, these results suggest that the proteins are not likely to be homologous and are very unlikely to be cross-reactive.

6.6 Conclusions

Trehalose intake was estimated at an average of 42.37 g/person/day. No signs of toxicity were recorded at levels of 5 g/kg over short and long periods of intake as evidenced by the studies presented in GRN 000045 and in the updated literature search in this GRAS notice. Current available studies show that trehalose is well tolerated at a 100 g/day dose, however an intake of trehalose of more than 30 g at one sitting can ellicit gastrointestinal discomfort in some populations (low trehalase activity, malabsorption disorders).

Based on the data and information presented herein, c-LEcta concludes that trehalose produced by enzymatic conversion of sucrose, meeting appropriate food-grade specifications and manufactured according to cGMP, is safe for use as a multipurpose food ingredient as presented in Section 1.3. c-LEcta has further concluded that sufficient data and information relevant to the safety of trehalose are publicly available. Therefore, and for the intended uses detailed in Part 3.1, trehalose produced by enzymatic conversion of sucrose can be concluded to be GRAS on the basis of scientific procedures.

Part 7. §170.255 List of Supporting Data and Information

A. List of acronyms

AAS	Atomic absorption spectroscopy
ACNFP	Advisory Committee on Novel Foods and Processes
ADI	Acceptable Daily Intake
ADME	absorption, distribution, metabolism, excretion
AOAC	AOAC International Association of Analytical Communities
BSA	Bovine Serum Albumin
bw	body weight
CDC	Center for Disease Control and Prevention
CFR	the Code of Federal Regulations
CFU/cfu	colony forming unit
cGLP	current good laboratory practice(s)
cGMP	current good manufacturing practice(s)
EFSA	European Food Safety Authority
EP	European Pharmacopoeia
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FCC	Food Chemical Codex
FCID	Food Commodity Intake Database
FDA	Food and Drug Administration
FD&C	Federal Food, Drug, and Cosmetic Act
FEMA	Federal Emergency Management Agency
FOIA	Freedom of Information Act
FSANZ	Food Standards Australia New Zealand
FSIS	The Food Safety and Inspection Service
g	gram(s)
GP	glucose phosphorylase
GmbH	Limited Liability Company in Germany
GRAS	Generally Recognized as Safe
GRN	GRAS Notifice
HACCP	Hazard Analysis and Critical Control Points
HPLC	High-Performance Liquid Chromatography
ICP	Inductively coupled plasma
ICP-AES	Inductively coupled plasma atomic emission spectroscopy
ISO	International Organization for Standardization
JECFA	Joint FAO/WHO Expert Committee on Food Additives
kDa	kilodaltons
kg	kilogram

M	Molar
µg	Micro gram
ml	mililiter
MS	Mass spectrometry
NA	Not applicable
ng	Nano gram
NHANES	National Health and Nutrition Examination Survey
PCR	Polymerase chain reaction
ppm	parts per million
SD	standard deviation
TP	trehalose phosphorylase
SP	sucrose phosphorylase
U	Units
US/ U.S.	United States
U.S.C	United States Code
USDA	United States Department of Agriculture
USP	United States Pharmacopeia
WHO	World Health Organization
WWEIA	What We Eat in America
w/w %	weight by weight percentage

B. List of References

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