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Safety evaluation of the use of the non-genetically modified *Hamamotoa singularis* strain YIT 10047 as a source of β -galactosidase

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Abstract

This assessment addresses the enzyme β -galactosidase which is not separated from the yeast cells used for its production. The β -galactosidase (β -D-galactoside galactohydrolase, EC 3.2.1.23) is produced with the non-genetically modified *Hamamotoa singularis* (formerly *Sporobolomyces singularis*) strain YIT 10047 by Yakult Pharmaceutical Industry Co., Ltd. The yeast cell suspension contains both live and dead yeast cells. It is intended to be used in the production of galacto-oligosaccharides (GOS). The final GOS products are free of viable cells of the *H. singularis*. Dietary exposure to the food enzyme total organic solids (TOS) was estimated to be up to 0.683 mg TOS/kg body weight (bw) per day in European populations. Genotoxicity tests of the cell suspension did not indicate a safety concern. The systemic toxicity was assessed by means of a repeated dose 90-day oral toxicity study in rats. The Panel identified a no observed adverse effect level of 296.25 mg TOS/kg bw per day, the highest dose tested. This results in a margin of exposure above 434. A search for the similarity of the amino acid sequence of the β -galactosidase to known allergens was made and no matches were found. The Panel considered that, under the intended conditions of use, the risk of allergic reactions by dietary exposure cannot be excluded, but the likelihood for this to occur is low. Based on the data provided, the Panel concluded that this yeast suspension used as a source of β -galactosidase does not give rise to safety concerns under the intended conditions of use.

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Keywords: food enzyme, β -D-galactoside galactohydrolase, β -Galactosidase, EC 3.2.1.23, *Hamamotoa singularis*, *Sporobolomyces singularis*, galacto-oligosaccharides

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[†] Deceased.

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1. Introduction

Article 3 of the Regulation (EC) No 1332/2008¹ provides definition for 'food enzyme' and 'food enzyme preparation'.

'Food enzyme' means a product obtained from plants, animals or microorganisms or products thereof including a product obtained by a fermentation process using microorganisms: (i) containing one or more enzymes capable of catalysing a specific biochemical reaction; and (ii) added to food for a technological purpose at any stage of the manufacturing, processing, preparation, treatment, packaging, transport or storage of foods.

'Food enzyme preparation' means a formulation consisting of one or more food enzymes in which substances such as food additives and/or other food ingredients are incorporated to facilitate their storage, sale, standardisation, dilution or dissolution.

Before January 2009, food enzymes other than those used as food additives were not regulated or were regulated as processing aids under the legislation of the Member States. On 20 January 2009, Regulation (EC) No 1332/2008 on food enzymes came into force. This Regulation applies to enzymes that are added to food to perform a technological function in the manufacture, processing, preparation, treatment, packaging, transport or storage of such food, including enzymes used as processing aids. Regulation (EC) No 1331/2008² established the European Union (EU) procedures for the safety assessment and the authorisation procedure of food additives, food enzymes and food flavourings. The use of a food enzyme shall be authorised only if it is demonstrated that:

- i) it does not pose a safety concern to the health of the consumer at the level of use proposed;
- ii) there is a reasonable technological need;
- iii) its use does not mislead the consumer.

All food enzymes currently on the European Union market and intended to remain on that market, as well as all new food enzymes, shall be subjected to a safety evaluation by the European Food Safety Authority (EFSA) and approval via an EU Community list.

The 'Guidance on submission of a dossier on food enzymes for safety evaluation' (EFSA CEF Panel, 2009) lays down the administrative, technical and toxicological data required.

1.1. Background and terms of reference as provided by the requestor

1.1.1. Background as provided by the European Commission

Only food enzymes included in the European Union (EU) Community list may be placed on the market as such and used in foods, in accordance with the specifications and conditions of use provided for in Article 7(2) of Regulation (EC) No 1332/2008 on food enzymes.

Five applications have been introduced by the Association of manufacturers and formulators of enzyme products (AMFEP) for the authorisation of the food enzyme Bacillolysins from *Bacillus subtilis* and by the companies "Meiji Seika Pharma Co., Ltd." for the authorisation of the food enzyme Polygalacturonase from *Talaromyces cellulolyticus/Talaromyces pinophilus*, "Yakult Pharmaceutical Industry Co., Ltd." for the authorisation of the food enzyme Beta-galactosidase from *Sporobolomyces singularis* (YIT 10047), and "Bioresco Ltd." for the authorisation of the food enzymes Cyclomaltodextrin glucotransferase from a genetically modified strain of *E. coli* K12 (WCM105xpCM703) and Cyclomaltodextrin glucanotransferase from a genetically modified strain of *E. coli* K12 (WCM105xpCM6420).

Following the requirements of Article 12.1 of Regulation (EC) No 234/2011³ implementing Regulation (EC) No 1331/2008, the Commission has verified that the five applications fall within the scope of the food enzyme Regulation and contain all the elements required under Chapter II of that Regulation.

¹ Regulation (EC) No 1332/2008 of the European Parliament and of the Council of 16 December 2008 on Food Enzymes and Amending Council Directive 83/417/EEC, Council Regulation (EC) No. 1493/1999, Directive 2000/13/EC, Council Directive 2001/112/EC and Regulation (EC) No 258/97. OJ L 354, 31.12.2008, pp. 7–15.

² Regulation (EC) No 1331/2008 of the European Parliament and of the Council of 16 December 2008 establishing a common authorisation procedure for food additives, food enzymes and food flavourings. OJ L 354, 31.12.2008, pp. 1–6.

³ Commission Regulation (EU) No. 234/2011 of 10 March 2011 implementing Regulation (EC) No 1331/2008 of the European Parliament and of the Council establishing a common authorisation procedure for food additives, food enzymes and food flavourings. OJ L 64, 11.03.2011, pp. 15–24.

1.1.2. Terms of Reference

The European Commission requests the European Food Safety Authority to carry out the safety assessments on the food enzymes Bacillolysin from *Bacillus subtilis*, Polygalacturonase from *Talaromyces cellulolyticus*/*Talaromyces pinophilus*, Beta-galactosidase from *Sporobolomyces singularis* (YIT 10047), Cyclomalto-dextrin glucanotransferase from a genetically modified strain of *E. coli* K12 (WCM105xpCM703) and Cyclomalto-dextrin glucanotransferase from a genetically modified strain of *E. coli* K12 (WCM105xpCM6420) in accordance with Article 17.3 of regulation (EC) No 1332/2008 on food enzymes.

1.2. Interpretation of the Terms of Reference

The present scientific opinion addresses the European Commission's request to carry out the safety assessment of food enzyme β -galactosidase from *Sporobolomyces singularis* strain YIT 10047.

Recent data identified the production microorganism as *Hamamotoa singularis* (Section 3.1). Therefore, this name will be used in this opinion instead of *Sporobolomyces singularis*.

2. Data and methodologies

2.1. Data

The applicant has submitted a dossier in support of the application for authorisation of the food enzyme β -galactosidase from *Hamamotoa singularis* (formerly *Sporobolomyces singularis*) strain YIT 10047.

Additional information was requested from the applicant on 2 October 2019, 19 May 2020 and 9 September 2022. The replies were received on 21 April 2020, 29 July 2022 and 14 September 2022, respectively (see 'Documentation provided to EFSA').

2.2. Methodologies

The assessment was conducted in line with the principles described in the EFSA 'Guidance on transparency in the scientific aspects of risk assessment' (EFSA, 2009) and following the relevant guidance documents of the EFSA Scientific Committee.

The 'Guidance on the submission of a dossier on food enzymes for safety evaluation' (EFSA CEP Panel, 2009) as well as the 'Statement on characterisation of microorganisms used for the production of food enzymes' (EFSA CEP Panel, 2019) have been followed for the evaluation of the application with the exception of the exposure assessment, which was carried out in accordance with the updated 'Scientific Guidance for the submission of dossiers on food enzymes' (EFSA CEP Panel, 2021a).

3. Assessment

This assessment focuses on the enzyme β -galactosidase. This enzyme is used in the form of a whole cell suspension, thus, not separated from the yeast cells used for its production. The yeast cell suspension contains both live and dead yeast cells. It is intended to be used in the production of galacto-oligosaccharides (GOS).

IUBMB nomenclature	β -galactosidase
Systematic name	β -D-galactoside galactohydrolase
Synonyms	Lactase; β -D-lactosidase
IUBMB No	EC. 3.2.1.23
CAS No	9031-11-2
EINECS No	232-864-1

β -Galactosidases typically catalyse the hydrolysis of terminal non-reducing β -D-galactose residues in β -D-galactosides. The specific enzyme under application also acts as a β -D-glucosidase and has a high trans-galactosylation activity. When lactose is used as a substrate, one lactose molecule is hydrolysed to galactose and glucose, while a second lactose molecule acts as recipient for trans-galactosylation, resulting in the formation of a trisaccharide. Higher molecular mass galacto-oligosaccharides are produced as the reaction proceeds.

3.1. Source of the food enzyme

The production strain for the food enzyme is a non-genetically modified yeast *Hamamotoa singularis* (formerly *Sporobolomyces singularis*) strain YIT 10047,⁴ which is deposited in the International Patent Organism Depository of Japan with deposit number FERM P-18817.⁵ Strain YIT 10047 was obtained from the parental strain *H. singularis* ATCC 24193 by chemical mutagenesis and selection of high β -galactosidase activity and the absence of catabolite repression (Ishikawa et al., 2005).

The production strain was taxonomically identified as *H. singularis* by sequence analysis of the internal transcribed spacer regions (ITS).⁶

H. singularis is a ballistospore-producing yeast belonging to the basidiomycota, originally isolated from insect frass. A literature search made by the applicant in 2015 and updated in 2020 found only a few publications, none of which indicated reports of metabolites of possible concern produced by *Sporobolomyces* or the related genera *Rhodotorula*.⁷

3.2. Production of the cell suspension

The cell suspension containing β -galactosidase is manufactured with food safety procedures based on Hazard Analysis and Critical Control Points and in accordance with current good manufacturing practice.⁸

The yeast cells are grown as a pure culture using a typical industrial medium in a submerged, batch fermentation system with conventional process controls in place. When a predetermined concentration of β -galactosidase is reached, the fermentation is stopped and the cells are separated from the culture medium by centrifugation. Yeast cells are then washed and again collected by centrifugation. The resulting yeast concentrate is adjusted with water to meet a specification of [REDACTED]. The applicant provided information on the identity of the substances used to control the fermentation and in the subsequent downstream processing of the food enzyme.⁹

The Panel considered that sufficient information has been provided on the manufacturing process to exclude issues of concern.

3.3. Characteristics of the cell suspension

3.3.1. Properties of the β -galactosidase

The β -galactosidase is a glycoprotein consisting of a single polypeptide chain of [REDACTED] amino acids.¹⁰ The molecular mass of the mature protein, calculated from the amino acid sequence, is [REDACTED] kDa.

The in-house determination of the activity of the β -galactosidase is based on the release of *o*-nitrophenol from *o*-nitrophenyl- β -D-galactopyranoside (reaction conditions: [REDACTED]). One Unit of activity is defined as quantity of the enzyme required to release 1 μ mol of *o*-nitrophenol per minute under the conditions of the assay.¹¹

Two methods were used to characterise the temperature and pH profiles of the β -galactosidase. The first was presented as relative hydrolytic activity curves under different conditions of temperature and pH, and was assumed to be based on measurements using the in-house method described above. The second data set was based on determining trans-galactosylation (production of galacto-oligosaccharides) by high-performance liquid chromatography (HPLC). Both measurements showed similar results, i.e. a temperature optimum around [REDACTED] and a pH optimum around [REDACTED]. Thermostability was tested after a pre-incubation of the food enzyme for 30 min at different temperatures. Under the conditions [REDACTED] of the applied temperature stability assay, β -galactosidase activity decreased above [REDACTED], showing no residual activity above [REDACTED]. At higher pH (pH 6.0), β -galactosidase activity was maintained up to [REDACTED].¹²

⁴ Also named as 7B6 in the dossier.

⁵ Additional information July 2022/Appendix 6.

⁶ Additional data April 2020/Table 1 and Appendix 1.

⁷ Technical dossier/pp. 39–41 & Addition data April 2020/pp. 4–5.

⁸ Additional data April 2020/Appendix 3.

⁹ Technical dossier/pp. 45–46/Annex 6 & Additional data April 2020/p. 6.

¹⁰ Technical dossier/pp. 12–13 & Additional data April 2020/Appendix 7.

¹¹ Technical dossier/pp. 18–19/Annex 4.

¹² Technical dossier/pp. 19–23 and Annex 5.

3.3.2. Chemical parameters of the yeast cell suspension

Data on the chemical parameters of the yeast cell suspension were provided for four batches used in GOS manufacturing, of which one batch was used for the toxicological tests (batch 4).¹³ The mean total organic solids (TOS) was 3.2% and the mean enzyme activity/TOS ratio was 138 U/g TOS.

Table 1: Compositional data of the manufacturing batches of the yeast cell suspension

Parameters	Unit	Batches			
		1	2	3	4 ^(a)
β-Galactosidase activity	U/g ^(b)	4.3	4.4	4.3	4.4
Protein	%	0.9	0.9	0.9	0.9
Ash	%	0.2	0.2	0.2	0.2
Water	%	96.6	96.6	96.5	96.9
Total organic solids (TOS)^(c)	%	3.2	3.2	3.3	2.9
Activity/g TOS	U/g TOS	134	138	130	152

(a): Batch used for the toxicological studies.

(b): Unit/g: U/g (see Section 3.3.1).

(c): TOS calculated as 100% – % water – % ash.

3.3.3. Purity of the yeast cell suspension

The lead content in all four batches of the cell suspension was below 0.05 mg/kg,¹⁴ which complies with the specification for lead as laid down in the general specifications for enzymes used in food processing (FAO/WHO, 2006). In addition, cadmium, mercury and arsenic contents were below the limits of detection (LoD) of the employed methods.^{15,16}

All four batches of the yeast cell suspension complied with the microbiological criteria (for total coliforms, *Escherichia coli* and *Salmonella*) as laid down in the general specifications for enzymes used in food processing (FAO/WHO, 2006). Counts of *Staphylococcus*, yeasts and filamentous fungi in all four batches were below the specifications (100 CFU/mL).^{17,18} No antimicrobial activity was detected in any of the tested batches.¹⁹

The presence of citrinin, ochratoxin A, aflatoxins B₁, B₂, G₁, G₂, patulin, sterigmatocystin, nivalenol, deoxynivalenol, zearalenone and fumonisins B₁, B₂ was examined in three batches of the yeast cell suspension (Batches 1, 2, 3 in Table 1) and all were below the LoD of the applied analytical methods.^{20,21} Adverse effects caused by the possible presence of other secondary metabolites of concern are addressed by the toxicological examination of the cell suspension.

The Panel considered that the information provided on the purity of the cell suspension is sufficient.

3.3.4. Viable cells of the production strain

The applicant stated that the cell suspension contains both live and dead yeast cells.²²

3.4. Toxicological data

A battery of toxicological tests including a bacterial gene mutation assay (Ames test), an *in vitro* mammalian cell micronucleus test and a repeated dose 90-day oral toxicity study in rats have been

¹³ Additional data April 2020/Appendix 4; Additional data July 2022/Appendix 5.

¹⁴ Technical dossier/pp. 16–17 and Annex 1; Additional data April 2020/Appendix 4; Additional data July 2022/Appendix 5.

¹⁵ Technical dossier/pp. 16–17 & Annex 1; Additional data April 2020/Appendix 4; Additional data July 2022/Appendix 5.

¹⁶ LoDs: Pb = 0.05 mg/kg; Cd, Hg = 0.01 mg/kg each; As = 0.1 mg/kg.

¹⁷ Technical dossier/pp. 16–17 and Annex 1; Additional data April 2020/Appendix 4; Additional data July 2022/Appendix 5; Additional data September 2022.

¹⁸ The production strain shows slow growth under the conditions of the ISO standard used for yeasts and filamentous fungi and is not expected to be detected.

¹⁹ Additional data April 2020/Appendix 6.

²⁰ Technical dossier/pp. 13–15 and Annex 3.

²¹ LoDs: citrinin = 0.1 ppm, ochratoxin A; aflatoxins (B₁, B₂, G₁, G₂) = 0.5 ppb each; patulin = 0.01 ppm; sterigmatocystin = 20 ppb; deoxynivalenol = 0.2 ppm; zearalenone = 50 ppb; fumonisins (B₁, B₂) = 0.1 ppm each.

²² Technical dossier/page 18.

provided.²³ Despite its slightly higher activity per unit TOS, batch 4 (Table 1) used in these studies was considered representative of the batches used for GOS manufacturing.

3.4.1. Genotoxicity

The Panel noted that the genotoxicity tests were performed with the yeast cell suspension containing live cells. The use of cell suspension was considered acceptable to test secondary metabolites possibly released by this yeast. Since the yeast cells are removed by microfiltration under the intended conditions of use, the lack of testing the cell lysate was not considered a limitation.

3.4.1.1. Bacterial reverse mutation test

A bacterial reverse mutation assay (Ames test) was performed with four strains of *Salmonella* Typhimurium (TA98, TA100, TA1535 and TA1537) and *Escherichia coli* WP2uvrA according to Organisation for Economic Co-operation and Development (OECD) Test Guideline 471 (OECD, 1997) and following good laboratory practice (GLP).²⁴

On the basis of the results of a dose finding test, five concentrations of the test item (6.2, 12.5, 25, 50 and 100 μ L yeast suspension/plate) were tested in triplicate in the presence or absence of metabolic activation (S9-mix), applying the pre-incubation method.

Precipitation of the test item was observed at the highest concentration tested. No growth inhibition was observed in any of the test conditions. No biologically relevant increase in the number of revertant colonies above the control values was observed in any strain tested, with or without S9-mix.

The Panel concluded that the yeast suspension did not induce gene mutations in bacteria under the test conditions of the study.

3.4.1.2. *In vitro* mammalian cell micronucleus test

The *in vitro* mammalian cell micronucleus test was carried out according to OECD Draft Guideline 487 (OECD, 2016) and following GLP.²⁵

A single experiment was performed with duplicate cultures of the human lymphoblastoid TK6 cell line. In a range-finding test, the 50% cell-growth inhibition concentration of the test item was 14.9 μ L/mL in the short-term treatment without metabolic activation (S9-mix), 14.6 μ L/mL in the short-term treatment with S9-mix and 15.9 μ L/mL in the long-term treatment without S9-mix.

Based on these results, in the main experiment, the cells were exposed and scored for the frequency of binucleated cells with micronuclei (MNBN) at 20.0, 18.0, 16.0 and 14.0 μ L yeast suspension/mL in the short-term treatment (4 h exposure and 20 h recovery period) without S9-mix, 18.0, 16.0, 14.0, 12.0 and 10.0 μ L yeast suspension/mL in the short-term treatment with S9-mix and 18.0, 14.0, 10.0, 6.00 and 2.00 μ L yeast suspension/mL in the 24 h continuous treatment without S9-mix.

Precipitates were observed at all the concentrations tested. The cell growth inhibition observed at the highest concentrations tested was 54%, 56% and 58% in the short-term treatment without S9-mix, in the short-term treatment with S9-mix and in the continuous treatment, respectively. The frequency of MNBN was not statistically significantly different to the negative controls at all concentrations tested.

The Panel concluded that the yeast suspension did not induce an increase in the frequency of MNBN under the test conditions applied in this study.

3.4.2. Repeated dose 90-day oral toxicity study in rats

The repeated dose 90-day oral toxicity study was performed in accordance with OECD Test Guideline 408 (OECD, 2018) and following GLP.²⁶ Groups of 10 male and 10 female Sprague–Dawley (CrI:CD(SD)) rats received by gavage the yeast suspension in doses of 2.5, 5 and 10 mL/kg body weight (bw) per day, corresponding to 74.0, 148.1 and 296.25 mg TOS/kg bw per day. Controls received the vehicle (water for injection).

No mortality was observed.

The feed consumption was statistically significantly decreased on day 1 of administration in mid-dose males (–8%), on day 28 in low- and mid-dose males (–7%; –7%) and on day 35 in low-dose

²³ Additional information July 2022.

²⁴ Additional information July 2022/Appendix 4.

²⁵ Additional information July 2022/Appendix 3.

²⁶ Additional data July 2022/Appendix 2.

males (–3%). The Panel considered the changes as not toxicologically relevant as they were only recorded sporadically, they were only observed in one sex, there was no dose–response relationship and there was no statistically significant change in the final feed consumption, body weight or body weight gain.

The haematological investigation revealed a statistically significant increase in monocyte count in mid-dose females (+42%). The Panel considered the change as not toxicologically relevant, as it was only observed in one sex, there was no dose–response relationship, the change was small and there was no change in white blood cell count.

The clinical chemistry investigation revealed a statistically significant increase in total cholesterol in mid-dose females (+28%). The Panel considered the change as not toxicologically relevant, as it was only observed in one sex and there was no dose–response relationship.

Statistically significant changes in organ weights included an increase in the absolute thyroid gland weight in high-dose males (+18%), an increase in the relative pituitary gland weight in mid-dose males (+14%), an increase in the relative adrenal gland weight in mid-dose males (+20%) and an increase in the absolute spleen weight in mid-dose females (+17%). The Panel considered the changes as not toxicologically relevant as they were only observed in one sex (all parameters), there was no dose–response relationship (spleen, pituitary and adrenal glands) and there were no histopathological changes in thyroid, pituitary glands, adrenal glands and spleen.

No other statistically significant or biologically relevant differences to controls were reported.

The Panel identified a no observed adverse effect level (NOAEL) of 296.25 mg TOS/kg bw per day, the highest dose tested.

3.4.3. Allergenicity

The allergenicity assessment considers only the food enzyme and not any carrier nor other excipient, which may be used in the final formulation.

The potential allergenicity of the β -galactosidase produced with the *H. singularis* YIT 10047 was assessed by comparing its amino acid sequence with those of known allergens according to the 'Scientific opinion on the assessment of allergenicity of GM plants and microorganisms and derived food and feed of the Scientific Panel on Genetically Modified Organisms' (EFSA GMO Panel, 2010). Using higher than 35% identity in a sliding window of 80 amino acids as the criterion, no match was found.²⁷

No information is available on oral and respiratory sensitisation or elicitation reactions of this β -galactosidase or to cells of *H. singularis*.

A few case reports are available that describe allergic reactions upon oral exposure to β -galactosidase in individuals respiratorily sensitised to β -galactosidase (Stöcker et al., 2016; Voisin and Borici-Mazi, 2016). Some studies have shown that adults with occupational asthma caused by an enzyme used in food can commonly ingest the corresponding allergen without acquiring clinical symptoms of food allergy (Cullinan et al., 1997; Brisman, 2002; Poulsen, 2004; Armentia et al., 2009).

Yeast extract, a known source of allergens, is present in the medium fed to the microorganisms.²⁸ However, during the fermentation process, this product will be degraded and utilised by the microorganisms for cell growth, cell maintenance and production of enzyme protein. In addition, the yeast biomass and fermentation solids are removed. Taking into account the fermentation process and the downstream processing, the Panel considered that no potentially allergenic residues are present in the final product.

The Panel considered that, under the intended conditions of use, the risk of allergic reactions upon dietary exposure to this food enzyme cannot be excluded, but the likelihood of such reactions to occur is low.

3.5. Dietary exposure

3.5.1. Intended use of the food enzyme

The applicant states that the suspension of cells of *Hamamotoa singularis* YIT 10047 or any preparation derived from the cell suspension will be used only in-house for the production of GOS and

²⁷ Additional data April 2020/Appendix 7.

²⁸ Technical dossier/p. 45–46.

will not be commercially distributed to third parties for sale or use in the production of other food ingredients.²⁹

The yeast cell is used as a source of β -galactosidase in which [redacted] kg of the yeast cell suspension – standardised to [redacted], is added to [redacted] kg lactose solution which includes [redacted] kg lactose.³⁰ This corresponds to a use level of 400 mg TOS/kg lactose.

A detailed description and a flowchart depicting the manufacturing process steps of the galacto-oligosaccharide products (syrup or powder format) have been provided.³¹ In this process, the yeast cell suspension is added to purified lactose extracted from whey, which results in the conversion of lactose to GOS. The reaction is monitored by HPLC to analyse the oligosaccharide composition. When a predefined composition is obtained, the solution is heated at 85–90°C for 10 min to stop the reaction. The solution is then cooled to a temperature of 40–50°C and adjusted to pH 6.0. A second enzyme (a β -galactosidase from *Kluyveromyces lactis*) is added to hydrolyse the unreacted lactose and again the reaction is stopped by heat treatment.

Subsequently, the reaction product is subjected to decolouration by treatment with activated carbon, filtration to remove cell debris and insoluble proteins, demineralisation with ion-exchange and microfiltration to remove any contaminating microorganisms. The purified GOS solution is finally concentrated by vacuum evaporation and UV sterilised to obtain a syrup, or alternatively, is dried under vacuum to obtain a powder. Such downstream processing is expected to remove TOS from the final GOS products. As a proxy for the TOS residues derived from the cells of the production strain, the amount of total nitrogen in three lots of GOS manufactured with the yeast cell suspension using the combustion method were found to be 0.003 g/100 g, which is slightly above the LoD.³²

No viable cells of the production strain were detected in three syrup batches, each tested in triplicate, when plated and incubated for 7 days.³³

3.5.2. Dietary exposure estimation

The Panel considered the analytical evidence indicated that residual amounts of TOS are largely removed during the production of GOS from lactose (see Section 3.5.1). However, given the added uncertainty that the food enzyme is not isolated from the production organism, the limited information available on the biology of the production organism itself and that the predominant consumption of the final food product is by infants and toddlers, the Panel considered it prudent to make a dietary exposure estimation.

Chronic exposure to the food enzyme-TOS was calculated by combining the maximum recommended use level with individual consumption data (EFSA CEP Panel, 2021a). The estimation involved selection of relevant food categories and application of technical conversion factors (EFSA CEP Panel, 2021b). Exposure from all FoodEx categories was subsequently summed up, averaged over the total survey period (days) and normalised for body weight. This was done for all individuals across all surveys, resulting in distributions of individual average exposure. Based on these distributions, the mean and 95th percentile exposures were calculated per survey for the total population and per age class. Surveys with only 1 day per subject were excluded and high-level exposure/intake was calculated for only those population groups in which the sample size was sufficiently large to allow calculation of the 95th percentile (EFSA, 2011).

Table 2 provides an overview of the derived exposure estimates across all surveys. Detailed mean and 95th percentile exposure to the food enzyme-TOS per age class, country and survey, as well as contribution from each FoodEx category to the total dietary exposure are reported in Appendix A – Tables 1 and 2. For the present assessment, food consumption data were available from 41 dietary surveys (covering infants, toddlers, children, adolescents, adults and the elderly), carried out in 22 European countries (Appendix B). The highest dietary exposure was estimated to be about 0.683 mg TOS/kg bw per day in infants at the 95th percentile.

²⁹ Additional data April 2020/p. 8.

³⁰ Technical dossier/p.57.

³¹ Technical dossier/Figure 8.

³² Additional data July 2022/Appendix 1, LoD = 0.001 g/100 g.

³³ Additional data April 2020/Appendix 2, LoD = 1 CFU/ml.

Table 2: Summary of estimated dietary exposure to food enzyme-TOS in six population groups

Population group	Estimated exposure (mg TOS/kg body weight per day)					
	Infants	Toddlers	Children	Adolescents	Adults	The elderly
Age range	3–11 months	12–35 months	3–9 years	10–17 years	18–64 years	≥ 65 years
Min–max mean (number of surveys)	0.090–0.299 (11)	0.012–0.130 (15)	0.003–0.009 (19)	0.001–0.003 (21)	0.001–0.003 (22)	0–0.004 (22)
Min–max 95th (number of surveys)	0.305–0.683 (9)	0.063–0.301 (13)	0.008–0.047 (19)	0.003–0.010 (20)	0.002–0.009 (22)	0.001–0.014 (21)

3.5.3. Uncertainty analysis

In accordance with the guidance provided in the EFSA opinion related to uncertainties in dietary exposure assessment (EFSA, 2006), the following sources of uncertainties have been considered and are summarised in Table 3.

Table 3: Qualitative evaluation of the influence of uncertainties on the dietary exposure estimate

Sources of uncertainties	Direction of impact
Model input data	
Consumption data: different methodologies/representativeness/underreporting/misreporting/no portion size standard	+/-
Use of data from food consumption surveys of a few days to estimate long-term (chronic) exposure for high percentiles (95th percentile)	+
Possible national differences in categorisation and classification of food	+/-
Model assumptions and factors	
Exposure to food enzyme-TOS was always calculated based on the recommended maximum use level	+
Selection of broad FoodEx categories for the exposure assessment	+
Use of recipe fractions in disaggregation FoodEx categories	+/-
Use of technical factors in the exposure model	+/-

+: uncertainty with potential to cause overestimation of exposure.

-: uncertainty with potential to cause underestimation of exposure.

The conservative approach applied to the exposure estimate to food enzyme-TOS, in particular assumptions made on the occurrence and use levels of this specific food enzyme, is likely to have led to overestimation of the exposure.

3.6. Margin of exposure

A comparison of the NOAEL (296.25 mg TOS/kg bw per day) from the 90-day rat study with the derived exposure estimates of 0–0.299 mg TOS/kg bw per day at the mean and of 0.001–0.683 mg TOS/kg bw per day at the 95th percentile resulted in a margin of exposure of at least 434.

4. Conclusions

Based on the data provided and the derived margin of exposure, the Panel concluded that the cell suspension of the non-genetically modified *Hamamotoa singularis* strain YIT 10047 used as a source of β -galactosidase does not give rise to safety concerns under the intended conditions of use.

Documentation provided to EFSA

- 1) β -Galactosidase from a concentrate of *Sporobolomyces singularis* YIT 10047 (YC-Y) - A Submission to the European Food Safety Authority (EFSA) requesting consideration for inclusion in the EU positive list. March 2015. Submitted by Spherix Consulting, Inc. for Yakult Pharmaceutical Industry Co., Ltd.

- 2) Additional information. April 2020. Submitted by Spherix Consulting, Inc. for Yakult Pharmaceutical Industry Co., Ltd.
- 3) Additional information. July 2022. Submitted by Spherix Consulting, Inc. for Yakult Pharmaceutical Industry Co., Ltd.
- 4) Additional information. September 2022. Submitted by Spherix Consulting, Inc. for Yakult Pharmaceutical Industry Co., Ltd.

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Abbreviations

bw	body weight
CAS	Chemical Abstracts Service
CEF	EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
CEP	EFSA Panel on Food Contact Materials, Enzymes and Processing Aids
CFU	Colony Forming Units
DRF	Dose-range finding
EC	European Commission and Enzyme Commission
EINECS	European Inventory of Existing Commercial Chemical Substances
EU	European Union
FAO	Food and Agricultural Organisation of the United Nations
GLP	Good Laboratory Practice
GMM	Genetically Modified Microorganism
GMO	Genetically Modified Organism
GMP	Good Manufacturing Practice
HACCP	Hazard Analysis and Critical Control Points
HPLC	High Performance Liquid Chromatography
IUBMB	International Union of Biochemistry and Molecular Biology
JECFA	Joint FAO/WHO Expert Committee on Food Additives
kDa	KiloDalton
LoD	Limit of Detection
OECD	Organisation for Economic Cooperation and Development
TOS	Total Organic Solids
WHO	World Health Organisation

Appendix A – Dietary exposure estimates to the food enzyme–TOS in details

Information provided in this appendix is shown in an Excel file (downloadable <https://efsa.onlinelibrary.wiley.com/doi/10.2903/j.efsa.2022.7650#support-information-section>).

The file contains two sheets, corresponding to two tables.

Table 1: Mean and 95th percentile exposure to the food enzyme–TOS per age class, country and survey.

Table 2: Contribution of food categories to the dietary exposure to the food enzyme–TOS per age class, country and survey.

Appendix B – Population groups considered for the exposure assessment

Population	Age range	Countries with food consumption surveys covering more than 1 day
Infants	From 12 weeks on up to and including 11 months of age	Bulgaria, Cyprus, Denmark, Estonia, Finland, France, Germany, Italy, Latvia, Portugal, Slovenia
Toddlers	From 12 months up to and including 35 months of age	Belgium, Bulgaria, Cyprus, Denmark, Estonia, Finland, France, Germany, Hungary, Italy, Latvia, Netherlands, Portugal, Slovenia, Spain
Children	From 36 months up to and including 9 years of age	Austria, Belgium, Bulgaria, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Italy, Latvia, Netherlands, Portugal, Spain, Sweden
Adolescents	From 10 years up to and including 17 years of age	Austria, Belgium, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Italy, Latvia, Netherlands, Portugal, Romania, Slovenia, Spain, Sweden
Adults	From 18 years up to and including 64 years of age	Austria, Belgium, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Netherlands, Portugal, Romania, Slovenia, Spain, Sweden
The elderly^(a)	From 65 years of age and older	Austria, Belgium, Cyprus, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Netherlands, Portugal, Romania, Slovenia, Spain, Sweden

(a): The terms 'children' and 'the elderly' correspond, respectively, to 'other children' and the merge of 'elderly' and 'very elderly' in the Guidance of EFSA on the 'Use of the EFSA Comprehensive European Food Consumption Database in Exposure Assessment' (EFSA, 2011).