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SCIENTIFIC OPINION

Safety evaluation of the food enzyme microbial collagenase from the genetically modified *Streptomyces violaceoruber* strain pCol

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Abstract

The food enzyme microbial collagenase (EC 3.4.24.3) is produced with the genetically modified *Streptomyces violaceoruber* strain pCol by Nagase (Europa) GmbH. The genetic modifications do not give rise to safety concerns. The food enzyme is free from viable cells of the production organism and its DNA. It is intended to be used in two food manufacturing processes: the production of modified meat and fish products and the production of protein hydrolysates from meat and fish proteins. The dietary exposure to the food enzyme–total organic solids (TOS) was estimated to be up to 1.098 mg TOS/kg body weight (bw) per day in European populations. Genotoxicity tests 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 940 mg TOS/kg bw per day, the highest dose tested, which, when compared with the estimated dietary exposure, resulted in a margin of exposure of at least 856. 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

collagenase, EC 3.4.24.3, food enzyme, genetically modified microorganism, microbial collagenase, *Streptomyces violaceoruber*

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 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 companies "Chr. Hansen" for the authorisation of the food enzyme Endothiapepsin from a genetically modified strain of *Cryphonectria parasitica* (strain DSM-29549); "Nagase (Europa) GmbH." for the authorisation of the food enzymes L-Ascorbate oxidase from *Cucurbita pepo* and *Cucurbita moschata*; and Microbial collagenase from a genetically modified strain of *Streptomyces violaceoruber* (strain pCol); "Novozymes A/S" for the authorisation of the food enzyme Inulinase from *Aspergillus niger* (strain NZYM-KF); and "Danisco US Inc" for the authorisation of the food enzyme Endo-1,3(4)-beta-glucanase from a genetically modified strain of *Bacillus Subtilis* (DP-Ezm28).

Following the requirements of Article 12.1 of Regulation (EC) No 234/2011[3] 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.

1.1.2 | Terms of Reference

The European Commission requests the European Food Safety Authority to carry out the safety assessments on the food enzymes Endothiapepsin from a genetically modified strain of *Cryphonectria parasitica* (strain DSM-29549), L-Ascorbate oxidase from *Cucurbita pepo* and *Cucurbita moschata*, Microbial collagenase from a genetically modified strain of *Streptomyces violaceoruber* (strain pCol); Inulinase from *Aspergillus niger* (strain NZYM-KF) and Endo-1,3(4)-beta-glucanase from a genetically modified strain of *Bacillus Subtilis* (DP-Ezm28) in accordance with article 17.3 of Regulation (EC) No 1332/2008 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, 11.3.2011, 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 microbial collagenase from the genetically modified *S. violaceoruber* strain pCol.

2 | DATA AND METHODOLOGIES

2.1 | Data

The applicant has submitted a dossier in support of the application for authorisation of the food enzyme microbial collagenase from a genetically modified *S. violaceoruber* strain pCol. The dossier was updated on 31 March 2016.

Additional information was requested from the applicant during the assessment process on 20 January 2023 and received on 17 July 2023 (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	Microbial collagenase
Systematic name	–
Synonyms	*Clostridium histolyticum* collagenase; clostridiopeptidase A; collagenase A; collagenase I
IUBMB no	EC 3.4.24.3
CAS no	9001-12-1
EINECS no	232-582-9

Microbial collagenases catalyse the hydrolysis of peptide bonds in collagen, resulting in the generation of peptides. The enzyme under assessment is intended to be used in two food manufacturing processes as described in the EFSA guidance (EFSA CEP Panel, 2023): the production of modified meat and fish products and the production of protein hydrolysates from meat and fish proteins.

3.1 | Source of the food enzyme

The enzyme is produced with the genetically modified bacterium *Streptomyces violaceoruber* strain pCol, which is deposited [redacted] with the deposit number [redacted].[4]

The production strain was identified as *Streptomyces violaceoruber* by average nucleotide identity (ANI) analysis of the whole genome sequence (WGS) of the production strain [redacted].[5] The WGS of the production strain was searched for the presence of antimicrobial resistance genes, using two regularly updated databases with an [redacted]. Several antimicrobial resistance genes of possible concern were identified.[6]

[4]Technical Dossier/2nd submission/Annex 3.2 Att.1.

[5]Technical Dossier/2nd submission/Annex 3.2 Att.7.

[6]Technical Dossier/Additional information/July 2023/Answer to Annex Q1.

3.1.1 | Characteristics of the parental and recipient microorganisms

The recipient microorganism [redacted].[7]

3.1.2 | Characteristics of introduced sequences

The sequence encoding the collagenase (*col*) was derived [redacted].

3.1.3 | Description of the genetic modification process

The production strain *S. violaceoruber* strain pCol was developed [redacted].[8] [redacted].[9]

3.1.4 | Safety aspects of the genetic modification

The technical dossier contains all necessary information on the donor organism and the genetic modification process.

The production strain *S. violaceoruber* pCol [redacted].[10]

The production strain contains the [redacted], which confers resistance towards an antimicrobial and is considered a hazard.

3.2 | Production of the food enzyme

The food enzyme is manufactured according to the Food Hygiene Regulation (EC) No 852/2004,[11] with food safety procedures based on Hazard Analysis and Critical Control Points, and in accordance with current Good Manufacturing Practice.[12]

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 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. The food enzyme was then formulated as a liquid or solid food enzyme preparation.[13] 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.[14,15]

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.

[7]Technical Dossier/Additional information/July 2023/Answer to Annex Q1.
[8]Technical Dossier/2nd submission/Annex 3.2-GMM dossier p. 16.
[9]Technical Dossier/2nd submission/Annex 3.2 Att. 5.
[10]Technical Dossier/2nd submission/Annex 3.2 Att. 6.
[11]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.
[12]Technical dossier/Annex A4.4 & add info January 2016 Annex A4.3.
[13]Technical dossier/COL petition dossier p. 40 Figure 11 & p. 42 Table 6.
[14]Technical dossier/COL petition dossier p. 41 Table 5.
[15]Technical dossier/Additional information/July 2023/Answer to Annex Q6.

3.3 | Characteristics of the food enzyme

3.3.1 | Properties of the food enzyme

The collagenase is a single polypeptide chain of 865 amino acids. The molecular mass of the mature protein, calculated from the amino acid sequence, is around 92.4 kDa.[16] The food enzyme was analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A consistent protein pattern was observed across all batches. The gels showed a major protein band migrating between 75 and 100 kDa marker bands, consistent with the expected mass of the enzyme.[17] The food enzyme was tested for lipase and α-amylase activities, and lipase activity was detected.[18] No other enzyme activities were reported.

The in-house determination of collagenase activity is based on the hydrolysis of carbobenzoxyglycyl-prolyl-leucyl-glycyl-proline (reaction conditions: pH 8, 30°C, 10 min) and determined by measuring the release of the dipeptide Gly-Pro spectrophotometrically at 570 nm after reaction with ninhydrin. The collagenase activity is expressed in Units/g (U/g).[19] One U is defined as the amount of enzyme that causes an increase in ninhydrin colouration corresponding to 1 µmol of glycine per minute.[20]

The food enzyme has a temperature optimum around 50°C (pH 8) and a pH optimum around pH 8 (30°C). The thermostability was tested after a pre-incubation of the food enzyme for 60 min at different temperatures (pH 8). The enzyme activity decreased above 35°C, showing no residual activity above 60°C.[21]

3.3.2 | Chemical parameters

Data on the chemical parameters for three batches used for commercialisation and one used for toxicological studies are shown in Table 1. The mean total organic solids (TOS) of the batches used for commercialisation was 21.1% and the mean enzyme activity/TOS ratio was 12.8 U/mg TOS. Prior to drying, the food enzyme was stabilised with dextrin.

TABLE 1 Composition of the food enzyme preparation.[22]

		Batches			
Parameters	**Unit**	**1**	**2**	**3**	**4**[a]
Collagenase activity	U/g[b]	2710	2690	2670	10,287
Protein	%	2.54	3.04	2.65	25.9
Ash	%	0.57	0.51	0.58	1.65
Water	%	4.4	4.0	4.3	4.4
Dextrin	%	74.15	74.15	74.15	_
Total organic solids (TOS)[c]	%	20.9	21.4	21.0	93.95
Activity/mg TOS	U/mg TOS	13.0	12.6	12.7	10.9

[a]Batch used for the toxicological studies.
[b]U: Collagenase Unit (see Section 3.3.1).
[c]TOS calculated as 100% – % water – % ash – % dextrin.

3.3.3 | Purity

The lead content in three commercial batches and in the batch used for toxicological studies was below 0.1 mg/kg, 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, arsenic and mercury contents were below the limits of detection (LoDs) of the employed methods.[23,24] For cadmium, the mean concentrations of three commercial batches and in the toxicological batch were 0.02 and 0.09 mg/kg, respectively.[25] The Panel considered these concentrations as not of concern.

[16]Technical dossier/Additional information/July 2023/Answer to Annex Q8.
[17]Technical dossier/Additional information/July 2023/Answer to Annex Q8.
[18]Technical dossier/Annex A2.4 (Confidential), A2.5 & A7.11.
[19]Technical dossier/Annex A2.5.
[20]Technical dossier/COL petition (CONF Rev. 1) dossier p. 28 & Annex A2.5.
[21]Technical dossier/COL petition (CONF Rev. 1) dossier pp. 27–29 Figure 4 & 5.
[22]Technical dossier/Additional information/July 2023/Answer to Annex Q9-A2.1; Answer to Annex Q10-A.7.1.1.
[23]LoDs: Pb = 0.1 mg/kg; As = 1 mg/kg; Cd = 0.01 mg/kg; Hg = 0.01 mg/kg.
[24]Technical dossier/COL petition dossier Table 7 p. 45, Annexes A2.1, A2.2, A2.3, A7.11 and Add info January 2016 Annex A2.5.
[25]Technical dossier/Additional information/July 2023/Answer to Annex Q9- A2.1 and Answer to Annex Q10-A.7.1.1.

The food enzyme preparation 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). In addition, the absence of *Staphylococcus aureus* (negative in 1 g) and sulfur-reducing anaerobes (< 30 CFU/g) was also reported.[26] No antimicrobial activity was detected in any of the tested batches.[27]

The presence of aflatoxins B1, B2, G1, G2 was examined in the commercial batches and all were below the LoD of the applied analytical method.[28,29]

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

3.3.4 | Viable cells and DNA of the production strain

The absence of viable cells of the production strain in the food enzyme was demonstrated in three independent batches of the liquid enzyme product, each analysed in triplicate. [redacted]. No colonies were produced. A positive control was included.[30]

The absence of recombinant DNA in the food enzyme was demonstrated by polymerase chain reaction (PCR) analysis of three batches of the dried food enzyme, each tested in triplicate. No DNA was detected with primers that would amplify [redacted], with a LoD of 1 ng spiked DNA/g food enzyme. [redacted] in the DNA extraction protocol.[31]

3.4 | Toxicological data

A battery of toxicological tests has been provided, including a bacterial reverse mutation test (Ames test), an in vitro mammalian micronucleus test and a repeated dose 90-day oral toxicity study in rats.

The batch 4 (Table 1) has a comparable activity/TOS value to those of the commercial batches and was considered suitable as a test item.

3.4.1 | Genotoxicity

3.4.1.1 | *Bacterial reverse mutation test – First study*

A bacterial reverse mutation test (Ames test) was performed according to the Organisation for Economic Co-operation and Development (OECD) Test Guideline 471 (OECD, 1997a) and following Good Laboratory Practice (GLP).[32]

Four strains of *Salmonella* Typhimurium (TA98, TA100, TA1535 and TA1537) and *Escherichia coli* WP2*uvrA* were used with or without metabolic activation (S9-mix), applying the pre-incubation method. A dose-finding test was conducted at a range of concentrations from 19.5 to 5000 µg/plate. Two separate experiments were carried out in triplicate, using five concentrations of the food enzyme ranging from 156 to 5000 µg/plate, corresponding to 146, 294, 587, 1174, 2349 and 4698 µg TOS/plate.

Cytotoxicity observed as growth inhibition was seen at the highest concentration tested in all strains, with or without S9-mix. When compared to controls, for strain TA1535 a doubling of revertant counts were seen in the first test with S9-mix and in the second test both with and without S9-mix. A slight but concentration-related increase in revertant counts compared to controls were observed for TA100 and TA1535 in both the first and second experiment, although a doubling of revertant counts were only seen for TA1535.

Upon treatment with the food enzyme, the authors concluded that there was a biologically relevant increase in the number of revertant colonies above the control values for strain TA1535 with or without S9-mix. This positive response is considered by the author to be a result of free amino acid (histidine) in the food enzyme tested.

3.4.1.2 | *Bacterial reverse mutation test – Second study*

To confirm the results for TA100 and TA1535 in the first study, a second study was conducted with these two strains. It was performed according to the OECD Test Guideline 471 (OECD, 1997a) and Thompson et al., 2005, following GLP.[33]

In a first experiment, the cells were exposed to the food enzyme using the pre-incubation method and, in a second experiment, using the treat and wash method, both with or without S9-mix. The cells were exposed to five concentrations from 1000 to 5000 µg/plate, corresponding to 940, 1880, 2820, 3760 and 4698 µg TOS/plate. No cytotoxicity was observed

[26]Technical dossier/Annexes A2.1, A2.2, A2.3, A7.11 and Add info January 2016 Annex A2.5.
[27]Technical dossier/COL petition dossier Table 7 p. 45, Annexes A2.1, A2.2, A2.3, A7.11 and Add info January 2016 Annex A2.5.
[28]LoD: aflatoxins B1, B2, G1 and G2 = 1 µg/kg each.
[29]Technical dossier/Add info January 2016 Annex A2.5.
[30]Technical dossier/Additional information/July 2023/Answer to Annex Q4.
[31]Technical dossier/Additional information/July 2023/Answer to Annex Q3.
[32]Technical dossier/Annex A7.1.2 COLL Ames (preincubation).
[33]Technical dossier/Annex A7.1.2 COLL Ames (Treat & Wash).

at any of the test conditions. A concentration-related increase in revertant counts was seen in the first experiment, but not reaching a doubling of colonies compared to controls, both for TA100 and TA1535. In the treat and wash test, no increase in revertant counts of biological relevance was seen for TA100 or TA1535 under the test conditions applied.

The Panel concluded that the enzyme collagenase did not induce gene mutations under the conditions applied in the present study.

3.4.1.3 | *In vitro mammalian cell gene mutation assay*

The in vitro mammalian cell gene mutation test was carried out in mouse lymphoma TK cells (L5178Y $tk^{+/-}$, clone 3.7.2C) according to the OECD Test Guideline 476 (OECD, 1997b) and following GLP.[34] In the range-finding experiment, cells were treated with the food enzyme with or without metabolic activation (S9-mix) at up to 5000 μg/mL. Based on the range findings, in the main experiment, cells were treated with six concentrations of food enzyme from 156 to 5000 μg/mL (corresponding to 146, 294, 587, 1174, 2349 and 4698 μg TOS/mL) for 3 h with and without S9-mix, and for 24 h without S9-mix.

An increase of mutation frequency exceeding the Global Evaluation Factor (OECD, 2015) was observed in the 24-h treatment at the highest concentration tested. The rate of small colonies was dominant (~ 75%), suggesting a clastogenic effect of the food enzyme. To confirm the positive result in the 24-h treatment without S9-mix, a test was performed with six concentrations of 2010 to 5000 μg/mL (corresponding to 1888, 2264, 2715, 3262, 3918, 4698 μg/mL). This test did not show any increase of the mutation frequency exceeding the global evaluation factor.

The Panel concluded that the food enzyme microbial collagenase did not induce gene mutations in mammalian cells under the conditions applied in the present study.

3.4.1.4 | *In vitro mammalian cell micronucleus test*

The in vitro mammalian cell micronucleus test was carried out according to the OECD Test Guideline 487 (OECD, 2016) and following GLP.[35] The experiment was carried out in duplicate cultures of human peripheral blood lymphocytes in the presence and absence of metabolic activation (S9-mix). The cytokinesis-block technique was applied.

In a range-finding study, concentrations of 125, 250, 500, 1000 and 2000 μg/mL of food enzyme were tested in a short-term treatment (4 h exposure and 20 h recovery period) with and without S9-mix, and in a long-term treatment (24-h exposure without recovery period). No signs of cytotoxicity were observed. Test item precipitation was noted at concentrations of 1000 and 2000 μg/mL in the absence and presence of metabolic activation (4- or 24-h exposure). On the basis of these results, the cell cultures were exposed to the food enzyme and scored for the frequency of binucleated cells with micronuclei (MNBN) at concentrations of 250, 500, 1000 and 2000 μg/mL (corresponding to 235, 470, 940 and 1880 μg TOS/mL) in the short-term treatment with and without S9-mix, and in the long-term treatment.

No cytotoxicity was seen either in the short-term treatment with or without S9-mix or in the long-term treatment. The frequency of MNBN was not statistically significantly different from the negative controls at any concentrations tested.

The Panel concluded that the food enzyme microbial collagenase did not induce an increase in the frequency of MNBNs under the test conditions applied in this study.

3.4.1.5 | *In vivo micronucleus test*

The in vivo micronucleus test was carried out according to the OECD Test Guideline 474 (OECD, 1997c) and following GLP.[36]

The food enzyme was tested for its ability to induce micronuclei (MN) in the polychromatic erythrocytes (PCEs) of the bone marrow of treated Crl:CD(SD)SPF rats. Based on the result of a range-finding study where no clinical signs or toxicity was observed, groups of five rats were administered doses of 500, 1000 or 2000 mg /kg body weight (bw) per day for two consecutive days, corresponding to 470, 940 or 1880 mg TOS /kg bw per day. Negative (water) and positive (mitomycin C) control groups were included.

Rats treated with the food enzyme exhibited %PCE values and mean frequencies of micronucleated polychromatic erythrocytes (MNPCEs) that were similar to and not statistically different from those seen in the concurrent vehicle control. No historical control data was included in the study report. The positive control demonstrated a significant increase in MNPCE.

The Panel concluded that the food enzyme microbial collagenase did not induce an increase in the MN frequency in vivo under the test conditions applied in this study. However, no information on bone marrow exposure was available from the study report and, therefore, the study was considered inconclusive.

Conclusions on genotoxicity

The food enzyme microbial collagenase was tested in a basic battery of in vitro genotoxicity studies. The test item in the presence or absence of S9 mix did not induce gene mutations in bacteria (four strains of *Salmonella* Typhimurium, TA1535, TA1537, TA98 and TA100 and one strain of *Escherichia coli,* WP2 *uvrA*), nor gene mutations in mammalian cells. The food enzyme did not induce chromosomal damage, evaluated as micronuclei frequency in human peripheral blood lymphocytes.

Based on these results, the Panel concluded that there is no concern for genotoxicity for the food enzyme microbial collagenase.

[34]Technical dossier/Annex A7.1.4 COLL MLA.

[35]Technical dossier/Additional information July 2023/Answer to Annex Q12.

[36]Technical dossier/Annex A7.1.5 COLL Micronucleus test.

3.4.2 | Repeated dose 90-day oral toxicity study in rodents

The repeated dose 90-day oral toxicity study was performed in accordance with the OECD Test Guideline 408 (OECD, 1998) and following GLP.[37] Groups of 10 male and 10 female Sprague–Dawley (Crl:CD(SD)) rats received the food enzyme in doses of 62.5, 250 or 1000 mg/kg bw per day by gavage, corresponding to 58.8, 235 or 940 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 42 of administration in low-dose males (−6%) and increased on day 21 in mid-dose females (+11%). The Panel considered these changes as not toxicologically relevant, as they were only recorded sporadically, there was no consistency between the changes in males and females, there was no dose–response relationship, there was no statistically significant change in the final food consumption and there was no statistically significant changes in the body weight or the body weight gain.

In the functional observations, a statistically significant decrease in the rearing count was observed in week 1 of administration in low-dose females (−33%) and in week 3 of administration in mid-dose males (−60%). The Panel considered the changes as not toxicologically relevant, as they were only recorded sporadically, there was no dose–response relationship and there were no changes in functional observations.

The haematological investigation revealed a statistically significant decrease in the mean corpuscular volume (MCV) and the mean corpuscular haemoglobin (MCH) in high-dose males (−2.8% and −3.0%, respectively). The Panel considered the changes as not toxicologically relevant as they were only observed in one sex (both parameters), the changes were small (both parameters), there were no changes in other relevant parameters (in red blood cell parameters, both parameters) and the changes were within the historical control values (both parameters).

The clinical chemistry investigation revealed a statistically significant decrease in creatinine and in chloride in high-dose males (−14% and −2%, respectively). The Panel considered the changes as not toxicologically relevant, as they were only observed in one sex (both parameters), the changes were small (both parameters), there were no changes in other relevant parameters (both parameters), there were no histopathological changes in the kidneys (both parameters) and the changes were within the historical control values (creatinine).

The urinalysis revealed a statistically significant decrease in the one-day excretion of sodium in high-dose males (−26%). The Panel considered the change as not toxicologically relevant, as it was only observed in one sex, there were no changes in other relevant parameters and there were no histopathological changes in the kidneys.

Statistically significant changes in organ weights included an increase in the absolute and the relative kidney weight in high-dose males (+14% and +13%, respectively). The Panel considered the changes as not toxicologically relevant, as they were only observed in one sex, the changes were small and there were no histopathological changes in the kidneys.

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

The Panel identified a no observed adverse effect level (NOAEL) of 940 mg TOS/kg bw per day, the highest dose tested.

3.4.3 | 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 enzyme microbial collagenase with the *Streptomyces violaceoruber* strain pCol 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.[38]

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

No allergic reactions following dietary exposure to any microbial collagenase have been reported in the literature.[39]

[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 fungal 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.

[37]Technical dossier/Annex A7.1.6 COLL 13Weeks subchronic.

[38]Technical dossier/2nd submission/p. 60/Annex 7.3.

[39]Technical dossier/Additional information July 2023/Answer to Annex Q13.

3.5 | Dietary exposure

3.5.1 | Intended use of the food enzyme

The food enzyme is intended to be used in two 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.[40]

Food manufacturing process[a]	Raw material (RM)	Maximum recommended use level (mg TOS/kg RM)[b]
Processing of meat and fish products		
• Production of modified meat and fish products	Meat, fish	**36.36**
	Sausage casing	**36.36**
• Production of protein hydrolysates from meat and fish proteins	Collagen	**1566.54**

[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.

In the production of modified meat and fish products, the food enzyme is added to meat[41] to hydrolyse collagen in the connective tissues for tenderising purposes.[42] The food enzyme is used also to treat sausage casing made of animal intestines[43] before stuffing.[44,45] The food enzyme–TOS remains in the final foods.

In the production of protein hydrolysates from meat and fish proteins, the food enzyme is added to collagen at the beginning of the food manufacturing process.[46] The resulting collagen hydrolysates are added to different foods or food supplements.[47] The food enzyme–TOS remains in the final foods.

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 food enzyme is inactivated in both of the food manufacturing processes listed in Table 2.

3.5.2 | 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, 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 in 26 European countries (Appendix B). The highest dietary exposure was estimated to be about 1.098 mg TOS/kg bw per day in toddlers at the 95th percentile.

[40]Technical dossier/Additional information July 2023/Answer to Annex Q16.

[41]Technical dossier/fig. 12.

[42]Technical dossier/p. 47.

[43]Technical dossier/Additional information July 2023/Annex Q16.

[44]Technical dossier/fig. 13.

[45]Technical dossier/Additional information July 2023/Answer 15.

[46]Technical dossier/fig. 14.

[47]Technical dossier/p. 47.

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)	0.042–0.245 (12)	0.133–0.353 (15)	0.151–0.357 (19)	0.046–0.239 (21)	0.034–0.135 (22)	0.021–0.129 (22)
Min–max 95th percentile (number of surveys)	0.152–0.603 (9)	0.317–1.098 (13)	0.367–1.065 (19)	0.138–0.789 (20)	0.108–0.500 (22)	0.084–0.486 (21)

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	+
Use of recipe fractions in disaggregation FoodEx categories	+/–
Use of technical factors in the exposure model	+/–

Abbreviations: +, 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.

3.6 | Margin of exposure

The comparison of the NOAEL (940 mg TOS/kg bw per day) from the 90-day rat study with the derived exposure estimates of 0.021–0.357 mg TOS/kg bw per day at the mean and from 0.084–1.098 mg TOS/kg bw per day at the 95th percentile resulted in a margin of exposure of at least 856.

4 | CONCLUSIONS

Based on the data provided and the derived margin of exposure, the Panel concluded that the food enzyme microbial collagenase produced with the genetically modified *S. violaceoruber* strain pCol does not give rise to safety concerns under the intended conditions of use.

The CEP Panel considered the food enzyme free from viable cells of the production organism and recombinant DNA.

5 | DOCUMENTATION AS PROVIDED TO EFSA

Request for the authorization of three collagenase preparations from Streptomyces violaceoruber pCol for use as food processing aids. December 2015. Submitted by Nagase (Europa) GmbH.

Additional information. July 2023. Submitted by Nagase (Europa) GmbH. The dossier was updated in March 2016.

ABBREVIATIONS

ANI	average nucleotide identity
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
MCH	mean corpuscular haemoglobin
MCV	mean corpuscular volume
MN	micronuclei
MNBN	bi-nucleated cells with micronuclei
MNPCE	micronucleated polychromatic erythrocytes
NOAEL	no observed adverse effect level
OECD	Organisation for Economic Cooperation and Development
PCEs	polychromatic erythrocytes
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[a], Serbia[a], 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[a], Serbia[a], Spain, Sweden
Adolescents	From 10 years up to and including 17 years of age	Austria, Belgium, Bosnia and Herzegovina[a], Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Italy, Latvia, Montenegro[a], Netherlands, Portugal, Romania, Serbia[a], Slovenia, Spain, Sweden
Adults	From 18 years up to and including 64 years of age	Austria, Belgium, Bosnia and Herzegovina[a], Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Montenegro[a], Netherlands, Portugal, Romania, Serbia[a], Slovenia, Spain, Sweden
The elderly[b]	From 65 years of age and older	Austria, Belgium, Cyprus, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Montenegro[a], Netherlands, Portugal, Romania, Serbia[a], Slovenia, Spain, Sweden

[a]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.

[b]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).