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Safety and efficacy of Axtra® XAP 104 TPT (endo-1,4-xylanase, protease and alpha-amylase) as a feed additive for chickens for fattening, laying hens and minor poultry species

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Abstract

Following a request from the European Commission, the EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) was asked to deliver a scientific opinion on the safety and efficacy of Axtra® XAP 104 TPT as a zootechnical feed additive (digestibility enhancers) for poultry species. The additive contains three enzyme activities (endo-1,4-beta-xylanase, protease and alpha-amylase) produced by three different genetically modified strains. Viable cells and recombinant DNA of the strains producing the protease and amylase were not detected in the final product. Owing to the insufficient data, uncertainty remained on the presence in the additive of viable cells of the strain producing the xylanase. The results obtained in the genotoxicity and subchronic oral toxicity studies performed with the three fermentation products did not indicate safety concerns resulting from the fermentation products used in the formulation/manufacturing. However, uncertainties remain on the suitability of the test item used in the studies conducted with the xylanase; therefore, the Panel was not in the position to conclude on the toxicological potential of AXTRA® XAP 104 TPT. Consequently, the Panel could not conclude on the safety of the additive for the target species, consumers and users. Owing to the uncertainty on the presence of viable cells of one of the production strains in the additive, the Panel could not conclude on the safety for the environment. The FEEDAP Panel concluded that AXTRA® XAP 104 TPT is efficacious in chickens for fattening, chickens reared for laying and minor poultry species up to the point of lay at the level of 2,000 U xylanase, 200 U amylase and 4,000 U protease per kg feed. Owing to the lack of sufficient data, the Panel could not conclude on the efficacy of the additive for laying hens.

Keywords: zootechnical additives, digestibility enhancers, safety, efficacy, xylanase, protease, alpha-amylase, poultry

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1. Introduction

1.1. Background and Terms of Reference

Regulation (EC) No 1831/2003[1] establishes the rules governing the Community authorisation of additives for use in animal nutrition. In particular, Article 4(1) of that Regulation lays down that any person seeking authorisation for a feed additive or for a new use of a feed additive shall submit an application in accordance with Article 7.

The European Commission received a request from Danisco (UK) Ltd[2] for authorisation of the product Axtra® XAP 104 TPT (endo-1,4-beta-xylanase, alpha-amylase and protease), when used as a feed additive for chickens for fattening, laying hens and minor poultry species (category: zootechnical additives; functional group: digestibility enhancers).

According to Article 7(1) of Regulation (EC) No 1831/2003, the Commission forwarded the application to the European Food Safety Authority (EFSA) as an application under Article 4(1) (authorisation of a feed additive or new use of a feed additive). EFSA received directly from the applicant the technical dossier in support of this application. The particulars and documents in support of the application were considered valid by EFSA as of 13 December 2017.

According to Article 8 of Regulation (EC) No 1831/2003, EFSA, after verifying the particulars and documents submitted by the applicant, shall undertake an assessment in order to determine whether the feed additive complies with the conditions laid down in Article 5. EFSA shall deliver an opinion on the safety for the target animals, consumer, user and the environment and on the efficacy of the product Axtra® XAP 104 TPT (endo-1,4-beta-xylanase, alpha-amylase and protease), when used under the proposed conditions of use (see Section 3.1.5).

1.2. Additional Information

The additive Axtra® XAP 104 TPT (endo-1,4-beta-xylanase, alpha-amylase and protease) is not authorised in the European Union.

2. Data and methodologies

2.1. Data

The present assessment is based on data submitted by the applicant in the form of a technical dossier[3] in support of the authorisation request for the use of Axtra® XAP 104 TPT as a feed additive.

EFSA has verified the European Union Reference Laboratory (EURL) report as it relates to the methods used for the control of the active substances in animal feed. The Executive Summary of the EURL report can be found in Annex A.[4]

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of Axtra® XAP 104 TPT (endo-1,4-beta-xylanase, alpha-amylase and protease) is in line with the principles laid down in Regulation (EC) No 429/2008[5] and the relevant guidance documents: Guidance on zootechnical additives (EFSA FEEDAP Panel, 2012a), Technical guidance: Tolerance and efficacy studies in target animals (EFSA FEEDAP Panel, 2011a), Technical Guidance for assessing the safety of feed additives for the environment (EFSA, 2008), Guidance on studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012b) and Guidance on the risk assessment of genetically modified microorganisms and their products intended for food and feed use (EFSA GMO Panel, 2011b).

[1] Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. OJ L 268, 18.10.2003, p. 29.
[2] Danisco Animal Nutrition, Market house, Ailesbury Court, high street, SN8 1AA. Marlborough (Wiltshire), United Kingdom.
[3] FEED dossier reference: FAD-2017-0053.
[4] The full report is available on the EURL website: https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2017-0053-axtra_xap_104_tpt.pdf
[5] Commission Regulation (EC) No 429/2008 of 25 April 2008 on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the preparation and the presentation of applications and the assessment and the authorisation of feed additives. OJ L 133, 22.5.2008, p. 1.

3. Assessment

This assessment deals with the safety and efficacy of Axtra® XAP 104 TPT (endo-1,4-beta-xylanase, alpha-amylase and a protease) as a zootechnical additive (functional group: digestibility enhancers) for chickens for fattening and reared for laying and laying hens and minor poultry species.

3.1. Characterisation

3.1.1. Characterisation of the production organisms

The three enzymes of the additive are produced by three genetically modified microorganisms deposited at the American Type Culture Collection (ATCC). The endo-1,4-beta-xylanase (Enzyme Commission Number 3.2.1.8; xylanase) is produced by *Trichoderma reesei* PTA-5588, the alpha-amylase (EC Number 3.2.1.1; amylase) is produced by *Bacillus licheniformis* SD-6525 and the protease (EC Number 3.4.21.62) is produced by *Bacillus subtilis* SD-2107.[6]

3.1.1.1. *Trichoderma reesei* ATCC PTA-5588 – production strain of xylanase

The taxonomic identification of the recipient strain [redacted] as *T. reesei* was confirmed by [redacted][7] The Panel notes that taxonomic identification performed with the production strain (*T. reesei* ATCC PTA-5588) would be preferred for the assessment. [redacted][8] The genetic modification of the production strain was fully described and assessed in a previous evaluation (EFSA, 2007b). The production strain has not been subject to any further genetic modification.

Since some *Trichoderma* species are known to be capable of producing various mycotoxins and antifungal metabolites, the recipient strain ([redacted]) was tested for its ability to produce mycotoxins: trichothecenes (trichodermin, trichodermol and harzianum A) or gliotoxin were not detected in the supernatant of culture of the recipient strain.[9] The Panel notes that the test with the production strain would have been preferred.

3.1.1.2. *Bacillus licheniformis* ATCC SD-6525 – production strain of alpha-amylase

The taxonomic identification of the recipient strain [redacted] as *B. licheniformis* was confirmed [redacted][10] However, the analysis was not conducted on the production strain (ATCC SD-6525). The Panel notes that taxonomic identification performed with the production strain (*B. licheniformis* ATCC SD-6525) would be preferred for the assessment *B. licheniformis* ATCC SD-6525.

[redacted][11] The production strain is reported to be resistant to chloramphenicol, a relevant antimicrobial [redacted][12] and its susceptibility to other antimicrobials was not tested. Therefore, uncertainty remains on the susceptibility of the production strain to relevant antimicrobials. The applicant briefly described the search of the whole genome sequence (WGS) of the production strain *B. licheniformis* ATCC SD-6525[13] [redacted][14] for the presence of antimicrobial resistance (AMR) [redacted]

[6] Technical dossier/Section II/Annex II.20.
[7] Technical dossier/Supplementary information June 2019/Annex_1a.
[8] Technical dossier/Section II/Annex II.13.
[9] Technical dossier/Section II/Annex II 17.
[10] Technical dossier/Supplementary information June 2019/Annex_1b.
[11] Technical dossier/Supplementary information June 2019/Annex_2a and Annex_2a1.
[12] Technical dossier/Section II/Annex II.14.
[13] Technical dossier/Supplementary information June 2019/Annex_2a.
[14] Technical dossier/Supplementary information June 2019/Annex_2a2.

[redacted]. It was reported that no matches to proteins known to be associated with any antimicrobial resistance were identified apart from the [redacted] introduced in ATCC SD-6525 [redacted].

The toxigenic potential of the production strain *B. licheniformis* ATCC SD-6525 was assessed according to the Guidance on the characterisation of microorganisms used as feed additives or as production organisms (EFSA FEEDAP Panel, 2018).[15] [redacted] *B. licheniformis* ATCC SD-6525 is considered to be not toxigenic.

Information relating to the genetically modified Bacillus licheniformis producing alpha-amylase[16]

[redacted]

[15] Technical dossier/Supplementary information June 2019/Annex_3a and Annex_3a1.
[16] Technical dossier/Section II/Appendix II.14 and supplementary information June 2019/annex 5a.
[17] Technical dossier/Supplementary information June 2019/Annex_5b.
[18] Technical dossier/Supplementary information June 2019/Annex_5a1.
[19] Technical dossier/Section II/Annex.14.
[20] Technical dossier/Supplementary information June 2019/Annex_5c1 and Reply_SIn.

[redacted]

3.1.1.3. *Bacillus subtilis* ATCC SD-2107 – production strain of protease

The taxonomic identification of an intermediate strain [redacted] as *B. subtilis* was confirmed [redacted][10] [redacted] The Panel notes that taxonomic identification performed with the production strain (*B. subtilis* ATCC SD-2107) would be preferred for the assessment. [redacted] The genetic modification of the production strain was fully described and assessed in a previous evaluation (EFSA, 2009). The production strain has not been subject to any further genetic modification.

The susceptibility of the production strain *B. subtilis* ATCC SD-2107 to the antibiotics recommended by the FEEDAP Panel (EFSA FEEDAP Panel, 2018) was tested by broth microdilution [redacted][21] [redacted] Therefore, B. subtilis ATCC SD-2107 is considered susceptible [redacted] but resistant to [redacted], a relevant antimicrobial. [redacted]

The toxigenic potential of the production strain B. subtilis ATCC SD-2107 was investigated [redacted][22] [redacted] B. subtilis ATCC SD-2107 is considered to be not toxigenic.

3.1.2. Manufacturing process

Each of the three enzymes (xylanase, amylase and protease) that are declared as main activities in the additive Axtra® XAP 104 TPT is produced separately by [redacted] fermentation processes with the corresponding production strain. [redacted] The [redacted] is produced with a genetically modified strain [redacted] and the assessment of the strain and resulting product is provided in Annex B. [redacted]

In the fermentation process of the protease, a mixture containing [redacted][23] Therefore, a full risk assessment would be required [redacted]

[21] Technical dossier/Supplementary information June 2019/Annex_2b and Annex_2b1.
[22] Technical dossier/Supplementary information June 2019/Annex_3b and Annex_3b1.
[23] Technical dossier/Supplementary information June 2019.

[redacted][24]

3.1.3. Characterisation of the additive

Axtra® XAP 104 TPT is available in granulated form with a guaranteed minimum activity per gram of product of 20,000 xylanase units (U),[25] 2,000 amylase U[26] and 40,000 protease U.[27] Other enzyme activities are present in the additive, [redacted][28]

The batch-to-batch variation was studied in five batches[29] and the mean values per gram of product were 28,508 xylanase U (ranging from 26,700 to 30,900 U/g; coefficient of variation (CV) of 5.0%), 2,722 amylase U (ranging from 2,500 to 2,800; CV of 4%) and 56,000 protease U (ranging from 52,200 to 58,400; CV of 4%).

[redacted][30]

[31]

Three batches of Axtra® XAP 104 TPT were analysed for chemical and microbiological contamination and antimicrobial activity.[32] The analyses of chemical contamination included arsenic (< 0.1 mg/kg), cadmium (< 0.01 mg/kg), lead (< 0.05 mg/kg) and mercury (< 0.005 mg/kg). The levels of mycotoxins, including total aflatoxins (< 5 μg/kg), ochratoxin (< 2 μg/kg), zearalenone (< 25 μg/kg), deoxynivalenol (< 0.5 mg/kg) and fumonisin (< 100 μg/kg), were also determined. Microbiological analysis included total viable counts (10 colony-forming units (CFU)/g), coliforms (< 10 CFU/g), *Escherichia coli* and *Salmonella* spp. (not detected in 25 g).

No antimicrobial activity was detected in three batches of Axtra® XAP 104 TPT analysed with the diffusion test. The reference strains used were: *Staphylococcus aureus* ATCC 6538, *Streptococcus pyogenes* ATCC 12344, *Bacillus cereus* ATCC 2, *Bacillus circulans* ATCC 4516, *Escherichia coli* ATCC 11229 and *Serratia marcescens* ATCC 14041.[33]

Particle size distribution (laser diffraction) and the dusting potential (Stauber Heubach method) were studied in three batches.[34] Particles below 650 μm diameter were 90%, particles below 400 μm were 10% and no particles were detected below 282 μm (mean particle size of 509 μm). The dusting potential ranged from 5 to 15 mg/m^3. The product has a bulk density 1,400 kg/m^3.[35]

3.1.3.1. Presence of the production strains in the additive

The analyses should be in line with the requirements established in the Guidance on the risk assessment of genetically modified microorganisms and their product intended for food and feed use (EFSA GMO Panel, 2011).

The presence of the production strains (*T. reesei* ATCC PTA-5588, *B. subtilis* ATCC SD 2107 and *B. licheniformis* ATCC SD 6525) was tested in three batches of the additive, analysed in duplicate for the fungi and in quadruplicate for the *Bacillus* strains.[36] [redacted]

[24] Technical dossier/Supplementary information February 2020/Annexes SI_2 and SI_3.
[25] One xylanase unit is the amount of enzyme that releases 0.48 μmol of reducing sugar equivalents from wheat arabinoxylans per minute at pH 4.2 and 50°C.
[26] One amylase unit is the amount of enzyme that releases 0.20 μmol of glucosidic linkages from a maltoheptasoide substrate per minute at pH 8.0 and 40°C.
[27] One protease unit is the amount of enzyme that releases 2.3 μg of phenolic compound from a casein substrate per minute at pH 10.0 and 50°C.
[28] Technical dossier/Section II/Annex II.40 and Supplementary information June 2019.
[29] Technical dossier/Section II/Annex II.2.
[redacted]
[31] Technical dossier/Section II/Annex II.4.
[32] Technical dossier/Section II/Annex II.3.
[33] Technical dossier/Section II/Appendix II.22.
[34] Technical dossier/Section II/Annex II.9 and II.11.
[35] Technical dossier/Section II/Annex II.10.
[36] Technical dossier/Section II Annexes II.3 and II.8 and Supplementary information June 2019/Annex_9a, Annex_9b and Annex_9c.

[redacted] Viable cells of the strains *B. licheniformis* ATCC SD 6525 and *B. subtilis* SD 2107 were not detected in the final additive. Uncertainty remains on the presence of viable cells of *T. reesei* ATCC 5888 due to the insufficient number of replicated analysis.

The presence of recombinant DNA from the three production strains was analysed in three batches of the final product.[37] [redacted] The analyses showed no amplification in the samples [redacted] Further data on the presence of recombinant DNA from *B. licheniformis* SD-6525 was provided for three batches of the intermediate concentrate [redacted][38] [redacted] The analyses showed no amplification in the samples.

3.1.3.2. Presence of other microbial strains related to the manufacturing process/product

[redacted] The analyses done [redacted] do not allow to conclude on the absence of viable cells [redacted]

The absence of viable cells and recombinant DNA of the production strain [redacted] was demonstrated [redacted] (see Annex B).

3.1.4. Stability and homogeneity

The shelf-life of three batches of Axtra® XAP 104 TPT was studied at 25°C (60% relative humidity (RH)) and 40°C (75% RH).[39] Samples of the additive were stored in their commercial packaging for up to 9 months. Mean recovery of xylanase/amylase/protease activity after 9 months at 25°C was of 99/83/95%. Mean recovery of xylanase/amylase/protease activity after 3 months at 40°C was 78/57/86% and after 9 months was 44/13/45%.

Three batches of the additive were added to a vitamin-mineral complete premixture for poultry (including choline chloride), to provide xylanase/amylase/protease 609/59/1,212 U/g premixture.[40] Samples were stored in closed plastic containers at 25°C (60% RH) for up to 6 months. Mean recovery of xylanase/amylase/protease activity after 6 months at 25°C was 99/102/97%.

Three batches of the additive were mixed in a complete feed (mash form) based on maize and soya bean meal (xylanase/amylase/protease 2,000/200/4,000 U/kg).[41] Samples were kept in closed paper bags at 25°C (60% RH) for 3 months. Mean enzyme activity recovery of xylanase/amylase/protease after 3 months was 77/48/104%. The mash feed was pelleted at 95°C.[42] Mean enzyme activity recovery of xylanase/amylase/protease after pelleting was 84/101/102%. Samples of the pelleted feed were stored in closed paper bags at 25°C for 3 months.[43] Mean enzyme activity recovery of the activities present after pelleting for xylanase/amylase/protease after 3 months storage was 85/73/95%.

The capacity of the additive to homogeneously distribute was studied in mash feed by analysing 10 subsamples of a batch. The coefficient of variation was 8% for xylanase and amylase and 7% for protease.[41]

3.1.5. Conditions of use

Axtra® XAP 104 TPT is proposed to be used as a zootechnical additive (functional group: digestibility enhancers) in feed for chickens for fattening, chickens reared for laying, laying hens and

[37] Technical dossier/Supplementary information June 2019/Annex_10a, Annex_10b and Annex_10c.
[38] Technical dossier/Supplementary information June 2019/Annex_2a5 and 5c2.
[39] Technical dossier/Section II/Annex II.34.
[40] Technical dossier/Section II/Annex II.35.
[41] Technical dossier/Section II/Annex II.36.
[42] Technical dossier/Section II/Annex II.38.
[43] Technical dossier/Section II/Annex II.37.

all minor poultry species at a minimum level of 1,000 xylanase U, 100 amylase U and 2,000 protease U per kg feed (50 mg additive/kg feed).

3.2. Safety

3.2.1. Safety aspects of the production organisms

The assessment of the genetic modification of the *T. reesei* ATCC PTA-5588 was performed in a previous evaluation (EFSA, 2007b) and the Panel concluded that the genetic modification does not raise any safety concern. The production strain has not been subject to any further genetic modification and no new information has been made available that would lead the Panel to reconsider its previous conclusion. The recombinant DNA of the strain was not detected in the additive, but uncertainty remains on the presence of viable cells in the product due to the limited data submitted.

Regarding the other two production strains, *B. licheniformis* ATCC SD 6525 and *B. subtilis* ATCC SD 2107, their parental strains belong to species considered to qualify for the qualified presumption of safety (QPS) approach to safety assessment (EFSA, 2007a; EFSA BIOHAZ Panel, 2020). This approach requires the identity of the strain to be conclusively established and evidence that the strain lacks toxigenic potential and does not show acquired resistance to antibiotics of human and veterinary importance and for genetically modified strains the safety of the genetic modification needs to be established. The identification of the two strains has been conclusively established at species level and lack of toxigenic potential has been confirmed. The two strains are resistant to [redacted], a relevant antimicrobial, [redacted] However, viable cells and recombinant DNA of these two bacterial strains were not detected in the final product. Therefore, the use of *B. licheniformis* ATCC SD 6525 and *B. subtilis* ATCC SD 2107 in the production of the enzymes contained in the final product does not raise safety concerns as regards the genetic modification of the production strains.

3.2.2. Toxicological studies

The applicant provided toxicological tests for the evaluation of the fermentation products that provide the declared main enzyme activities in the additive: xylanase, protease and amylase. [redacted]

3.2.2.1. Xylanase from *T. reesei* ATCC PTA-5888

Bacterial reverse mutation assay

In order to investigate the potential of the enzyme preparation containing xylanase to induce gene mutations in bacteria, the Ames test was performed according to OECD Test Guideline 471 (1997) [redacted][44] The Panel concluded that the test item did not induce gene mutations in bacteria under the experimental conditions employed in this study.

In vitro *chromosomal aberration test*

A chromosomal aberrations test was performed according to OECD Test Guideline 473 [redacted][45]

[44] Technical dossier/Section III/Annex III.3.
[45] Technical dossier/Section III/Annex III.6.

[redacted] The Panel concluded that the test item did not induce chromosome damage in cultured human peripheral blood lymphocytes under the experimental conditions employed in this study.

Subchronic oral toxicity study

[redacted] The study was conducted in compliance with OECD guideline 408.[46] [redacted] From this study, a no observed adverse effect level (NOAEL) [redacted] the highest dose tested, was identified.

Considerations on the test item

[redacted][47]

3.2.2.2. Amylase from *Bacillus licheniformis* ATCC SD-6525

The parental strain belongs to a species considered to qualify for the qualified presumption of safety (QPS) approach to safety assessment (EFSA, 2007a; EFSA BIOHAZ Panel, 2020). The genetic modification is not expected to have an impact on the toxicological profile of the production strain and data supporting the lack of toxigenicity for the production strain has been provided. Therefore, from this point of view, the production strain is presumed as safe. The applicant provided some toxicological studies to support the safety of the product.

Bacterial reverse mutation assay

In order to investigate the potential of the enzyme preparation containing amylase to induce gene mutations in bacteria, the Ames test was performed according to OECD Test Guideline 471 [redacted][48]

[redacted]

[46] Technical dossier/Section III/Annex III.9.
[47] Technical dossier/Supplementary information June 2019/Annex 14a.
[48] Technical dossier/Section III/Annex III.4.

[redacted] The Panel concluded that the test item did not induce gene mutations in bacteria under the experimental conditions employed in this study.

In vitro *chromosome aberration test in human lymphocytes*

A chromosomal aberrations test was performed according to OECD Test Guideline 473 (1997) [redacted][49] The Panel concluded that the test item did not induce chromosome damage in cultured human peripheral blood lymphocytes under the experimental conditions employed in this study.

Subchronic oral toxicity study

[redacted][50] The study was conducted in GLP and compliance with OECD guideline 408.

[redacted] From this study, an NOAEL [redacted] the highest dose tested, was identified.

3.2.2.3. Protease from *Bacillus subtilis* ATCC SD 2107

The parental strain belongs to a species considered to qualify for the qualified presumption of safety (QPS) approach to safety assessment (EFSA, 2007a; EFSA BIOHAZ Panel, 2020). The genetic modification is not expected to have an impact on the toxicological profile of the production strain and data supporting the lack of toxigenicity has been provided. Therefore, from this point of view, the production strain is presumed as safe. The applicant provided the following toxicological studies to support the safety of the product.

Bacterial reverse mutation assay

In order to investigate the potential of the enzyme preparation containing protease to induce gene mutations in bacteria, the Ames test was performed [redacted][51]

[49] Technical dossier/Section III/Annex III.7.
[50] Technical dossier/Section III/Annex III.10.
[51] Technical dossier/Section III/Annex III.5a to III.5c.

[redacted] The Panel concluded that the test item did not induce gene mutations in bacteria under the experimental conditions employed in this study.

Chromosomal aberration test

A chromosomal aberrations test was performed [redacted] to evaluate the potential [redacted] induce chromosome damage in human lymphocytes.[52] [redacted] The Panel concluded that the test item did not induce chromosome damage in cultured human peripheral blood lymphocytes under the experimental conditions employed in this study.

Subchronic oral toxicity study

[redacted][53]

From this study, a no observed adverse effect level (NOAEL) of [redacted], the highest dose tested, was identified.

Considerations on the test item

[redacted][54]

3.2.2.4. Conclusions on the toxicological studies

The three products of fermentation used for the preparation of the additive showed no genotoxicity potential in a battery of tests addressing gene mutation, and numerical and structural chromosome aberrations. The results obtained in subchronic oral toxicity studies raised no concerns regarding the products. The results from the protease and amylase enzyme products do support the presumption of safety of the fermentation products resulting from the two production strains *B. licheniformis* ATCC SD 6525 and *B. subtilis* ATCC SD 2107, respectively. However, the Panel cannot conclude on the suitability

[52] Technical dossier/Section III/Annex III.8.
[53] Technical dossier/Section III/Annex III.11.
[54] Technical dossier/Supplementary information June 2019/Annex 14b.

of the test item used in the tests submitted for the assessment of the fermentation product containing the xylanase, and consequently, no conclusion can be drawn from those studies.

Regarding [redacted], the results obtained in the toxicological tests submitted (see Annex B) showed no safety concerns. [redacted]

3.2.3. Safety for the target species

3.2.3.1. Safety for chickens for fattening

The applicant submitted two tolerance trials, one of which was not considered further due to the high mortality of the birds [redacted][55]

In the second trial,[56] [redacted]

The results of the study showed no adverse effects of the additive in chickens for fattening up to 30-fold the minimum recommended dose of 1,000 xylanase U, 100 amylase U and 2,000 protease U per kg feed.

3.2.3.2. Safety for laying hens

[redacted][59]

[55] Technical dossier/Section III/Annex III.1.

[56] Technical dossier/Supplementary information June 2019/Annex 12a to 12d and supplementary information February 2020/ Annex SI_8.

[redacted]

[59] Technical dossier/Section III/Annex III.2.

[redacted]

Feeding the laying hens with the additive up to 30-fold the minimum recommended dose of 1,000 xylanase U, 100 amylase U and 2,000 protease U per kg feed, did not have negative effects on the laying performance, or on biochemical and haematological blood parameters.

Conclusions for the target species

The tolerance studies provided in chickens for fattening and laying hens showed that animals tolerated up to 30 times the minimum recommended level of 1,000 xylanase U, 100 amylase and 2,000 protease U/kg feed. Therefore, the Panel concludes that Axtra® XAP 104 TPT is safe for chickens for fattening and laying hens animals under the proposed conditions of use. This conclusion can be extended to chickens reared for laying. Considering the wide margin of safety shown, the FEEDAP Panel extrapolates the conclusion to minor poultry species for fattening or laying.

The Panel notes that uncertainty remains regarding the test item used in the toxicological studies of the xylanase, which prevent the Panel reaching a conclusion on the toxicological safety, including genotoxicity of the xylanase component of the additive. This uncertainty regarding genotoxicity has an impact also on the safety of the additive for target animals, especially laying hens and minor species for laying. Therefore, the Panel is not in a position to conclude on the safety of Axtra® XAP 104 TPT for the target species.

3.2.4. Safety for the consumer

The results obtained in the genotoxicity and subchronic oral toxicity studies performed with the test items did not indicate safety concerns resulting from the fermentation products used in the formulation/manufacturing. However, uncertainties remain on the suitability of the test item used in the studies conducted with the xylanase which may not reflect the fermentation product currently used in the formulation of the additive, and consequently, no conclusions can be drawn regarding the safety of the additive for the consumers.

3.2.5. Safety for user

No specific data were provided to address the safety for the users. In the absence of such data, the FEEDAP Panel cannot conclude on the potential of the additive to be irritant to the skin and eyes or on its skin-sensitising properties.

Owing to the nature of the active substances, the additive should be considered a respiratory sensitiser, the dusting potential is negligible; therefore, the likelihood of exposure is low.

Uncertainty remains as regards the suitability of the test item in the toxicological studies submitted, including the genotoxicity potential, for the fermentation product that contains the xylanase. The results from those tests are relevant for the users who may be in direct contact with it. Since no conclusion was drawn from the studies, the FEEDAP Panel cannot conclude on the safety of the additive for the user.

[redacted]

3.2.6. Safety for the environment

Viable cells and recombinant DNA of the genetically modified strains of *B. licheniformis* ATCC SD 6525 and *B. subtilis* ATCC SD 2107 were not detected in the final product. Owing to the insufficient number of replicated analysis, uncertainty remains on the presence of viable cells of the genetically modified strain *T. reesei* ATCC 5888 in the final additive. The data on the [redacted] product indicate that viable cells and recombinant DNA of the production strain would not be present in the additive.

The active substances present in the additive are proteins and as such will be degraded/inactivated during the passage through the digestive tract of animals. Therefore, no risks to the environment are expected from the active compounds. However, uncertainty remains on the presence of viable cells of the genetically modified strain *T. reesei* ATCC 5888 in the additive. Therefore, the Panel cannot conclude on the safety of the additive for the environment.

3.3. Efficacy

3.3.1. Efficacy for chickens for fattening

Five efficacy trials were submitted. Two of the studies were not further considered due to the high mortality registered in one case ([redacted])[62] or the low performance of the birds in the other case (30% below the performance objectives).[63]

For the other three trials, the details of the study design are given in the Table 1 and the results in Table 2. In all trials, 1-day-old male Ross 308 birds were used and were kept under study for at least 35 days. In the three studies, the birds received either a non-supplemented diet (control) or a diet containing the additive to provide the minimum recommended level of 1,000 U xylanase, 100 U amylase and 2,000 U protease per kg feed. Two of the studies included also a further treatment with double the minimum recommended dose. The confirmation of the enzyme activities is presented in Table 1. The health and mortality were monitored throughout the study and the body weight and feed intake were recorded. Feed to gain ratio was calculated. The data were analysed with an ANOVA (pen basis) and group means were compared with Tukey (trial 2) or Duncan (trial 3) tests. The significance level was set at $p < 0.05$.

Table 1: Experimental design of the efficacy trials performed in chickens for fattening

Trial	Total no. of animals (animals × replicate) replicates × treatment	Breed sex (duration)	Composition feed (Form)	Enzyme activities xylanase/ amylase/protease (U/kg feed)	
				Intended	Analysed
1[64]	480 (20) 12	Ross 308 Males (35 days)	Maize, wheat, soya bean meal (pelleted)	0/0/0 1,000/100/2,000	–/–/– 1,135/103/2,116
2[65]	1,056 (22) 16	Ross 308 Males (42 days)	Maize, soya bean meal, wheat middling (mash)	0/0/0 1,000/100/2,000 2,000/200/4,000	–/–/– 1,105/114/2,436 2,427/216/6,000
3[66]	1,500 (50) 10	Ross 308 Males (42 days)	Wheat, maize, soya bean meal (mash)	0/0/0 1,000/100/2,000 2,000/200/4,000	–/–/– 1,154/126/2,688 2,092/269/5,163

The mortality was low and not different between the groups. The birds that received the additive at the minimum recommended level showed compared to the control improvements in the final body weight (trial 3) and on the feed to gain ratio (trial 1). In trial 2, birds receiving the additive at double the minimum recommended dose showed, compared to the control, improvements in the final body weight and the feed to gain ratio. Therefore, the results of the studies showed that the additive has the potential to be efficacious in chickens for fattening at the level of 2,000 xylanase U, 200 amylase U and 4,000 protease U per kg feed.

[62] Technical dossier/Section IV/Annex IV.2
[63] Technical dossier/Section IV/Annexes IV.3.1 and IV.3.2.
[64] Technical dossier/Section IV/Annex IV.4.1 and IV.4.2.
[65] Technical dossier/Supplementary information June 2019/Annex 16a.
[66] Technical dossier/Supplementary information June 2019/Annex 16b.

Table 2: Effects of Axtra® XAP 104 TPT on the performance and mortality of chickens for fattening

Trial	Enzyme activity xylanase/ amylase/protease (U/kg feed)	Feed intake (g)(1)	Final body weight (g)	Feed to gain ratio	Mortality and culling (%)
1	0/0/0	3,644	2,408	1.54[a]	2.5
	1,000/100/2,000	3,639	2,438	1.52[b]	3.6
2	0/0/0	93.3	2,516[b]	1.58[a]	3.1
	1,000/100/2,000	95.1	2,581[ab]	1.57[ab]	4.5
	2,000/200/4,000	94.4	2,599[a]	1.55[b]	3.4
3	0/0/0	101	2,748[b]	1.57	1.4
	1,000/100/2,000	103	2,852[a]	1.55	1.0
	2,000/200/4,000	102	2,830[a]	1.56	2.0

a,b: Mean values within a trial and within a column with a different superscript are significantly different $p < 0.05$.
(1): Total feed intake for trial 1 and daily feed intake for trials 2 and 3.

3.3.2. Efficacy for laying hens

Three trials were provided, one short term and two long term.

The short-term trial was a balance trial[67] conducted with 96 21-week-old Lohmann-Brown layers which were caged in groups of two and distributed to four dietary treatments (12 replicates per treatment). A basal diet (Gross energy content ~14.5 MJ/kg feed) based on maize and soya bean meal was either not supplemented (control) or supplemented with the additive to provide xylanase/amylase/protease 1,000/100/2,000, 1,500/150/3,000 or 2,000/200/4,000 U per kg feed (enzyme activities were confirmed by analysis). The feed was offered *ad libitum* from weeks 21–25 in mash form and contained titanium dioxide as an external marker. Laying performance of the hens was measured and a balance study was performed on the last 4 days under study. Feed and excreta samples were analysed for different parameters including the content of energy to determine the content of metabolisable energy in the diets. An ANOVA was done with the data and the mean groups were compared with Tukey test. One hen died during the study. The results of the balance trial showed a significantly higher metabolisable energy content of the diets in the hens fed the additive. Values of metabolisable energy content (nitrogen corrected) were 10.9, 11.5, 11.8 and 11.9 MJ/kg feed for the control, 1,000/100/2,000, 1,500/150/3,000 and 2,000/200/4,000 U per kg feed, respectively. No differences were observed in the laying performance of the hens during the experimental period.

The other two studies were designed as long-term trials and were done in the same place, same dates and with diets with very similar composition.[68] In each trial, a total of 240 21-week-old Isa Brown laying hens were distributed in cages of five hens and the cages were allocated to two dietary treatments (24 replicates per treatment). In each study, two basal diets (depending on the stage, I or II) based on wheat and soya bean meal were either not supplemented (control) or supplemented with the additive to provide xylanase/amylase/protease 1,000/100/2,000 U per kg feed (enzyme activities were confirmed by analysis). Diets were offered for 38 weeks in mash form. Health and mortality were checked throughout the study. Hens were weighed at the beginning and at the end of the trial. Egg production was monitored daily and the weight of the eggs was measured on all eggs laid on one day per month. The egg mass produced was estimated from the eggs produced and the average egg weight measured once per month. Feed intake was measured throughout the study and feed to egg mass ratio was calculated. At the end of the trial, yolk colour, albumen height and shell thickness were measured. An ANOVA was done with the data and the significance level was set at $p < 0.05$.

Mortality in the study was acceptable. The results showed that in the two trials, the hens receiving the additive at the minimum recommended dose gained less weight than the control group and showed a significantly better feed to egg mass ratio (mortality corrected) (Table 3). In the second of the trials, feeding the hens with the additive resulted in a lower feed intake. No differences were observed in any of the other parameters. [redacted]

[67] Technical dossier/Section IV/Annex IV.1.1.
[68] Technical dossier/Section IV/Annex IV.5 and IV.6 and Supplementary information June 2019/Annex 18.

[redacted][69] Taking into consideration this and the fact that the trials took place in the same place and time, the Panel considers that the two studies are not independent and therefore would count as a single study.

The hens fed the additive at the recommended dose showed a better utilisation of the energy present in the diets and improvements on the feed to egg mass ratio in the long-term study provided. In the absence of a third study with significant and positive effects the FEEDAP Panel cannot conclude on the efficacy of Axtra® XAP 104 TPT in laying hens.

Table 3: Effect of Axtra® XAP 104 TPT on the performance of laying hens

Trial	Treatments	Body weight change (g/hen)	Daily feed intake (g/hen)	Laying rate (%)	Egg weight (g)	Daily egg mass per hen (g/hen)	Feed to egg mass	Mortality (%)
1	0/0/0 1,000/100/2,000	258[a] 188[b]	122 120	93.5 92.9	63.1 63.8	58.8 59.3	2.07[a] 2.03[b]	0.9 3.8
2	0 1,000/100/2,000	290[a] 206[b]	123[a] 119[b]	92.2 92.1	63.0 63.3	58.1 58.3	2.13[a] 2.06[b]	6.7 5.0

a,b: Values within one column for the same study with different superscripts are significantly different ($p < 0.05$).

3.3.2.1. Conclusions on efficacy

Based on the results obtained in the efficacy trials, the FEEDAP Panel concludes that Axtra® XAP 104 TPT is efficacious in chickens for fattening at the level of 2,000 U xylanase, 200 U amylase and 4,000 U protease per kg feed (double the minimum recommended dose). The conclusion on the efficacy is extended to chickens reared for laying. Since the mode of action of the enzymes is well known and can be assumed to be the same in poultry species, the conclusion is extrapolated to minor poultry species for fattening or reared for laying/breeding.

The FEEDAP Panel cannot conclude on the efficacy of the product in laying hens or in other poultry species for laying because a third evidence is missing.

3.4. Post-market monitoring

The FEEDAP Panel considers that there is no need for specific requirements for a post-market monitoring plan other than those established in the Feed Hygiene Regulation[71] and Good Manufacturing Practice.

4. Conclusions

The FEEDAP Panel notes that:

- The tolerance studies in chickens and laying hens showed that the additive is well tolerated at 30× the minimum recommended dose (or 15× the efficacious dose in chickens),
- The toxicological studies submitted did not indicate a concern of the test items used for the consumers,
- The additive is a respiratory sensitiser, but in the absence of data, no conclusions can be reached regarding skin/eye irritancy and skin sensitisation,

[69] Technical dossier/Supplementary information February 2020/Annex SI_9.
[70] Sources of information included, Dierick and Decuypere (1994), Bach Knudsen (1997), Pedersen et al. (2014) and CVB Veevoedertabel (2019).
[71] Regulation (EC) No 183/2005 of the European Parliament and of the Council of 12 January 2005 laying down requirements for feed hygiene. OJ L 35, 8.2.2005, p. 1.

- No concerns for the environment are expected from the active substances used in the formulation of the additive,

However, the following limitations have been identified in the current assessment, namely:

- uncertainty remains on the presence of viable cells of the genetically modified strain *Trichoderma reesei* ATCC 5888,
- uncertainty remains on the characterisation of the [redacted] used in the manufacturing process of the protease and their presence in the final additive,
- uncertainty remains regarding the test item used in the toxicological studies of the xylanase, which prevent the Panel reaching a conclusion on the safety, including the endpoint of genotoxicity, of the xylanase component of the additive.

Considering the uncertainties above, the FEEDAP Panel is not in a position to conclude on the safety of Axtra® XAP 104 TPT for the target species, consumers, users and the environment.

The FEEDAP Panel concludes that Axtra® XAP 104 TPT is efficacious in chickens for fattening, chickens reared for laying and minor poultry species up to the point of lay at the level of 2,000 xylanase U, 200 amylase U and 4,000 protease U per kg feed (double the minimum recommended dose). Owing to the lack of enough data, the Panel cannot conclude on the efficacy of the additive for laying hens.

5. Documentation as provided to EFSA/Chronology

Date	Event
06/10/2017	Dossier received by EFSA. Axtra® XAP 104 TPT for poultry species. Submitted by Danisco (UK) Ltd.
30/10/2017	Reception mandate from the European Commission
13/12/2017	Application validated by EFSA – Start of the scientific assessment
13/02/2018	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. *Issues: methods of analysis*
13/03/2018	Comments received from Member States
14/03/2018	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. *Issues: characterization, safety and efficacy*
30/04/2018	Reception of supplementary information from the applicant - EURL
23/05/2018	Reception of the Evaluation report of the European Union Reference Laboratory for Feed Additives
16/07/2018	Clarification teleconference during the risk assessment with the applicant
19/06/2019	Reception of supplementary information from the applicant - Scientific assessment re-started
02/10/2019	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. *Issues: characterization and efficacy*
13/12/2019	Clarification teleconference during the risk assessment with the applicant
13/02/2020	Reception of supplementary information from the applicant - Scientific assessment re-started
25/05/2020	Opinion adopted by the FEEDAP Panel. End of the Scientific assessment.

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Abbreviations

FEEDAP	Additives and Products or Substances used in Animal Feed
ATCC	American Type Culture Collection
EURL	European Union Reference Laboratory
AMR	Antimicrobial resistance
CV	Coefficient of variation
RH	Relative humidity
QPS	Qualified presumption of safety
NOAEL	No observed adverse effect level

Annex A – Executive Summary of the Evaluation Report of the European Union Reference Laboratory for Feed Additives on the Method(s) of Analysis for Axtra® XAP

In the current application, authorisation is sought under Article 4 (1) for *Axtra® XAP 104 TPT* under the category/functional group (4 a) 'zootechnical additive'/'digestibility enhancers', according to the classification system of Annex 1 of Regulation (EC) No 1831/2003. The authorisation is sought for the use of the *feed additive* for chickens for fattening and reared for laying, laying hens and all minor poultry species.

According to the Applicant, *Axtra® XAP 104 TPT* is a preparation containing *endo-1,4*-beta-*xylanase*, alpha-*amylase* and *protease.* The Applicant expressed the enzyme activities in different units defined as follows:

- one unit of *endo-1,4*-beta-*xylanase* activity (UX) is the amount of enzyme, which liberates 0.48 micromoles per minute of reducing sugars, expressed as xylose equivalents, from a wheat arabinoxylan substrate at pH 4.2 and 50°C;
- one unit of alpha-*amylase* activity (UA) is the amount of enzyme required to release, in the presence of an excess of alpha-glucosidase, 0.20 micromoles per minute of glucosidic linkages, expressed as p-nitrophenol equivalents, from a maltoheptasoide substrate at pH 8.0 and 40°C; and
- one unit of *protease* activity (UP) is the amount of enzyme which liberates 2.3 micrograms per minute of phenolic compounds, expressed as tyrosine equivalents, from a casein substrate at pH 10.0 and 50°C.

According to the Applicant, *Axtra® XAP 104 TPT* has a guaranteed minimum enzyme activity of 20,000 UX/g *endo-1,4*-beta-*xylanase,* 2,000 UA/g alpha-*amylase* and 4,000 UP/g *protease.* The product is intended to be incorporated directly in *feedingstuffs* or through *premixtures* with the following proposed minimum enzyme activities in *feedingstuffs*: 1,000 UX/kg for *endo-1,4*-beta-*xylanase;* 100 UA/kg for alpha-*amylase* and 2,000 UP/kg for *protease*.

For the quantification of the active substances in the *feed additive, premixtures* and *feedingstuffs*, the Applicant submitted three single laboratory validated and further verified colorimetric methods, based on the enzymatic hydrolysis:

- by xylanase of an azurine cross-linked wheat arabinoxylan substrate at pH 4.2 and 50°C for the determination of *endo-1,4*-beta-*xylanase*;
- by amylase of an azurine cross-linked starch polymer substrate at pH 8.0 and 40°C for the determination of alpha-*amylase*; and
- by protease of a dyed cross-linked casein substrate at pH 10.0 and 50°C for the determination of *protease*.

Based on the performance characteristics available, the EURL recommends for official control the proposed single-laboratory validated and further verified colorimetric methods for the quantification of the three enzymes in the *feed additive, premixtures* and *feedingstuffs.*

Further testing or validation of the methods to be performed through the consortium of National Reference Laboratories as specified by Article 10 (Commission Regulation (EC) No 378/2005) is not considered necessary.

Annex B – Characterisation of the [redacted] used [redacted]

In the manufacturing process, [redacted][72] [redacted][73] [redacted][74]

A.1. Description of the genetic modification

[redacted][75] [redacted][76] [redacted][77]

[72] Technical dossier/Supplementary information June 2019/Annex_7k.
[73] Technical dossier/Supplementary information June 2019/Annex_7b.
[74] Technical dossier/Supplementary information February 2020/Annex SI_5.
[75] Technical dossier/Supplementary information June 2019/Annex 7d and supplementary information February 2020.
[76] Technical dossier/Supplementary information June 2019/Annex_7e.
[77] Technical dossier/Supplementary information June 2019/Annex_7f.

[redacted]

The production strain [redacted] *is a genetically modified strain* [redacted] *The sequences introduced during the genetic modification raised no concerns.* [redacted] *However, viable cells and recombinant DNA of the production strain were not detected* [redacted] *no concerns regarding the genetic modification of the production strain* [redacted].

[redacted]

Bacterial reverse mutation assay

In order to investigate the potential of the [redacted] to induce gene mutations in bacteria, the Ames test was performed according to OECD Test Guideline 471 [redacted][80] [redacted] *The Panel concluded that the test item did not induce gene mutations in bacteria under the experimental conditions employed in this study.*

In vitro *mammalian chromosome aberration test*

A chromosomal aberrations test was performed according to OECD Test Guideline 473 [redacted][81] [redacted]

[78] Technical dossier/Supplementary information February 2020/Annex SI_7.
[79] Technical dossier/Supplementary information February 2020/Annex SI_6.
[80] Technical dossier/Supplementary information June 2019/Annex 7l.
[81] Technical dossier/Supplementary information June 2019/Annex 7m.

[redacted] The Panel concluded that the test item did not induce chromosome damage in cultured human peripheral blood lymphocytes under the experimental conditions employed in this study.

Subchronic oral toxicity study

A 90-day oral toxicity study was conducted. [redacted][82] The study was conducted according to OECD guideline 408, [redacted] There were no effects of treatment on clinical observations, body weight or food intake or any other endpoint investigated in the study. [redacted]

Conclusions on the toxicological tests

The results of the toxicological tests do not raise concerns from the fermentation product obtained from [redacted]

[82] Technical dossier/Supplementary information June 2019/Annex 7n.