

ADOPTED: 15 September 2022

doi: 10.2903/j.efsa.2022.7576

Safety evaluation of the food enzyme urease from the non-genetically modified *Limosilactobacillus reuteri* strain 48/72

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Abstract

The food enzyme urease (urea amidohydrolase EC 3.5.1.5) is produced with the non-genetically modified *Limosilactobacillus reuteri* strain 48/72 by Nagase (Europa) GmbH. The food enzyme is intended to be used in brewing processes for the production of Japanese sake. Dietary exposure to the food enzyme–total organic solids (TOS) was estimated to be up to 0.009 mg TOS/kg body weight per day in European populations. The production strain of the food enzyme fulfils the requirements for the Qualified Presumption of Safety approach to safety assessment. As no other concerns arising from the manufacturing process have been identified, the Panel considered that toxicological tests were not needed for the assessment of this food enzyme. A search for similarity of the amino acid sequence of the food enzyme to known allergens was made and no match was found. The Panel notes that the food enzyme contains a known allergen. Therefore, allergenicity cannot be excluded. Based on the data provided, the Panel concludes that this food enzyme does not give rise to safety concerns under the intended conditions of use, except for individuals sensitised to the identified allergen.

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Keywords: food enzyme, urease, urea amidohydrolase, EC 3.5.1.5, *Limosilactobacillus reuteri*

Requestor: European Commission

Question number: EFSA-Q-2016-00102

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Legal notice: 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.

Declarations 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.

Acknowledgments: The Panel wishes to acknowledge all European competent institutions, Member State bodies and other organisations that provided data for this scientific output.

Suggested citation: EFSA CEP Panel (EFSA Panel on Food Contact Materials, Enzymes and Processing Aids), Lambré C, Barat Baviera JM, Bolognesi C, Cocconcelli PS, Crebelli R, Gott RM, Grob K, Lampi E, Mengelers M, Mortensen A, Rivière G, Steffensen I-L, Tlustos C, Van Loveren H, Vernis L, Zorn H, Glandorf B, Herman L, Aguilera J, Andryszkiewicz M, Liu Y, di Piazza G and Chesson A, 2022. Scientific Opinion on the safety evaluation of the food enzyme urease from the non-genetically modified *Limosilactobacillus reuteri* strain 48/72. EFSA Journal 2022;20(10):7576, 12 pp. <https://doi.org/10.2903/j.efsa.2022.7576>

ISSN: 1831-4732

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The EFSA Journal is a publication of the European Food Safety Authority, a European agency funded by the European Union.



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1. Introduction

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

'Food enzyme' means a product obtained from plants, animals or 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 1331/2008² established the European Union (EU) procedures for the safety assessment and the authorisation procedure of food additives, food enzymes and food flavourings. The use of a food enzyme shall be authorised only if it is demonstrated that:

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

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

1.1. Background and Terms of Reference as provided by the requestor

1.1.1. Background as provided by the European Commission

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

Five applications have been introduced by the companies "Danisco US Inc." for the authorisation of the food enzyme Cellulase from *Penicillium funiculosum* (strain DP-Lzc35), "Advanced Enzyme Technologies Ltd." for the authorisation of the food enzyme Triacylglycerol lipase from a genetically modified strain of *Aspergillus niger agg* (strain FL 108SC), "Avances Bioquimicos Alimentacion, S.L." for the authorisation of the food enzyme Catalase from porcine livers and "Nagase (Europa) GmbH" for the authorization of the food enzymes 1,4-alpha-glucan branching enzyme from *Geobacillus stearothermophilus* (strain TRBE14) and Urease from *Lactobacillus fermentum* (strain 48/72).

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

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

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

³ Commission Regulation (EU) No 234/2011 of 10 March 2011 implementing Regulation (EC) No 1331/2008 of the European Parliament and of the Council establishing a common authorisation procedure for food additives, food enzymes and food flavourings. OJ L 64, 11.3.2011, pp. 15–24.

1.1.2. Terms of Reference

The European Commission requests the European Food Safety Authority to carry out the safety assessments on the food enzymes Cellulase from *Penicillium fumiculosum* (strain DP-Lzc35), Triacylglycerol lipase from a genetically modified strain of *Aspergillus niger agg* (strain FL108SC), Catalase from porcine livers, 1,4-alpha-glucan branching enzyme from *Geobacillus stearothermophilus* (strain TRBE14) and Urease from *Lactobacillus fermentum* (strain 48/72) in accordance with Article 17.3 of Regulation (EC) No 1332/2008 on food enzymes.

1.2. Interpretation of the Terms of Reference

The present scientific opinion addresses the European Commission's request to carry out the safety assessment of food enzyme urease from *Lactobacillus fermentum* (strain 48/72). Recent data identified the production microorganism as *Limosilactobacillus reuteri* (Section 3.1). Therefore, the name *Limosilactobacillus reuteri* will be used in this opinion.

2. Data and Methodologies

2.1. Data

The applicant has submitted a dossier in support of the application for authorisation of the food enzyme urease from *Lactobacillus fermentum* (strain 48/72).

Additional information was requested from the applicant during the assessment process on 11 October 2021 and 12 May 2022 and was subsequently provided (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, 2009b) 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, 2009a) as well as the 'Statement on characterisation of microorganisms used for the production of food enzymes' (EFSA CEP Panel, 2019) have been followed for the evaluation of the application with the exception of the exposure assessment, which was carried out in accordance with the updated 'Scientific Guidance for the submission of dossiers on food enzymes' (EFSA CEP Panel, 2021a).

3. Assessment

IUBMB nomenclature	Urease
Systematic name	Urea amidohydrolase
Synonyms	
IUBMB No	EC 3.5.1.5
CAS No	9,002-13-5
EINECS No	232-656-0

Ureases catalyse the hydrolysis of the amide linkage in urea, releasing ammonia and carbon dioxide. The enzyme is intended to be used in brewing processes for the production of Japanese sake.

3.1. Source of the food enzyme

The urease is produced with the bacterium *Limosilactobacillus reuteri* strain 48/72, which is deposited at the Biological Resource Center of the National Institute of Technology and Evaluation (NBRC, Japan) as *Lactobacillus fermentum*, with deposit number [REDACTED]⁴. The production strain was identified as *L. reuteri* by whole genome sequence (WGS) analysis, [REDACTED]⁵.

⁴ Technical dossier/Additional information April 2022/Answer to Question 1.

⁵ Technical dossier/Additional information April 2022/Answer to Question 2.

L. reuteri 48/72 was obtained from the parental strain *L. reuteri* TK1214 (designated as *L. fermentum* TK1214) [REDACTED].⁶ [REDACTED]

The species *L. reuteri* is included in the list of organisms for which the Qualified Presumption of Safety (QPS) (EFSA BIOHAZ Panel, 2020) may be applied, provided that the absence of acquired antimicrobial resistance (AMR) genes are verified for the specific strain used.⁷ WGS analysis of the production strain against two maintained databases with criteria > 80% similarity and 70% coverage did not identify known genes encoding AMR.⁵ Therefore, the production strain is considered to qualify for the QPS status.

3.2. Production of the food enzyme

The food enzyme is manufactured according to the Food Hygiene Regulation (EC) No 852/2004⁸, with food safety procedures based on hazard analysis and critical control points, and in accordance with current good manufacturing practice.⁹

The production strain is grown as a pure culture using a typical industrial medium in a submerged [REDACTED]¹⁰ fermentation system with conventional process controls in place. After completion of the fermentation and release of the intracellular enzyme by treatment with lysozyme, the solid biomass is removed from the fermentation broth by filtration. The filtrate containing the enzyme is then further purified and concentrated, including an ultrafiltration step in which enzyme protein is retained, while most of the low molecular mass material passes the filtration membrane and is discarded. Finally, the food enzyme is formulated with lactose and freeze-dried.¹¹ 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.¹²

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

Urease consists of three polypeptide chains (α , β and γ) of 573, 125 and 90 amino acids, respectively. The molecular masses of each subunit, calculated from their amino acid sequences, are 61.8, 14.2 and 9.8 kDa, respectively. The food enzyme was analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis. A consistent protein pattern was observed across all batches. The gels showed a single major protein band corresponding to an apparent molecular mass of about 62 kDa, consistent with the expected mass of the α subunit. The protein profile also included bands of lesser staining intensity.¹⁴ The food enzyme was tested for protease, amylase and lipase activities. Protease and amylase activities were detected.¹⁵

The in-house determination of urease activity is based on the hydrolysis of urea (reaction conditions: pH 4.0, 37°C, 30 min). The enzymatic activity is determined by measuring the release of ammonia by a colorimetric assay at 640 nm. Urease activity is expressed in units/g (U/g). One unit is defined as the amount of enzyme that releases 1 μ mol of ammonia per minute under the assay conditions.¹⁶

The food enzyme has a temperature optimum around 60°C (pH 4.0) and a pH optimum around pH 3.5 (37°C). Thermostability was tested after pre-incubation of the food enzyme for 10 min at different

⁶ Technical dossier/Annex A3.2.

⁷ Available online: <https://zenodo.org/record/3336268#.X8pXR2hKiUn>

⁸ 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.

⁹ Technical dossier/Annex A4 and Additional information April 2022/Answer to Question 3.

¹⁰ Technical dossier/Additional information April 2022.

¹¹ Technical dossier/pages 29–34.

¹² Technical dossier/page 31 and Additional information April 2022/Answer to Question 5.

¹³ Technical dossier/Additional information April 2022/Answer to Question 6.

¹⁴ Technical dossier/Additional information April 2022/Answer to Question 7.

¹⁵ Technical dossier/page 25/Annex A2.3.

¹⁶ Technical dossier/page 23/Annex A2.4.

temperatures (pH 4.0). Urease activity decreased above 50°C, showing no residual activity above 80°C.¹⁷

3.3.2. Chemical parameters

Data on the chemical parameters of the food enzyme preparation were provided for three batches used for commercialisation (Table 1).¹⁸ The mean total organic solids (TOS) is 8.4% and the mean enzyme activity/TOS ratio is 38.3 U/mg TOS. Prior to drying, the food enzyme is stabilised with lactose.

Table 1: Composition of the food enzyme preparation

Parameters	Unit	Batches		
		1	2	3
Urease activity	U/g batch ^(a)	3,160	3,470	3,030
Protein	%	NA ^(b)	NA	NA
Ash	%	0.5	0.9	0.7
Water	%	0.5	1.3	0.7
Stabiliser (lactose)	%	89.8	89.6	90.7
Total organic solids (TOS) ^(c)	%	9.2	8.2	7.9
Activity/mg TOS	U/mg TOS	34.3	42.3	38.4

(a): U: Units (see Section 3.3.1).

(b): NA: not analysed.

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

3.3.3. Purity

The lead content in the three commercial batches was below 5 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, the levels of arsenic, cadmium and mercury were below the limits of detection (LoDs) of the employed methods.^{19,20}

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). No antimicrobial activity was detected in any of the tested batches.²⁰

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

3.4. Toxicological data

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

3.4.1. Allergenicity

The allergenicity assessment considers only the food enzyme and not any carrier or other excipient, which may be used in the final formulation.

The potential allergenicity of the urease from *L. reuteri* 48/72 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.¹³

¹⁷ Technical dossier/pages 23–24.

¹⁸ Technical dossier/page 22/Annex A2.1. and Additional information April 2022/Answer to Question 8.

¹⁹ LoDs: Pb = 0.05 mg/kg; As = 1 mg/kg; Cd, Hg = 0.01 mg/kg each.

²⁰ Technical dossier/page 23/Annex A2.1.

No information is available on oral and respiratory sensitisation or elicitation reactions of this urease. The applicant conducted a literature search looking for possible allergenic effects of ureases and no relevant report was found.²¹

[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 of these materials are present in the food enzyme. The Panel also noted that a known allergen (egg white lysozyme) is used during the downstream processing of the food enzyme and is likely to be present in the final product.

The Panel considered that, under the intended conditions of use, the risk of allergic sensitisation and elicitation reactions upon dietary exposure to this food enzyme, particularly in individuals sensitised to egg white lysozyme, cannot be excluded.

3.5. Dietary exposure

3.5.1. Intended use of the food enzyme

The food enzyme is currently used in brewing processes for the producing Japanese sake at the maximum use levels of 0.39 mg TOS/L sake.²²

During the production of the Japanese sake, the food enzyme is added to raw rice ferment before filtration.²³ Urease is used to hydrolyse urea that is produced by yeast during fermentation. The hydrolysis of urea prevents the formation of ethyl carbamate. The food enzyme–TOS remains in sake.

Based on data provided on thermostability (see Section 3.3.1), it is expected that the urease is inactivated during pasteurisation. This was confirmed [REDACTED]

[REDACTED]. The urease activity was fully inactivated by pasteurisation alone.^{24,25}

3.5.2. Dietary exposure estimation

Japanese sake is not a commonly consumed alcoholic beverage in the EU. It is not identifiable as a specific FoodEx category in the Comprehensive European Food Consumption Database.²⁶ To obtain actual consumption data of the Japanese sake from European consumers, having considered that sake is made by a brewing process, the Panel decided to substitute the consumption data of sake with those of beer.

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

Table 2 provides an overview of the derived exposure estimates across all surveys. Detailed mean and 95th percentile exposure to the food enzyme–TOS per age class, country and survey, as well as contribution from each FoodEx category to the total dietary exposure are reported in Appendix A – Tables 1 and 2. For the present assessment, food consumption data were available from 41 dietary surveys (covering infants, toddlers, children, adolescents, adults and the elderly), carried out in 22 European countries (Appendix B). The highest dietary exposure was estimated to be about 0.009 mg TOS/kg bw per day in adults at the 95th percentile.

²¹ Technical dossier/Annex A7.1.

²² Technical dossier/Additional information June 2022.

²³ Technical dossier/Figure 13 and Additional information April 2022/Answer to question 10.

²⁴ Technical dossier/Annex 5.2, LoD = 0.022 DUN/mL.

²⁵ Technical dossier/Additional information April 2022/Answer to question 12.

²⁶ Available online: <https://www.efsa.europa.eu/en/data-report/food-consumption-data>

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

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

3.5.3. Uncertainty analysis

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

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

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

TOS: total organic solids.

+: uncertainty with potential to cause overestimation of exposure.

-: uncertainty with potential to cause underestimation of exposure.

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

3.6. Margin of exposure

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

4. Conclusions

Based on the data provided, the Panel concludes that the food enzyme urease produced with the *Limosilactobacillus reuteri* strain 48/72 does not give rise to safety concerns under the intended conditions of use, except for individuals sensitised to egg white lysozyme.

5. Documentation as provided to EFSA

Request for the authorization of a urease preparation from a non GM *Lactobacillus fermentum* 48/72 for use as a food processing aid. February 2016. Submitted by Nagase (Europa) GmbH.

Additional information. April 2022. Submitted by Nagase (Europa) GmbH.

Additional information. June 2022. Submitted by Nagase (Europa) GmbH.

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Abbreviations

AMR	antimicrobial resistance
CAS	Chemical Abstracts Service
CEF	EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
CEP	EFSA Panel on Food Contact Materials, Enzymes and Processing Aids
EINECS	European Inventory of Existing Commercial Chemical Substances
FAO	Food and Agricultural Organization of the United Nations
GMM	genetically modified microorganism
GMO	genetically modified organism
IUBMB	International Union of Biochemistry and Molecular Biology
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LoD	limit of detection
PCR	polymerase chain reaction
QPS	Qualified Presumption of Safety
SDS–PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
TOS	total organic solids
WHO	World Health Organization
WGS	whole genome sequence

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

Information provided in this appendix is shown in an excel file (downloadable <https://efsa.onlinelibrary.wiley.com/doi/10.2903/j.efsa.2022.7576#support-information-section>).

The file contains two sheets, corresponding to two tables.

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

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

Appendix B – Population groups considered for the exposure assessment

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

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