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SCIENTIFIC OPINION

Safety evaluation of the food enzyme bacillolysin from the non-genetically modified *Bacillus amyloliquefaciens* strain AE-NP

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Abstract

The food enzyme bacillolysin (EC 3.4.24.28) is produced with the non-genetically modified *Bacillus amyloliquefaciens* strain AE-NP by Amano Enzyme Inc. The production strain meets the requirements for the qualified presumption of safety (QPS) approach to safety assessment. The food enzyme is intended to be used in 14 food manufacturing processes. Since residual amounts of total organic solids (TOS) are removed in three manufacturing processes, dietary exposure was calculated only for the remaining 11 food manufacturing processes in which the food enzyme-TOS is retained. It was estimated to be up to 35.251 mg TOS/kg body weight (bw) per day in European populations. As the production strain qualifies for the QPS approach and no issue of concern arising from the production process of the food enzyme were identified, the Panel considered that no toxicological studies other than the assessment of allergenicity were necessary. A search for the similarity of the amino acid sequence of the food enzyme to known allergens was made and no match was found. The Panel considered that the risk of allergic reactions by dietary exposure cannot be excluded, but the likelihood is low. Based on the data provided, the Panel concluded that this food enzyme does not give rise to safety concerns under the intended conditions of use.

KEYWORDS

bacillolysin, *Bacillus amyloliquefaciens*, *Bacillus* metalloendopeptidase, EC 3.4.24.28, food enzyme

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 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 1331/2008[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 1332/20082 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 Bacillolysin from *Bacillus amyloliquefaciens*, and the companies "Danisco US Inc." for the authorisation of the food enzymes Alpha-amylase from a genetically modified strain of *Bacillus licheniformis* (DP-Dzb44), Beta-galactosidase from a genetically modified strain of *Bacillus subtilis* (DP-Ezg29) and Endo-1,4-beta-xylanase from a genetically modified strain of *Bacillus subtilis* (DP-Ezd31), and "Intertek Scientific & Regulatory Consultancy" for the authorisation of the food enzyme Beta-Fructofuranosidase from *Aspergillus fijiensis* (strain ATCC® 20611™).

Following the requirements of Article 12.1 of Regulation (EC) No 234/2011[3] implementing Regulation (EC) No 1331/20083, the Commission has verified that the five 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 Alpha-amylase from a genetically modified strain of *Bacillus licheniformis* (DP-Dzb44), Bacillolysin from *Bacillus amyloliquefaciens*, Beta-galactosidase from a genetically modified strain of *Bacillus subtilis* (DP-Ezg29), Endo-1,4-beta-xylanase from a genetically modified strain of *Bacillus subtilis* (DP-Ezd31) and Beta-Fructofuranosidase from *Aspergillus fijiensis* (strain ATCC® 20611™) in accordance with Article 17.3 of Regulation (EC) No 1332/20082 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 food enzyme bacillolysin from *Bacillus amyloliquefaciens* 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-2015-00837. During a meeting between EFSA, the European Commission and the Association of Manufacturers and Formulators of Enzyme Products (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 joint dossier EFSA-Q-2015-00837. This data package, identified as EFSA-Q-2022-00573, concerns the food enzyme bacillolysin that is produced with a strain of *Bacillus amyloliquefaciens* (AE-NP) and submitted by Amano Enzyme Inc.

2 | DATA AND METHODOLOGIES

2.1 | Data

The applicant has submitted a dossier in support of the application for authorisation of the food enzyme bacillolysin from a non-genetically modified *Bacillus amyloliquefaciens* strain AE-NP.

Additional information was requested from the applicant during the assessment process on 30 May 2023 and 14 December 2023, and received on 27 September 2023 and 11 January 2024, respectively (see 'Documentation provided to EFSA').

Following the request for additional data sent by EFSA on 14 December 2023, the applicant requested a clarification teleconference on 10 January 2024, after which the applicant provided additional data on 11 January 2024.

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	Bacillolysin
Systematic name	–
Synonyms	*Bacillus* metalloendopeptidase; *Bacillus subtilis* neutral proteinase
IUBMB no	EC 3.4.24.28
CAS no	9080-56-2
EINECS no	232-991-2

Bacillolysins catalyse the hydrolysis of peptide bonds of proteins with broad specificity, releasing peptides and amino acids. The enzyme under assessment is intended to be used in 14 food manufacturing processes: processing of cereals and other grains for the production of (1) baked products, (2) brewed products, (3) distilled alcohol and (4) low-protein cereals; processing of dairy products for the production of (5) flavouring preparations and (6) modified milk proteins; processing of meat and fish products for the production of (7) modified meat and fish products and (8) protein hydrolysates; processing of plant- and fungal-derived products for the production of (9) plant-based analogues of milk and milk products, (10) tea and other herbal and fruit infusions, (11) edible oils from plant and algae and (12) protein hydrolysates; (13) processing of eggs and egg products and (14) processing of yeast and yeast products.

[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 bacillolysin is produced with the non-genetically modified bacterium *Bacillus amyloliquefaciens* strain AE-NP, which is deposited at the National Institute of Technology and Evaluation (NITE) Biological Resource Center (Japan), with the deposit number [redacted].[6] The production strain, [redacted], was identified as *B. amyloliquefaciens* by whole genome sequence (WGS) analysis, [redacted].[7]

The species *B. amyloliquefaciens* 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 and toxigenic activity are verified for the specific strain used (EFSA, 2007; EFSA BIOHAZ Panel, 2022). The production strain *B. amyloliquefaciens* AE-NP was found not to be cytotoxic to Vero cells using a lactate dehydrogenase (LDH) assay.[8] The WGS of the production strain was interrogated for the presence of antimicrobial resistance genes using two regularly updated databases with thresholds of > 70% identity and > 60% coverage. No genes of concern were identified.[9]

Therefore, the production strain was considered to meet the requirements for the QPS approach to safety assessment.

3.2 | Production of the food enzyme

The food enzyme is manufactured according to the Food Hygiene Regulation (EC) No 852/2004,[10] with food safety procedures based on Hazard Analysis and Critical Control Points and in accordance with current good manufacturing practice.[11]

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.[12] 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.[13]

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 mature bacillolysin is a single polypeptide chain of [redacted] amino acids.[14] The molecular mass of the mature protein, calculated from the amino acid sequence, is [redacted] kDa.[15] The food enzyme was analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.[16] A consistent protein pattern was observed across all batches. The gel showed a major protein band corresponding to an apparent molecular mass of about [redacted] kDa, consistent with the expected mass of the enzyme. No other enzyme activities were reported.[17]

The determination of bacillolysin activity is based on the hydrolysis of casein (reaction conditions: pH 7.0, 37°C, 60 min) and is determined by measuring the release of peptides, which are quantified spectrophotometrically at 660 nm after reaction with Folin's reagent. The bacillolysin activity is expressed in protein digestive activity units (U)/g. One unit is defined as the amount of enzyme that produces peptides equal to 1 µg of tyrosine per minute under the conditions of the assay.[18]

The food enzyme has a temperature optimum around 50°C (pH 7.0) and a pH optimum around pH 7.0 (37°C). Thermostability was tested after a pre-incubation of the food enzyme for 30 min at different temperatures (pH 7.0). The enzyme activity decreased above 30°C, showing no residual activity after pre-incubation above 65°C.

[6]Technical dossier/Annex 4.
[7]Technical dossier/Annex 5.
[8]Technical dossier/Annex 7.
[9]Technical dossier/Annex 6 and Additional data September 2023/Annex 1.
[10]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.
[11]Technical dossier/p. 44/Annex 8.1, Annex 8.2.
[12]Technical dossier/pp. 44–51/Annex 9.
[13]Technical dossier/Annex 10.
[14]Technical dossier/p. 34/Annex 12.
[15]Technical dossier/p. 34.
[16]Technical dossier/p. 33.
[17]Technical dossier/p. 35.
[18]Technical dossier/pp. 34–35/Annex 2.

3.3.2 | Chemical parameters

Data on the chemical parameters of the food enzyme were provided for three batches intended for commercialisation (Table 1).[19] The mean total organic solids (TOS) was 85.4% and the mean enzyme activity/TOS ratio was 2135 U/mg TOS.

TABLE 1 Composition of the food enzyme.

		Batches		
Parameters	**Unit**	**1**	**2**	**3**
Bacillolysin activity	U/g[a]	1,970,000	1,630,000	1,870,000
Protein	%	51.6	46.2	50.2
Ash	%	10.0	11.7	11.6
Water	%	3.2	3.6	3.8
Total organic solids (TOS)[b]	%	86.8	84.7	84.6
Activity/TOS ratio	U/mg TOS	2270	1924	2210

[a]U: Unit (see Section 3.3.1).
[b]TOS calculated as 100% – % water – % ash.

3.3.3 | Purity

The lead content in all batches was below 1 mg/kg,[20,21] which complies with the specification for lead as laid down in the general specifications for enzymes used in food processing (FAO/WHO, 2006).

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).[22] No antimicrobial activity was detected in any of the tested batches.[23]

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

3.4 | Toxicological data

As the production strain qualifies for the QPS approach of safety assessment and no issues of concern arising from the production process of the food enzyme were identified (see Sections 3.1, 3.2 and 3.3), the Panel considered that no toxicological studies other than the assessment of allergenicity were necessary (EFSA CEP Panel, 2021).

3.4.1 | Allergenicity

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

The potential allergenicity of the bacillolysin produced with the *B. amyloliquefaciens* strain AE-NP 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.[24]

No information was available on oral and respiratory sensitisation or elicitation reactions of this bacillolysin.

Faeces from the astigmatid mite (Acari: Acaridida) have been associated with respiratory allergy; the major allergen is suggested to be bacillolysin produced by *Bacillus cereus* parasitising acari (Erban et al., 2016). Several studies have shown that individuals respiratorily sensitised to an enzyme can ingest the allergen without acquiring clinical symptoms of food allergy (Armentia et al., 2009; Cullinan et al., 1997; Poulsen, 2004). In addition, no allergic reactions upon dietary exposure to any other bacillolysin have been reported in the literature.

[19]Technical dossier/p. 32/Annex 1, Annex 2, Annex 3.
[20]Technical dossier/p. 33/Annex 1, Annex 3.
[21]Limit of detection (LoD): Pb = 1 mg/kg.
[22]Technical dossier/p. 33/Annex 1, Annex 3.
[23]Technical dossier/p. 33/Annex 1, Annex 3.
[24]Technical dossier/pp. 66–67/Annex 12.

[redacted], products that may cause allergies or intolerances (listed in the Regulation (EU) No 1169/2011[25]), are used as raw materials. In addition, [redacted] known sources of allergens, are 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 cannot be excluded, but the likelihood is low.

3.5 | Dietary exposure

3.5.1 | Intended use of the food enzyme

The food enzyme is intended to be used in 14 food manufacturing processes at the recommended use levels summarised in Table 2.

TABLE 2 Intended uses and recommended use levels of the food enzyme as provided by the applicant.[26,27]

Food manufacturing process[a]	Raw material (RM)	Recommended use level (mg TOS/kg RM)[b]	
Processing of cereals and other grains			
• Production of baked products	Flour	**45.4**	
• Production of brewed products	Cereals	**227.2**	
• Production of distilled alcohol	Cereals	90.9	
• Low-protein cereal production[c]	Cereals	18,172.7	
Processing of dairy products			
• Production of flavouring preparation from dairy products	Dairy products	**45.4**	
• Production of modified milk proteins[28]	Milk proteins	**227.2**	Other uses
		13,629.5	Infant formulae
Processing of meat and fish products			
• Production of modified meat and fish products	Meat and fish products	**45.4**	
• Production of protein hydrolysates from meat and fish proteins[29]	Meat and fish proteins	**227.2**	
Processing of plant- and fungal-derived products			
• Production of plant-based analogues of milk and milk products	Cereals, nuts, pulses, seeds	**9.1**	
• Production of edible oils from plant and algae	Algal cells	1088.1	
• Production of tea and other herbal and fruit infusions	Tea leaves, herbs, fruits	**27.3**	
• Production of protein hydrolysates from plants and fungi[30]	Plant proteins	**227.2**	Other uses
		13,629.5	Infant formulae
Processing of eggs and egg products	Eggs	**45.4**	
Processing of yeast and yeast products	Yeast	**45.4**	

[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 represent the maximum recommended use levels which were used for calculation.

[c]This process is not included in the 'Food manufacturing processes and technical data used in the exposure assessment of food enzymes' (EFSA CEP Panel, 2023).

[25]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.

[26]Technical dossier/pp. 55–56.

[27]Technical dossier/Additional information September 23.

[28]Technical dossier/Additional information September 23/Answer 9.

[29]Technical dossier/Additional information September 23/Answer 9.

[30]Technical dossier/Additional information September 23/Answer 9.

In the production of baked products, the food enzyme is added to flour during the preparation of the dough or batter.[31] The bacillolysin cleaves peptide bonds in the gluten network, thus reducing the rigidity of the dough. The food enzyme-TOS remains in the final food products (e.g. bread, cakes, biscuits).

In the production of brewed products, the food enzyme is added to the grains during the mashing step for beer production[32] or during liquefaction and fermentation to produce sake and other cereal-based liquors.[33] The bacillolysin hydrolyses proteins of the cereals to release free amino nitrogen for the growth of yeast during fermentation. In addition, the partial degradation of protein promotes the clarity of beer. The food enzyme-TOS remains in the brewed products.

In the production of distilled alcohol, the food enzyme may be added to cereals during the slurry mixing, liquefaction, pre-saccharification or fermentation steps.[34] The bacillolysin hydrolyses proteins surrounding the starch granules, making them accessible to amylolytic enzymes for the conversion into fermentable sugars.[35] The food enzyme-TOS is not carried over to the final processed foods (EFSA CEP Panel, 2023).

In the production of low-protein cereals, the food enzyme is added to the cereals after the rinsing phase.[36] The proteolytic reaction of the food enzyme reduces the protein content of the final products.[37] The food enzyme-TOS is removed from the cereals by repeated washing. To substantiate this removal, the amount of proteins present after each washing step was measured spectrophotometrically and by means of SDS-PAGE analysis with Coomassie blue staining. The data showed that the amount of peptidase present in the cereals after the repeated washings is less than 1%.[38] These data were considered by the Panel as sufficient to confirm the removal of the food enzyme-TOS in these low-protein cereals.

In the production of flavouring preparations from dairy products, the food enzyme is added to dairy products (e.g. curd, cheese)[39] to enhance sensory properties. The food enzyme-TOS remains in these enzyme-modified dairy ingredients (EMDI), which are subsequently used as an ingredient to formulate a variety of foods such as processed cheese, cheese sauce, cheese powder, salad dressings and snacks.

In the production of modified milk proteins, the food enzyme can be added with or without other peptidases to milk proteins after the mixing with water.[40] The hydrolysis improves solubility and taste. The food enzyme-TOS remains in the modified milk proteins, which are ingredients of a variety of final foods (e.g. sport products, nutritional bars). Whey protein hydrolysates, which are ingredients of infant formulae, follow-on formulae and food for special medical purposes, are further purified by ultra- and nanofiltration.[41] The applicant provided analytical data to support the food enzyme-TOS removal in infant formulae. The amount of proteins before and after the ultrafiltration was measured spectrophotometrically in a food enzyme solution.[42] These data were considered by the Panel as not sufficient to confirm the absence of TOS in the infant formulae, considering that the majority of the TOS components, that have a low molecular mass, may pass through the ultrafiltration membrane. Therefore, the Panel opted for a conservative scenario by considering the full remaining of the food enzyme-TOS in infant formulae.

To manufacture protein hydrolysates, the food enzyme is added with or without other peptidases to a variety of partially purified proteins from plant (e.g. legumes and cereals) and animal (e.g. meat, collagen) materials[43] during hydrolysis.[44] Bacillolysin is used to achieve hydrolysis and enhance flavour of the resulting protein hydrolysates, which are subsequently used as ingredients in a variety of foods. The food enzyme-TOS remains in the final foods.

In the production of modified meat and fish products, the food enzyme is used to treat meat and fish to obtain protein concentrates that are subsequently used as ingredients in a variety of foods.[45] The food enzyme-TOS remains in the final foods.

In the production of plant-based analogues of milk and milk products, the food enzyme is added to the slurry of plant materials (e.g. oat flour) during the incubation step.[46] The hydrolysis by bacillolysin increases the yield and enhances the flavour. The food enzyme-TOS remains in these plant-based analogues of milk and milk products.

In the production of edible oils from algae, the food enzyme is added to the algae concentrate during the cell lysis step[47] to hydrolyse proteins of the algae cell wall.[48] The food enzyme-TOS is removed in the final processed oils by refining processes (EFSA CEP Panel, 2023).

[31]Technical dossier/Annex 11/p. 1.
[32]Technical dossier/Annex 11/p. 11.
[33]Technical dossier/ Additional information September 23/Answer 8.
[34]Technical dossier/Annex 11/p. 13.
[35]Technical dossier/ Additional information September 23/Answer 6.
[36]Technical dossier/ Annex 11/p. 15.
[37]Technical dossier/p. 81.
[38]Technical dossier/Additional information September 23/Annex 3–2.
[39]Technical dossier/Annex 11/p. 2.
[40]Technical dossier/Annex 11/p. 7.
[41]Technical dossier/Annex 11/p. 8.
[42]Technical dossier/ Additional information September 23/Annex 3–1.
[43]Technical dossier/ Additional information September 23/Answer 9.
[44]Technical dossier/Annex 11/p. 7.
[45]Technical dossier/Annex 11/pp. 4–6.
[46]Technical dossier/Annex 11/p. 10.
[47]Technical dossier/Annex 11/p. 14.
[48]Technical dossier/p. 80.

In processing of tea, herbal and fruit infusions, the food enzyme is added to tea leaves, herbs and fruits[49] to enhance the sensory properties of the infusions.[50] The food enzyme-TOS remains in the final products.

In the processing of eggs and egg products, the food enzyme is added to the whole liquid egg, to the egg white and to the egg yolk during the incubation step.[51] The hydrolysis by bacillolysin improves the sensory and technological properties of these products.[52] The food enzyme-TOS remains in the final egg products.

In yeast processing, the food enzyme is added during the autolysis and the incubation step.[53] The bacillolysin is used to hydrolyse the insoluble proteins, optimising the extraction process and improving the functional properties of the yeast products. The food enzyme-TOS remains in yeast products.

Based on data provided on thermostability (see Section 3.3.1) and the downstream processing steps applied in the food manufacturing processes, it is expected that this bacillolysin will be inactivated or removed in all 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 only for the 11 food manufacturing processes where the food enzyme-TOS remains 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).

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

TABLE 3 Summary of the 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)	0.315–9.549 (12)	0.670–4.675 (15)	0.184–1.351 (19)	0.084–0.769 (21)	0.272–0.734 (22)	0.264–0.508 (23)
Min–max 95th percentile (number of surveys)	1.086–35.251 (11)	1.543–15.788 (14)	0.380–2.542 (19)	0.179–1.447 (20)	0.641–1.842 (22)	0.522–1.008 (22)

in 26 European countries (Appendix B). The highest dietary exposure was estimated to be 35.251 mg TOS/kg bw per day in infants at the 95th percentile.

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.

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.

[49]Technical dossier/Annex 11/p. 14.
[50]Technical dossier/p. 80.
[51]Technical dossier/Annex 11/p. 10.
[52]Technical dossier/p. 83.
[53]Technical dossier/Annex 11/p. 9.

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	
For yeast processing, although only yeast extract is produced,[54] the food categories chosen for calculation cover also those containing mannoproteins resulting from the treatment of yeast cell walls	+
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	+/–
Exclusion of three processes from the exposure assessment: – Production of distilled alcohol – Production of low-protein cereals – Production of edible oils from plant and algae	–

Abbreviations: +, uncertainty with potential to cause overestimation of exposure; –, uncertainty with potential to cause underestimation of exposure.

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

3.6 | Margin of exposure

Since no toxicological assessment was considered necessary by the Panel, a margin of exposure was not calculated.

4 | CONCLUSIONS

Based on the data provided, the QPS status of the production strain and the absence of issues of concern arising from the production process, the Panel concluded that the food enzyme bacillolysin produced with the non-genetically modified *Bacillus amyloliquefaciens* strain AE-NP does not give rise to safety concerns under the intended conditions of use.

5 | DOCUMENTATION AS PROVIDED TO EFSA

Application for authorisation of Bacillolysin from *Bacillus amyloliquefaciens* AE-NP in accordance with Regulation (EC) No 1331/2008. 15 September 2022. Submitted by Amano Enzyme Inc.

Additional information. September 2023 and January 2024. Amano Enzyme Inc.

ABBREVIATIONS

AMR	Anti-microbial resistance
bw	body weight
CAS	Chemical Abstracts Service
CEP	EFSA Panel on Food Contact Materials, Enzymes and Processing Aids
EINECS	European Inventory of Existing Commercial Chemical Substances
FAO	Food and Agricultural Organisation 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
LDH	Lactate dehydrogenase
LoD	limit of detection
MOE	margin of exposure

[54]Technical dossier/ Additional information September 23/Answer to question 7.

QPS	qualified presumption of safety
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TOS	total organic solids
WGS	whole genome sequencing
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 Macedoni*, 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 included 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).