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SCIENTIFIC OPINION

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Safety evaluation of the food enzyme subtilisin from the non-genetically modified *Bacillus paralicheniformis* strain AP-01

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Abstract

The food enzyme subtilisin (EC 3.4.21.62) is produced with the non-genetically modified *Bacillus paralicheniformis* strain AP-01 by Nagase (Europa) GmbH. It was considered free from viable cells of the production organism. The food enzyme is intended to be used in five food manufacturing processes. Since residual amounts of food enzyme-total organic solids (TOS) are removed in one process, dietary exposure was calculated only for the remaining four food manufacturing processes. It was estimated to be up to 0.875 mg TOS/kg body weight per day in European populations. The production strain of the food enzyme has the capacity to produce bacitracin and thus failed to meet the requirements of the Qualified Presumption of Safety approach. Bacitracin was detected in the industrial fermentation medium but not in the food enzyme itself. However, the limit of detection of the analytical method used for bacitracin was not sufficient to exclude the possible presence of bacitracin at a level representing a risk for the development of antimicrobial resistant bacteria. A search for the similarity of the amino acid sequence of the food enzyme to known allergens was made and twenty-eight matches with respiratory allergens, one match with a contact allergen and two matches with food allergens (melon and pomegranate) were found. The Panel considered that the risk of allergic reactions upon dietary exposure to this food enzyme, particularly in individuals sensitised to melon or pomegranate, cannot be excluded, but would not exceed the risk of consuming melon or pomegranate. Based on the data provided, the Panel could not exclude the presence of bacitracin, a medically important antimicrobial, and consequently the safety of this food enzyme could not be established.

KEYWORDS

Bacillus paralicheniformis, EC 3.4.21.62, EFSA-Q-2022-00601, serine endopeptidase, subtilisin

CONTENTS

1 | INTRODUCTION

Article 3 of the Regulation (EC) No 1332/2008[1] provides definition for 'food enzyme' and 'food enzyme preparation'.

'Food enzyme' means a product obtained from plants, animals or micro-organisms or products thereof including a product obtained by a fermentation process using micro-organisms: (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 1331/2008[2] 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:

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

All food enzymes currently on the EU 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.

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 Union 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[1] on food enzymes.

Six applications have been introduced by the companies "Decernis, LLC", "Keller and Heckman LLP", the "Association of Manufacturers and Formulators of Enzyme Products (AMFEP)" and "Novozymes A/S" for the authorisation of the food enzymes Cyclomaltodextrin glucanotransferase from *Geobacillus stearothermophilus*, Dextranase from *Chaetomium gracile*, Subtilisin from *Bacillus licheniformis*, Mucorpepsin from *Rhizomucor miehei*, Animal rennet consisting of chymosin and pepsin from the abomasum of *Bos primigenius* (cattle), *Bubalus bubalis* (buffalo), *Capra aegagrus hircus* (goat) and *Ovis aries* (sheep), and Lipase from a genetically modified strain of *Aspergillus niger* (strain NZYM-DB), respectively.

Following the requirements of Article 12.1 of Regulation (EC) No 234/2011[3] implementing Regulation (EC) No 1331/2008[2], the Commission has verified that the six applications fall within the scope of the food enzyme Regulation and contains all the elements required under Chapter II of that Regulation.

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 Cyclomaltodextrin glucanotransferase from *Geobacillus stearothermophilus*, Dextranase from *Chaetomium gracile*, Subtilisin from *Bacillus licheniformis*, Mucorpepsin from *Rhizomucor miehei*, Animal rennet consisting of chymosin and pepsin from the abomasum of *Bos primigenius* (cattle), *Bubalus bubalis* (buffalo), *Capra aegagrus hircus* (goat) and *Ovis aries* (sheep), and Lipase from a genetically modified strain of *Aspergillus niger* (strain NZYM-DB) in accordance with Article 17.3 of Regulation (EC) No 1332/2008[1] on food enzymes.

[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.

1.2 | Interpretation of the Terms of Reference

The present scientific opinion addresses the European Commission's request to carry out the safety assessment of the food enzyme subtilisin from *Bacillus licheniformis* submitted by the Association of Manufacturers and Formulators of Enzyme Products (AMFEP).

The application was submitted initially as a joint dossier[4] and identified as the EFSA-Q-2015-00232. During a meeting between EFSA, the European Commission and AMFEP,[5] it was agreed that joint dossiers will be split into individual data packages.

The current opinion addresses one data package originating from the former joint dossier. This data package is identified as EFSA-Q-2022-00601 and concerns the food enzyme subtilisin produced with the *Bacillus licheniformis* strain AP-01 and submitted by Nagase (Europa) GmbH.

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

2 | DATA AND METHODOLOGIES

2.1 | Data

The applicant has submitted a dossier in support of the application for authorisation of the food enzyme subtilisin from a non-genetically modified *Bacillus licheniformis* strain AP-01.

Additional information, requested from the applicant during the assessment process on 02 May 2023, was received on 27 February 2024 (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, 2009a) 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, 2009b) 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. Additional information was requested in accordance with the updated 'Scientific Guidance for the submission of dossiers on food enzymes' (EFSA CEP Panel, 2021) and the guidance on the 'Food manufacturing processes and technical data used in the exposure assessment of food enzymes' (EFSA CEP Panel, 2023).

3 | ASSESSMENT

IUBMB nomenclature	Subtilisin
Systematic name	Serine endopeptidase
Synonyms	Alcalase, bacillopeptidase, alkaline proteinase, thermoase, subtilopeptidase
IUBMB No	EC 3.4.21.62
CAS No	9014-01-1
EINECS No	232-752-2

Subtilisins catalyse the hydrolysis of peptide bonds of proteins with a broad specificity, releasing peptides and amino acids. The enzyme under assessment is intended to be used in five food manufacturing processes as described in the EFSA guidance (EFSA CEP Panel, 2023): (1) processing of cereals and other grains for the production of cereal-based products other than baked; (2) processing of dairy products for the production of modified milk proteins; processing of plant- and fungal-derived products for the production of (3) edible oils from plant and algae and (4) protein hydrolysates; (5) processing of meat and fish products for the production of modified meat and fish.

[4]Commission Implementing Regulation (EU) No 562/2012 of 27 June 2012 amending Commission Regulation (EU) No 234/2011 with regard to specific data required for risk assessment of food enzymes Text with EEA relevance. OJ L 168, 28.6.2012, p. 21–23.

[5]The full detail is available at the https://www.efsa.europa.eu/en/events/event/ad-hoc-meeting-industry-association-amfep-joint-dossiers-food-enzymes.

3.1 | Source of the food enzyme

The subtilisin is produced with the non-genetically modified bacterium *Bacillus paralicheniformis* strain AP-1, which is deposited at the culture collection of National Institute of Technology and Evaluation (NITE Biological Resource Center (Japan)) as *B. licheniformis* with the deposit number NITE SD 00511.[6] The production strain was identified as *B. paralicheniformis* by phylogenomic and whole genome sequence (WGS) analysis, showing an average nucleotide identity [redacted] with respect to the type strain *B. paralicheniformis* KJ-16.[7,8,9]

The species *B. paralicheniformis* is included in the list of organisms for which the qualified presumption of safety (QPS) may be applied, provided that the absence of acquired antimicrobial resistance (AMR) genes, toxigenic activity and the inability to synthesise bacitracin are verified for the specific strain used (EFSA BIOHAZ Panel, 2020, 2022). WGS analysis of the strain did not show the presence of AMR genes of concern.[10,11] A cytotoxicity test made with culture supernatants indicated that the production strain *B. paralicheniformis* did not induce cell damage to Vero cells using the Lactate Dehydrogenase assay.[12] The WGS analysis showed the presence of genes involved in bacitracin biosynthesis and this important antimicrobial agent[13] was detected in the supernatant of an industrial culture of the production strain (620 mg/kg).[14,15] Therefore, the production strain cannot be considered to qualify for the QPS approach.

3.2 | Production of the food enzyme

The food enzyme is manufactured according to the Food Hygiene Regulation (EC) No 852/2004,[16] with food safety procedures based on Hazard Analysis and Critical Control Points, and in accordance with current Good Manufacturing Practice.[17]

The production strain is grown as a pure culture using a typical industrial medium in a submerged, batch fermentation system with conventional process controls in place. After completion of the fermentation, the solid biomass is removed from the fermentation broth by filtration. The filtrate containing the enzyme is then further purified and concentrated, including an ultrafiltration step in which enzyme protein is retained, while most of the low molecular mass material passes the filtration membrane and is discarded. Finally, the food enzyme was spray-dried prior to analysis.[18] 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.[19]

The Panel considered that sufficient information has been provided on the manufacturing process and the quality assurance system implemented by the applicant to exclude issues of concern.

3.3 | Characteristics of the food enzyme

3.3.1 | Properties of the food enzyme

The subtilisin is a single polypeptide chain of [redacted] amino acids.[20] The molecular mass of the mature protein, calculated from the amino acid sequence, is [redacted] kDa.[21] The food enzyme was analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. A consistent protein pattern was observed across all batches.[22] The gel showed a single major protein band corresponding to an apparent molecular mass of around [redacted] kDa. The food enzyme was tested for α-amylase and lipase activities and neither were detected.[23] No other enzyme activities were reported.

The in-house determination of subtilisin activity is based on the hydrolysis of casein (reaction conditions: pH 7.5, 30°C, 10 min) and determined by measuring the reaction of the released tyrosine with Folin–Ciocalteau's reagent, which is

[6]Technical Dossier/Subtilisin(AP)_Annexes_1-4/Annex 1.
[7]Technical Dossier/Subtilisin(AP)_Annexes_1-4/Annex 4/p. 5.
[8]Technical Dossier/Subtilisin(AP)_Annexes_1-4/Annex 2.
[9]Technical Dossier/Subtilisin(AP)_Annexes_1-4/Annex 3.
[10]Technical Dossier/Subtilisin(AP)_Annexes_1-4/Annex 4/p. 11.
[11]Additional information February 2024/ Answer to Annex Q2.a.
[12]Technical Dossier/Subtilisin(AP)_Annexes_5-16/Annex 5.
[13]WHO's List of Medically Important Antimicrobials: a risk management tool for mitigating antimicrobial resistance due to non-human use. Geneva: World Health Organization; 2024.
[14]Technical Dossier/Subtilisin(AP)_Annexes_1-4/Annex 4/p. 13.
[15]Additional information February 2024/Answer to Annex Q1.
[16]Regulation (EC) No 852/2004 of the European Parliament and of the Council of 29 April 2004 on the hygiene of food additives. OJ L 226, 25.6.2004, pp. 3–21.
[17]Technical Dossier/Subtilisin(AP)/p. 38/Annexes_5-16/Annex 6, Annex 7.
[18]Technical Dossier/Subtilisin(AP)/pp. 38-46/Annexes_5-16/Annex 8.
[19]Technical Dossier/Subtilisin(AP)/Annexes_5-16/Annex 9.
[20]Technical Dossier/Subtilisin(AP)/p. 46.
[21]Additional information February 2024/ Answer to Annex Q3.
[22]Technical Dossier/Subtilisin(AP)/p. 47/Annexes_5-16/Annex 14.
[23]Technical Dossier/Subtilisin(AP)/p. 48/Annexes_5-16/Annex 15.

detected spectrophotometrically. The enzyme activity is expressed in Proteolytic Unit of Nagase (PUN)/g. One PUN is defined as the amount of the enzyme that releases one μmol of tyrosine equivalent per minute under the assay conditions.[24]

The food enzyme has a temperature optimum around 65°C (pH 7.5) and a pH optimum around pH 10.0 (30°C). Thermostability was tested after a pre-incubation of the food enzyme for 30 min at different temperatures (pH 9.0). Enzyme activity decreased above 60°C, showing no residual activity above 65°C.[25]

3.3.2 | Chemical parameters

Data on the chemical parameters of the food enzyme were provided for three batches for commercialisation (Table 1).[26] The mean total organic solids (TOS) of the three food enzyme batches was 84.8% and the mean enzyme activity/TOS ratio was 1082 PUN/mg TOS.

TABLE 1 Composition of the food enzyme.

Parameters	Unit	Batches		
		1	2	3
Subtilisin activity	PUN/g[a]	951,000	992,000	810,000
Protein	%	62.4	59.3	53.3
Ash	%	12.4	10.5	10.3
Water	%	3.9	3.7	4.8
Total organic solids (TOS)[b]	%	83.7	85.8	84.9
Activity/TOS ratio	PUN/mg TOS	1136	1156	954

[a]PUN: Proteolytic Unit of Nagase (see Section 3.3.1).

[b]TOS calculated as 100% – % water – % ash.

3.3.3 | Purity

The lead content in the three commercial batches was below 0.05 mg/kg[27] 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, the concentration of arsenic was below the limit of detection (LoD) of the employed method.[28,29]

The food enzyme complies 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).[30] No antimicrobial activity was detected in any of the tested batches.[31]

Three food enzymes batches were analysed for the presence of bacitracin. In all samples, bacitracin was below the LoD of 5 mg/kg.[32]

The exposure to low concentrations of antimicrobials, including sub-inhibitory concentrations, may result in the selection of AMR bacteria (EFSA BIOHAZ Panel, 2021). For several antimicrobial agents, the lowest drug concentration that can result in enrichment of resistant bacteria, has been estimated. The predicted no effect concentration (PNEC) for bacitracin has been calculated by Bengtsson-Palme and Larsson (2016) to be 8 ng/mL. The Panel also noted that bacitracin may select for cross-resistance to colistin (Xu et al., 2018), a highest priority critically important antimicrobial.[33] The Panel considered that the LoD was insufficient to exclude the presence of bacitracin in the food enzyme at a concentration that would represent a risk for the development of resistance in bacteria.

The Panel considered that the information provided on the purity of the food enzyme was sufficient.

[24]Technical Dossier/Subtilisin(AP)/p. 48/Annexes_5-16/Annex 15.

[25]Technical Dossier/Subtilisin(AP)/pp. 48-49/Annexes_5-16/Annex 10.

[26]Technical Dossier/Subtilisin(AP)/pp. 50–51.

[27]Technical Dossier/Subtilisin(AP)/p. 51/Annexes_5-16/Annex 10; Additional information February 2024/Answer to Annex Q4.

[28]Technical Dossier/Subtilisin(AP)/p. 51/Annexes_5-16/Annex 10; Additional information February 2024/Answer to Annex Q4.

[29]LoDs: Pb = 0.05 mg/kg; As = 0.75 mg/kg.

[30]Technical Dossier/Subtilisin(AP)/p. 51/Annexes_5-16/Annex 10; Additional data February 2024/Answer to Annex Q4.

[31]Technical Dossier/Subtilisin(AP)/p. 51/Annexes_5-16/Annex 10; Additional data February 2024/Answer to Annex Q4.

[32]Additional information February 2024/Answer to Annex Q1, LoD = 5 mg/kg.

[33]WHO's List of Medically Important Antimicrobials: a risk management tool for mitigating antimicrobial resistance due to non-human use. Geneva: World Health Organization; 2024.

3.3.4 | Viable cells of the production strain

The absence of viable cells of the production strain in the food enzyme was demonstrated in three independent batches analysed in a total of nine technical replicates. [redacted]. No colonies were produced. A positive control was included.[34]

3.4 | Toxicological data

The production strain produces bacitracin, therefore, it does not meet the requirements for the QPS approach, which in principle would trigger the need for toxicological studies. However, in view of the risk identified in Section 3.3.3, the Panel considered it not justified to request the toxicological data to complete this section of the opinion.

3.4.1 | Allergenicity

The allergenicity assessment considered only the food enzyme and not any carriers or other excipients that may be used in the final formulation.

The potential allergenicity of the subtilisin produced with the *B. paralicheniformis* strain AP-01 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, twenty-eight matches with proteases annotated as respiratory allergens, two proteases classified as food allergens and one protease identified as a contact allergen were found. The matching food allergens were Cuc m 1, a subtilisin-like protease from *Cucumis melo* (melon) and Pun g 14, a chitinase III from *Punica granatum* (pomegranate), the matching contact allergen was Tri r 2. A match was also found with an alkaline protease from *Trichophyton rubrum* (Athlete's foot fungus).[35]

No information is available on oral and respiratory sensitisation or elicitation reactions of this subtilisin.[36]

No allergic reactions to oral ingestion of the contact allergen alkaline protease from *Trichophyton rubrum*, a fungus residing on the skin, are expected.

Several studies have shown that adults respiratorily sensitised to a food enzyme may be able to ingest the corresponding allergen without acquiring clinical symptoms of food allergy (Armentia et al., 2009; Cullinan et al., 1997; Poulsen, 2004).

Allergic reaction cannot be excluded in individuals allergic to melon and pomegranate.

[redacted], a product that may cause allergies or intolerances (listed in the Regulation (EU) No 1169/2011[37]), is used as a raw material. In addition, [redacted], known sources of allergens, are also present in the media fed to the microorganisms. However, during the fermentation process, these products will be degraded and utilised by the microorganisms for cell growth, cell maintenance and production of enzyme protein. In addition, the microbial biomass and fermentation solids are removed. Taking into account the fermentation process and downstream processing, the Panel considered that no potentially allergenic residues from these sources are present in the food enzyme.

The Panel considered that the risk of allergic reactions upon dietary exposure to this food enzyme, particularly in individuals sensitised to melon or pomegranate, cannot be excluded, but it would not exceed the risk of consuming melon or pomegranate.

3.5 | Dietary exposure

3.5.1 | Intended use of the food enzyme

The food enzyme is intended to be used in five food manufacturing processes at the recommended use levels summarised in Table 2.

[34]Additional information February 2024/ Answer to Annex Q2.b.

[35]Technical dossier/pp. 53-55/Annex 16.

[36]Additional information February 2024/ Answer to Annex Q5.

[37]Regulation (EU) No 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers, amending Regulations (EC) No 1924/2006 and (EC) No 1925/2006 of the European Parliament and of the Council, and repealing Commission Directive 87/250/EEC, Council Directive 90/496/EEC, Commission Directive 1999/10/EC, Directive 2000/13/EC of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and Commission Regulation (EC) No 608/2004.

TABLE 2 Intended uses and recommended use levels of the food enzyme as provided by the applicant.[38]

Food manufacturing process[a]	Raw material (RM)	Recommended use level (mg TOS/kg RM)[b]
Processing of cereals and other grains		
• Production of cereal-based products other than baked	Flour, rice	0.057–**0.57**
Processing of dairy products		
• Production of modified milk proteins	Whey protein concentrate (powder)[39]	**305**
Processing of plant- and fungal-derived products		
• Production of edible oils from plant and algae	Algal cells	2.32
• Production of protein hydrolysates from plants and fungi[40]	Soy protein, wheat protein	0.407–**4.07**
Processing of meat and fish products		
• Production of modified meat and fish products	Meat, fish[41]	**0.102**

Abbreviation: TOS, total organic solids.

[a]The name has been harmonised by EFSA according to the 'Food manufacturing processes and technical data used in the exposure assessment of food enzymes' (EFSA CEP Panel, 2023).

[b]The numbers in bold were used for calculation.

In the production of cereal-based products other than baked, the food enzyme is added to rice before cooking, or to flour during dough formation in noodles and pasta.[42] The subtilisin cleaves the peptide bonds in the gluten network, thus, improving rheology of the dough or the shelf life in cooked rice.[43] The food enzyme–TOS remain in the final products.

In the production of modified milk proteins, the food enzyme is added to whey protein concentrates[44] to achieve the desired degree of hydrolysis. The hydrolysis by subtilisin can also enhance the flavour of the resulting milk protein products (e.g. whey protein hydrolysates), which are subsequently used as ingredients in a variety of foods, including infant formula, follow-on formula and foods for special medical purposes. The food enzyme–TOS remain in the final foods.

In the production of edible oils from plant and algae, the food enzyme is added to the algae concentrate[45] alone or together with other peptidases to hydrolyse proteins of the algae cell wall.[46] The food enzyme–TOS are removed in the final processed foods by refining processes (EFSA CEP Panel, 2023).

In the production of modified meat and fish products, the food enzyme is added to meat or fish[47] to hydrolyse protein.[48] The food enzyme–TOS remain in the meat and fish products.

In the production of protein hydrolysates from plants and fungi, the food enzyme is added to plant proteins (e.g. wheat and soy proteins).[49] The subtilisin is used alone or together with other peptidases to achieve the desired degree of hydrolysis, to increase the yield and to enhance flavours. The food enzyme–TOS remain in these protein hydrolysates.

Based on data provided on thermostability (see Section 3.3.1) and the downstream processing step applied in the food processes, it is expected that the subtilisin is inactivated or removed in the food manufacturing processes listed in Table 2.

3.5.2 | Dietary exposure estimation

In accordance with the guidance document (EFSA CEP Panel, 2021), dietary exposure was calculated for the four food manufacturing processes where the food enzyme–TOS remain in the final foods.

Chronic exposure to the food enzyme–TOS was calculated by combining the maximum recommended use level with individual consumption data (EFSA CEP Panel, 2021). The estimation involved selection of relevant food categories and application of technical conversion factors (EFSA CEP Panel, 2023). 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).

[38]Additional information February 2024 / revised Table 9.

[39]Additional information February 2024/Answer to Annex Q6.

[40]Additional information February 2024/Answer to Annex Q8.

[41]Additional information February 2024/Answer to Annex Q7.

[42]Additional information February 2024/Answer to Annex Q9.

[43]Technical dossier/p. 66.

[44]Additional information February 2024/Answer to Annex Q6.

[45]Technical dossier/pp. 57–58.

[46]Technical dossier/p. 67.

[47]Additional information February 2024/Answer to Annex Q7.

[48]Technical dossier/p. 72.

[49]Additional information February 2024/Answer to Annex Q8.

Table 3 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 48 dietary surveys (covering infants, toddlers, children, adolescents, adults and the elderly), carried out in 26 European countries (Appendix B). The highest dietary exposure was estimated to be 0.875 mg TOS/kg bw per day in infants at the 95th percentile.

TABLE 3 Summary of the estimated dietary exposure to food enzyme–TOS in six population groups.

	Estimated exposure (mg TOS/kg body weight per day)					
Population group	**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)	0.004–0.353 (12)	0.009–0.235 (15)	0.005–0.014 (19)	0.002–0.009 (21)	0.001–0.010 (22)	0–0.007 (23)
Min–max 95th percentile (number of surveys)	0.015–0.875 (11)	0.031–0.643 (14)	0.010–0.062 (19)	0.006–0.027 (20)	0.003–0.036 (22)	0.001–0.011 (22)

Abbreviation: TOS, total organic solids.

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 4.

TABLE 4 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	+
For the production of cereal-based products other than baked, although only rice, pasta and noodles were mentioned by the applicant,[50] the food categories chosen for calculation covered also breakfast cereals	+
In the production of modified milk proteins, the calculation included not only whey protein hydrolysates but also milk protein isolates and concentrates	+
In the absence of analytical data to demonstrate the removal of the food enzyme–TOS in infant formulae, follow-on formulae and foods for special medical purposes,[51] these highly regulated formulae were included in the calculation.	+
Use of recipe fractions in disaggregation FoodEx categories	+/–
Use of technical factors in the exposure model	+/–
Exclusion of one process from the exposure assessment: - production of edible oils from plant and algae	–

Abbreviation: +, uncertainty with potential to cause overestimation of exposure; –, uncertainty with potential to cause underestimation of exposure; TOS, total organic solids.

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

The exclusion of one food manufacturing process from the exposure assessment was based on > 99% of TOS removal. This is not expected to have an impact on the overall estimate derived.

[50] Additional information February 2024/Answer to Annex Q9.

[51] Additional information February 2024/Answer to Annex Q6.

3.6 | Margin of exposure

In the absence of appropriate data, a margin of exposure could not be calculated.

4 | CONCLUSIONS

Based on the data provided, the Panel could not exclude the presence of bacitracin, a medically important antimicrobial, and consequently the safety of the food enzyme subtilisin produced with the non-genetically modified *B. paralicheniformis* strain AP-01 could not be established.

5 | DOCUMENTATION AS PROVIDED TO EFSA

Application for authorisation of food enzyme, Subtilisin from Bacillus licheniformis AP-01 in accordance with Regulation (EC) No 1331/2008. September 2022. Submitted by Nagase (Europa) GmbH.

Additional information. February 2024. Submitted by Nagase (Europa) GmbH.

ABBREVIATIONS

AMR	antimicrobial resistance
bw	body weight
CAS	chemical abstracts service
CEP	EFSA Panel on Food Contact Materials, Enzymes and Processing Aids
EC	European Commission
EINECS	European Inventory of Existing Commercial Chemical Substances
EU	European Union
FAO	Food and Agricultural Organization of the United Nations
GLP	good laboratory practice
GMO	genetically modified organism
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
PNEC	predicted no effect concentration
PUN	Proteolytic Unit of Nagase
QPS	qualified presumption of safety
TOS	total organic solids
WGS	whole genome sequence
WHO	World Health Organization

CONFLICT OF INTEREST

If you wish to access the declaration of interests of any expert contributing to an EFSA scientific assessment, please contact interestmanagement@efsa.europa.eu.

REQUESTOR

European Commission

QUESTION NUMBER

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NOTE

The full opinion will be published in accordance with Article 12 of Regulation (EC) No 1331/2008 once the decision on confidentiality will be received from the European Commission.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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APPENDIX A

Dietary exposure estimates to the food enzyme–TOS in details

Appendix A can be found in the online version of this output (in the 'Supporting information' section). The file contains two sheets, corresponding to two tables.

Table 1: Average 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, Spain
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, Republic of North Macedonia*, Serbia*, 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, Republic of North Macedonia*, Serbia*, Spain, Sweden
Adolescents	From 10 years up to and including 17 years of age	Austria, Belgium, Bosnia and Herzegovina*, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Italy, Latvia, Montenegro*, Netherlands, Portugal, Romania, Serbia*, Slovenia, Spain, Sweden
Adults	From 18 years up to and including 64 years of age	Austria, Belgium, Bosnia and Herzegovina*, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Montenegro*, Netherlands, Portugal, Romania, Serbia*, 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, Montenegro*, Netherlands, Portugal, Romania, Serbia*, Slovenia, Spain, Sweden

*Consumption data from these pre-accession countries are not reported in Table 3 of this opinion, however, they are included in Appendix B for testing purpose.

[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).