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Supporting document 1

Risk and technical assessment – Application A1347

Protein-glutamine glutaminase from *Bacillus licheniformis* (gene donor: *Chryseobacterium viscerum*) *for use as a processing aid*

Executive summary

Chr. Hansen Pty Ltd. has applied to amend the Australia New Zealand Food Standards Code (the Code) to permit the use of the enzyme protein-glutamine glutaminase (EC 3.5.1.44), from *Bacillus licheniformis* containing the protein-glutamine glutaminase gene from *Chryseobacterium viscerum*. The enzyme is proposed for use as a processing aid in the manufacture of dairy products, fermented milk products, milk proteins, analogues of milk and milk products, analogues of meat, yeast, yeast products, and protein hydrolysates from plants, fungi, meat and fish. It catalyses the deamidation of the gamma amide of protein-bound glutamine to form protein-bound L-glutamate, releasing ammonia.

The available evidence provides adequate assurance the proposed use of protein-glutamine glutaminase from *B. licheniformis* as a processing aid is technologically justified. Protein-glutamine glutaminase performs its technological function during food processing and, as such, meets the definition of a processing aid for the purposes of the Code.

The microbiological assessment undertaken by FSANZ did not identify any public health or safety concerns related to the use of *B. licheniformis* as a production organism for protein-glutamine glutaminase. Analysis of the production strain confirmed the presence and stability of the inserted DNA.

Bioinformatics analysis identified no significant homology between protein-glutamine glutaminase and any known toxins or allergens. A no observed adverse effect level (NOAEL) of 372 mg total organic solids (TOS)/kg bw/day was identified in a 90-day dietary toxicity study of protein-glutamine glutaminase in rats. The theoretical maximum daily intake (TMDI) of the enzyme was calculated to be 1.16 mg TOS/kg bw/day. A comparison of the NOAEL and the TMDI results in a margin of exposure (MOE) of approximately 300. Based on the reviewed data it is concluded that in the absence of any identifiable hazard an Acceptable Daily Intake (ADI) ‘not specified’ is appropriate.

FSANZ concludes that there are no public health and safety concerns associated with the use of protein-glutamine glutaminase from *Bacillus licheniformis* (gene donor: *C. viscerum*) in the quantity and form required to perform its typical function, consistent with Good Manufacturing Process.

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1 Introduction

Chr. Hansen Pty Ltd applied to Food Standards Australia New Zealand (FSANZ) to amend the Australia New Zealand Food Standards Code (the Code) to permit the use of the enzyme protein-glutamine glutaminase (EC 3.5.1.44) from *Bacillus licheniformis* containing the protein-glutamine glutaminase gene from *Chryseobacterium viscerum* as a processing aid.

Protein-glutamine glutaminase catalyses deamidation of gamma amide of protein-bound glutamine to form protein-bound L-glutamate, with the release of ammonia. It will be used as a processing aid in the production of dairy products, fermented milk products, milk proteins, analogues of milk and milk products, analogues of meat, yeast, yeast products, and protein hydrolysates from plants, fungi, meat and fish at the minimum level required to achieve the desired effect, in accordance with the principles of Good Manufacturing Practice (GMP).[1]

1.1 Objectives of the assessment

The objectives of this risk and technical assessment were to:

- determine whether the proposed purpose is solely a technological function and that the enzyme achieves its technological purpose as a processing aid in the quantity and form proposed to be used
- evaluate potential public health and safety concerns that may arise from the use of this enzyme as a processing aid by considering the:
  - safety and history of use of the production microorganism
  - safety of the enzyme preparation.

2 Food technology assessment

2.1 Identity of the enzyme

The applicant provided information regarding the identity of the enzyme and this has been verified using the IUBMB[2] enzyme nomenclature reference database (McDonald et al. 2009). Details of the identity of the enzyme are provided below.

Accepted IUBMB name:	protein-glutamine glutaminase
Systematic name:	protein-L-glutamine amidohydrolase
Other names/common names:	peptidoglutaminase II; glutaminyl-peptide glutaminase; destabilase; peptidylglutaminase II
Commercial name:	none provided in IUBMB database
IUBMB enzyme nomenclature:	EC 3.5.1.44

[1] GMP is defined in section 1.1.2—2 of the Code as follows: ***GMP*** *or* ***Good Manufacturing Practice****, with respect to the addition of substances used as food additives and substances used as processing aids to food, means the practice of:*
(a) limiting the amount of substance that is added to food to the lowest possible level necessary to accomplish its desired effect; and
(b) to the extent reasonably possible, reducing the amount of the substance or its derivatives that:
*(i) remains as a *component of the food as a result of its use in the manufacture, processing or packaging; and*
(ii) is not intended to accomplish any physical or other technical effect in the food itself;
(c) preparing and handling the substance in the same way as a food ingredient.

[2] International Union of Biochemistry and Molecular Biology.

CAS number: 62213-11-0

Reaction: protein L-glutamine + H_2O = protein L-glutamate + NH_3

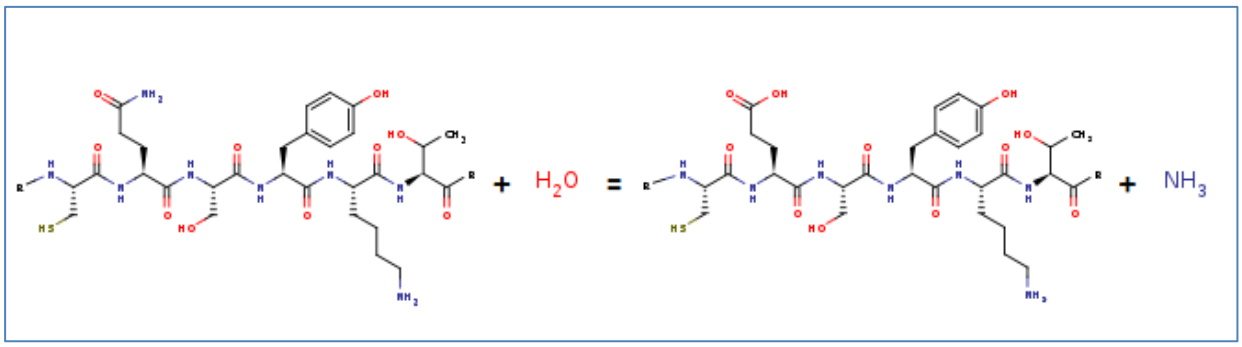

Figure 1: Reaction catalysed by protein-glutamine glutaminase (Source: BRENDA)

2.2 Manufacturing process

2.2.1 Production of the enzyme

Enzymes from microorganisms are typically produced by controlled fermentation followed by removal of the production microorganism, purification and concentration of the enzyme. Final standardisation with stabilisers, preservatives, carriers, diluents, and other approved food-grade additives and ingredients is carried out after the purification and concentration steps.

The formulated enzymes are referred to as enzyme preparations, which, depending upon the application in food, may be a liquid, semi-liquid or dried product. Enzyme preparations may contain either one major active enzyme that catalyses a specific reaction during food processing or two or more active enzymes that catalyse different reactions (FAO/WHO 2020a).

Protein-glutamine glutaminase is produced by submerged fed-batch pure culture fermentation of *Bacillus licheniformis.* The fermentation process comprises three operations: direct inoculation of the stock culture suspension into the seed fermenter, seed fermentation and main fermentation.

Once fermentation is complete, a recovery process involving multiple steps to separate the enzyme-containing culture medium from the *Bacillus licheniformis* biomass and other insoluble matter. It includes five steps including pre-treatment, primary separation, filtration, concentration, polish and germ filtration. The enzyme solution is then formulated either as a single enzyme preparation or mixed with other enzymes as a liquid or granular product.

The typical composition of the enzyme preparation is shown in Table 1.

Table 1: Typical composition of protein-glutamine glutaminase preparation

Component	Approximate %
Enzyme solids (Total organic solids)	3
Sodium chloride	10
Sucrose	40
Water	47

The applicant has stated the enzyme is manufactured in accordance with GMP and provided documentation regarding the compliance of their quality management system used in the manufacturing process with ISO 9001:2015. The resultant product meets the general specifications for enzyme preparations used in food processing as established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and Food Chemicals Codex (FCC) (see also section 2.2.2 of this report).

Information on the raw materials used in the production and recovery of the enzyme preparation is deemed confidential commercial information (CCI) under section 114 of the *Food Standards Australia New Zealand Act 1991* (the FSANZ Act) and can only be disclosed under certain circumstances in accordance with that Act. This information has been evaluated by FSANZ but cannot be disclosed in this report.

2.2.2 Specifications for identity and purity

There are international general specifications for enzyme preparations used in the production of food, established by JECFA in its Compendium of Food Additive Specifications and in FCC (14th edition), referenced in section S3—2 of Schedule 3 of the Code. Enzymes used as processing aids need to meet either of these specifications, or a relevant specification in section S3—3 of Schedule 3.

Schedule 3 of the Code includes specifications for arsenic and heavy metals (section S3—4) if they are not already detailed within specifications in sections S3—2 or S3—3.

The applicant provided analytical data for three representative standardised batches of the enzyme. Table 2 provides a comparison of the summary results of those analyses with international specifications established by JECFA and FCC, as well as those in the Code. Based on those results the enzyme met the relevant specifications.

Table 2: Analysis of representative standardised batches of enzyme compared to JECFA, Food Chemicals Codex, and Code specifications for enzymes

Test parameters	Test results	Specifications		
		JECFA	Food Chemicals Codex	The Code – section S3—4
Lead (mg/kg)	ND (LOQ <0.5)	≤5	≤5	≤2
Arsenic (mg/kg)	ND (LOQ <0.3)	-	-	≤1
Cadmium (mg/kg)	ND (LOQ <0.05)	-	-	≤1
Mercury (mg/kg)	ND (LOQ <0.05)	-	-	≤1
Coliforms (cfu/g)	<4	≤30	≤30	-
***Salmonella* (in 25 g)**	ND	Absent	Negative	-
***Escherichia coli* (in 25 g)**	ND	Absent	-	-
Antimicrobial activity	ND	Absent	-	-

cfu = colony forming units; ND = not detected; LOQ = limit of quantitation

2.3 Technological purpose

The protein-glutamine glutaminase preparation will be used as a processing aid in several food manufacturing processes to catalyse deamination of gamma-amide of protein-bound glutamine in the presence of water.

The applicant has stated that the enzyme has a wide range of functions, including:

- Reduced fouling in heat treatment equipment when producing heat-treated milk, milk-based barista products and plant-based analogues, and modified milk proteins
- Reduced curding and improved mouth feel of cooled ready-to-drink milk-based coffee products in heat-treated milk and milk-based barista and plant-based analogue products
- Improved protein solubility in production of modified milk protein, plant-based analogues of milk and milk products and protein hydrolysates from plants, fungi, meat and fish
- Increased yield in cheese production
- Improved heat tolerance of milk proteins
- Improved meat-like structure in plant-based meat analogues
- Improved texture in cheese, plant-based analogues of milk, meat and fish-based protein hydrolysates.

Literature on food industry applications of protein-glutamine glutaminase provided by the applicant and identified by FSANZ support the functions of increased solubility and improved emulsification, foaming and flavour (Liu et al. 2022; Martinez et al. 2022).

The applicant stated the enzyme preparation is used at the minimum level required to achieve the desired effect, in accordance with the principles of GMP. The highest use level of the enzyme preparation for solid food is 165 mg total organic solids (TOS)/kg raw material (plant and fungal proteins), and 66 mg TOS/ kg raw material (plant-based raw materials) for liquids.

Protein-glutamine glutaminase performs its technological function during food processing. The applicant states the enzyme exerts no function in the final food. This is due to either denaturation during heat treatment or removal during processing. As such, the enzyme meets the definition of a processing aid. Information on the physical and chemical properties of the enzyme preparation is summarised in Table 3.

Table 3: Protein-glutamine glutaminase enzyme preparation physical/chemical properties

Physical/chemical properties of commercial enzyme preparation	
Enzyme activity	25000 PGLU-A/g*
Appearance	Liquid
Temperature optimum	25-30°C
Thermostability	Enzyme activity up to 65°C; 10 min at 75°C causes inactivation
pH range and optimum	Range pH 3-8, with an optimum of pH 4

*PGLU-A: protein-glutamine glutaminase activity units. One unit is the amount of protein-glutamine glutaminase that converts 1.25 mM glutamine per minute under specified conditions.

2.4 Allergen considerations

The applicant provided product information which indicated the enzyme preparation does not contain known food allergens.

2.5 Food technology conclusion

The use of protein-glutamine glutaminase to catalyse the deamidation of gamma amide of protein-bound glutamine to form protein-bound L-glutamate during the the processing and production of certain foods is consistent with the typical function of the enzyme. The foods are dairy products, fermented milk products, milk proteins, analogues of milk and milk products, analogues of meat, yeast, yeast products, and protein hydrolysates from plants, fungi, meat and fish.

The stated benefits include increased solubility, improved emulsification and foaming and improved flavour. The evidence presented to support its proposed use provides adequate assurance that the use of the enzyme, in the quantity and form proposed to be used (which must be consistent with GMP), is technologically justified.

Protein-glutamine glutaminase performs its technological purpose during the manufacture of a wide range of food products and is not performing a technological purpose in the final food. This is due to various factors including denaturing during processing steps that involve high temperatures and/or removal in certain processing steps. It therefore functions as a processing aid for the purposes of the Code.

There are relevant identity and purity specifications for the enzyme in the Code, and the applicant has provided evidence that the enzyme meets these specifications.

3 Safety assessment

This safety assessment aims to evaluate potential public health and safety concerns that may arise from using this enzyme as a processing aid.

Some information relevant to this section is deemed CCI under section 114 of the FSANZ Act. This information has been evaluated by FSANZ but cannot be disclosed in this report.

3.1 Source microorganism

B. licheniformis has a long history of safe industrial use, particularly in the production of enzymes for food processing, dating back to 1972 (De Boer et al. 1994, Sewalt et al. 2018, Muras et al. 2021). *B. licheniformis* is a gram-positive spore-forming bacterial species used in a range of biotechnological applications, including the production of bioactive compounds that are used by aquaculture, agriculture, food, biomedicine, and pharmaceutical industries (Muras et al. 2021).

The European Food Safety Authority (EFSA) has granted *B. licheniformis* with qualified presumption of safety (QPS) status, with the qualification that the production strain be absent of toxigenic activity (EFSA BIOHAZ Panel et al. 2026). *B. licheniformis* also falls under Class 1 Containment under the European Federation of Biotechnology guidelines (Frommer et al. 1989).

B. licheniformis has been reported to be associated with foodborne illness from cooked meats, ice cream, cheese, raw milk, infant feed and prawns (Salkinoja-Salonen et al. 1999). However, the incidence of human infections and pathogenicity is rare and tends to be

limited to immune-compromised individuals (Haydushka et al. 2012, Logan 2012, Zou et al. 2024).

B. licheniformis is widely used to produce food-grade enzymes and other food products (Aslam et al. 2020). FSANZ has previously assessed the safety of *B. licheniformis* for several enzyme processing aids. Schedule 18 of the Code currently permits the use of the following *B. licheniformis* produced enzyme processing aids: serine proteinase (Application A1098), subtilisin (A1206), alpha-amylase (A1219), beta-amylase (A1220) and transglutaminase (A1275).

The applicant provided data that confirmed the production strain's identity as *B. licheniformis*. Using the safe strain concept (Pariza and Johnson 2001), the risk of toxin production by the production organism was determined to be very low. Any *B. licheniformis* spores present post-fermentation would be removed during the ultrafiltration stage in the manufacturing process. FSANZ evaluated the methodology and results of microbiological data from three batches of the final product confirming the absence of the production strain.

The microbiological assessment undertaken by FSANZ did not identify any public health or safety concerns related to the use of *B. licheniformis* as a production organism for protein-glutamine glutaminase.

3.2 Characterisation of the genetic modification to the production strain

3.2.1 Description of the DNA to be introduced and the method of transformation

The gene encoding the protein-glutamine glutaminase enzyme was synthesised *in vitro* based on the sequence from *Chryseobacterium viscerum* available in public databases. Data provided by the applicant and analysed by FSANZ confirmed the identity of the protein-glutamine glutaminase enzyme.

The gene was integrated into the *B. licheniformis* genome using standard molecular biology techniques. It was placed under control of a hybrid *Bacillus* promoter and a *Bacillus* terminator. The gene was integrated at specific integration sites in the host's genome.

The final production strain was selected based on rapid growth and high enzyme activity.

3.2.2 Characterisation of the inserted DNA

Next generation sequencing confirmed the insertion of the expression cassette in the genome at the specific integration sites. No antibiotic-resistance markers are present in the final production strain.

3.2.3 Stability of the inserted DNA

The inserted DNA that is integrated into the genome of the production strain is unable to replicate autonomously and unlikely to be transferred by conjugation to other organisms. Therefore, it is considered genetically stable.

To provide further evidence of the stability of the introduced gene, the applicant provided phenotypic data from large-scale fermentation of the production strain. These data confirmed that the inserted protein-glutamine glutaminase gene is expressed stably over multiple generations.

3.3 Safety of the enzyme

3.3.1 History of safe use

The enzyme was approved by Danish Veterinary and Food Administration in July 2025. It is used as a processing aid in countries where there are no restrictions of the use of enzyme processing aids.

3.3.2 Bioinformatic assessment of homology with known toxins

The applicant provided the results of a January 2025 search for sequence homology of protein-glutamine glutaminase from *B. licheniformis* to known toxins in the NCBI Identical Protein Groups resource[3]. No hits with an E-value < 0.00001 were identified, indicating that the enzyme has no significant structural homology with known toxins.

3.3.3 Toxicology data

The applicant provided study reports of a 13-week dietary study in rats, and two genotoxicity studies. All these studies were conducted using representative batches of the enzyme concentrate prior to the final stabilization and standardization steps applied to produce the commercial product.

Toxicology

The 13-week dietary study was conducted according to OECD Guideline 408 (as of June 2018) and was consistent with GLP. Details are provided in Appendix 1. The diets contained the protein-glutamine glutaminase at concentrations equivalent to 0, 1875, 3750 and 5625 ppm in terms of TOS. The overall mean achieved dosages were 0, 122, 244 and 372 mg TOS/kg bw/day in males and 0, 138, 292 and 447 mg TOS/kg bw/day in females.

There were no treatment-related effects on any parameters measured, and it was concluded that the no observed adverse effect level (NOAEL) was 5625 ppm TOS, equal to mean dosages of 372 mg TOS/kg bw/day for males and 447 mg TOS/kg bw/day for females.

Genotoxicity

Results of genotoxicity assays are summarised in Table 4. No evidence of mutagenicity, clastogenicity or aneugenicity of protein-glutamine glutaminase was observed.

[3] https://www.ncbi.nlm.nih.gov/ipg/

Table 4: Genotoxicity studies of protein-glutamine glutaminase enzyme concentrate

Test	Test system	Enzyme Concentrations	Result
Bacterial reverse mutation assay conducted in compliance with OECD TG 471	*Salmonella enteriditis* var. Typhimurium TA98, TA100, TA1535, TA1537, *Escherichia coli* WP2*uvr*A	Preliminary test: 5, 15, 50, 150 500,1500, 5000 µg TOS/mL Definitive test: 50, 150, 500, 1500, 5000 µg TOS/mL	Negative ± S9
In vitro mammalian micronucleus assay conducted in compliance with OECD TG 487	Human peripheral blood lymphocytes	750, 1500, 2000, 3000 µg TOS/mL	Negative ± S9

3.3.4 Potential for allergenicity

The applicant provided the results of a recent (January 2025) search of the COMprehensive Protein Allergen REsource (COMPARE)[4] for sequence homology of the protein-glutamine glutaminase enzyme to known allergens. No matches were identified.

3.3.5 Assessments by other regulatory agencies

No safety assessments by other regulatory agencies are available.

4 Dietary exposure assessment

The objective of the dietary exposure assessment was to review the budget method calculation presented by the applicant as a 'worst-case scenario' approach to estimating likely levels of dietary exposure, assuming that all of the TOS from the protein-glutamine glutaminase enzyme preparation remained in the food.

The budget method is a valid screening tool for estimating the theoretical maximum daily intake (TMDI) of a food additive (Douglass et al 1997). The calculation is based on physiological food and liquid requirements, the food additive concentration in foods and beverages, and the proportion of foods and beverages that may contain the food additive. The TMDI can then be compared to an ADI or a NOAEL to estimate a margin of exposure (MOE) for risk characterisation purposes. Whilst the budget method was originally developed for use in assessing food additives, it is also appropriate to use for estimating the TMDI for processing aids (FAO/WHO 2020b). The method is used by overseas regulatory bodies and the FAO/WHO Joint Expert Committee on Food Additives (JECFA) (FAO/WHO 2021) for dietary exposure assessments for processing aids.

In their budget method calculation, the applicant made the following assumptions:

- the maximum physiological requirement for solid food (including milk) is 25 g/kg body weight/day
- 50% of solid food is processed

[4] https://comparedatabase.org

- all processed solid foods contain a maximum of 20% protein
- all solid foods contain the highest use level of 165 mg TOS/kg in the raw material (plant and fungal proteins)
- the maximum physiological requirement for liquid is 100 mL/kg body weight/day (the standard level used in a budget method calculation for non-milk beverages)
- 25% of non-milk beverages are processed
- all processed beverages contain a maximum of 20% cereal ingredients
- the densities of non-milk beverages are ~1 g/mL
- all non-milk beverages contain the highest use level of 66 mg TOS/kg in the raw material (plant-based raw materials)
- all of the TOS from the enzyme preparation remains in the final food.

Based on these assumptions, the applicant calculated the TMDI of the TOS from the enzyme preparation to be 0.74 mg TOS/kg bw/day.

As assumptions made by the applicant differ from those that FSANZ would have made in applying the budget method, FSANZ independently calculated the TMDI using the following assumptions that are conservative and reflective of a first tier in estimating dietary exposure:

- The maximum physiological requirement for solid food (including milk) is 50 g/kg body weight/day (the standard level used in a budget method calculation where there is potential for the enzyme preparation to be in baby foods or general purpose foods that would be consumed by infants).
- FSANZ would generally assume 12.5% of solid foods contain the enzyme based on commonly used default proportions noted in the FAO/WHO Environmental Health Criteria (EHC) 240 Chapter 6 on dietary exposure assessment (FAO/WHO 2009). However, the applicant has assumed a higher proportion of 50% based on the nature and extent of use of the enzyme and therefore FSANZ has also used this proportion for solid foods as a worst-case scenario.

All other inputs and assumptions used by FSANZ remained as per those used by the applicant. The TMDI of the TOS from the enzyme preparation based on FSANZ's calculations for solid food and non-milk beverages is 1.16 mg TOS/kg bw/day.

Both FSANZ and the applicant's estimates of the TMDI will be overestimates of the dietary exposure given the conservatisms in the budget method. This includes that it was assumed that all of the TOS from the enzyme preparation remains in the final foods and beverages whereas the applicant has stated that it is denatured during processing and subsequently diluted or removed.

5 Discussion

The use of protein-glutamine glutaminase from *B. licheniformis,* produced using the gene from *C. viscerum*, as a processing aid in food is consistent with its known functions. In the presence of water, it will be used to deamidate protein-bound glutamine to form glutamate, with the release of ammonia. The enzyme is intended to be used in dairy products, fermented milk products, milk proteins, analogues of milk and milk products, analogues of meat, yeast, yeast products, and protein hydrolysates from plants, fungi, meat and fish, where it may confer benefits including improved solubility, foaming, emulsification and flavour.

Protein-glutamine glutaminase functions as a processing aid as defined in the Code and does not perform a technological purpose in the food for sale. The evidence presented to support its proposed use provides adequate assurance that the use of the enzyme, in the

quantity and form proposed to be used (which must be consistent with GMP), is technologically justified.

The microbiological assessment undertaken by FSANZ did not identify any public health or safety concerns related to the use of *B. licheniformis* as a production organism for protein-glutamine glutaminase. Analysis of the production strain confirmed the presence and stability of the inserted DNA.

No significant homology between the enzyme and any known toxins or allergens was identified. There was no evidence of genotoxicity of protein-glutamine glutaminase *in vitro* and no adverse effects were observed in a 90-day dietary toxicity study in rats. The NOAEL was 372 mg TOS/kg bw/day in male rats, the highest dose tested.

The reaction catalysed by the enzyme produces ammonia. The amount of ammonia or ammonium in the final food is insignificant compared to endogenous production levels and other sources in the diet and not considered to be of health concern.

The TMDI was calculated by FSANZ to be 1.16 mg TOS/kg bw/day. A comparison of the NOAEL and the TMDI results in a MOE of approximately 300.

Based on the reviewed data it is concluded that, in the absence of any identifiable hazard, an ADI ‘not specified’ is appropriate.

FSANZ concludes there are no safety concerns from the use of protein-glutamine glutaminase from *B. licheniformis* using the gene from *C. viscerum* in the quantity and form required to perform its typical function which must be consistent with GMP.

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Appendix 1: Toxicity study

13-week dietary toxicity study of protein-glutamine glutaminase from Bacillus licheniformis *in Han Wistar rats. Labcorp Early Development Laboratories Limited. Regulatory status: Conducted according to OECD Guideline 408 (as of June 2018); GLP.*

Rats were group-housed under standard laboratory conditions of environment and husbandry. They were assigned to groups of 10/sex/group and provided *ad libitum* with diets containing 0, 16,448, 32,895 or 49,343 ppm w/w protein-glutamine glutaminase, equivalent to 1875, 3750 and 5625 ppm in terms of TOS. Water was used as the diluent and the negative control article. Clinical observations were recorded daily and behaviour in an arena was assessed weekly. Individual body weights and the feed consumption per cage were recorded weekly. Ophthalmologic examinations were conducted on all rats prior to study start and on rats in the control and high dose groups in Week 12. A Functional Observational Battery (FOB) was conducted on all rats in Week 12, including sensory reactivity observations, grip strength, and motor activity. Blood was collected prior to scheduled killing at the end of the study for determination of haematology and clinical chemistry parameters, levels of clotting factors, and levels of thyroid hormones. Vaginal smears were collected from females for four consecutive days prior to scheduled kill for determination of the stage of the oestrus cycle. At the end of the in-life phase all rats were killed and subject to detailed gross necropsy. Fresh organ weights were recorded for the organs listed in the Guideline, and sperm was collected from all males. Organs and tissues were preserved as listed in the Guideline. All tissues from rats in the control group and the high dose group were examined microscopically.

The overall mean achieved dosages were 0, 122, 244 and 372 mg TOS/kg bw/day in males and 0, 138, 292 and 447 mg TOS/kg bw/day in females.

One male rat in the high dose group was euthanised in moribund condition in Week 10 of the study. The rat exhibited decreased activity, generalised pallor, piloerection, bruxism and bloodstained nares. The cause of morbidity was not determined, but because no similar clinical findings or lesions were found in any other rats, the morbidity in this rat was considered to be unrelated to treatment. At necropsy the majority of major organs were pale and there were dark contents in the small and large intestines. Microscopic findings included minimal focal tubular basophilia in the kidneys, minimal and focal glandular dilatation in the glandular stomach, minimal increased apoptosis of lymphocytes in the thymus, and decreased cellularity of the red pulp of the spleen.

All other rats survived to the end of the in-life phase and there were no treatment-related clinical observations. There were no treatment-related effects on bodyweights, bodyweight gains, feed consumption, ophthalmic findings, performance on the FOB, haematology including clotting factors, clinical chemistry, oestrus cycles of females, thyroid hormones, absolute or relative organ weights, gross necropsy findings or microscopic findings. It was concluded that the no observed adverse effect level was 5625 ppm TOS, equal to mean dosages of 372 mg TOS/kg bw/day for males and 447 mg TOS/kg bw/day for females.