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## Safety and efficacy of ENZY CARBOPLUS<sup>®</sup> (endo-1,4-beta-xylanase and endo-1,3(4)-beta-glucanase) as a feed additive for avian species, weaned piglets and minor weaned porcine species

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### Abstract

ENZY CARBOPLUS<sup>®</sup> is an additive available in liquid and solid forms that contains endo-1,4-beta-xylanase and endo-1,3(4)-beta-glucanase which are produced by two genetically modified strains of *Komagataella pastoris*. The Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) concluded that the production strains are safe and the genetic modifications raise no safety concerns. The Panel also concluded that the additive is safe for the target species at the recommended dosages and that its use as a feed additive raises no concern for consumers. The additive in either form is not a skin or eye irritant nor a dermal sensitiser; however, it is considered as a potential respiratory sensitiser. The production strains and their recombinant DNA were not detectable in the additive; moreover, the active substances of the additive will be degraded/inactivated during passage through the digestive tract of animals or in the environment, consequently, the Panel concluded that the additive poses no concerns to the environment. The Panel concluded that the additive has a potential to be efficacious as a zootechnical additive in chickens for fattening at 4,000 LXU and 375 LGU per kg feed, in turkeys for fattening at 1,400 LXU and 120 LGU per kg feed, and in weaned piglets at 700 LXU and 60 LGU per kg feed. The Panel extended these conclusions to chickens reared for laying and turkeys reared for breeding at the corresponding doses. Since the mode of action of xylanase and glucanase can be reasonably assumed to be the same within avian or within porcine species, the Panel extrapolated the conclusions on the efficacy to all avian species up to the point of lay and to all porcine species. Owing to the lack of data, no conclusions could be drawn on the efficacy of the additive in laying hens.

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## Summary

Following a request from the European Commission, the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) was asked to deliver a scientific opinion on the safety and the efficacy of ENZY CARBOPLUS® (endo-1,4-beta-xylanase and endo-1,3(4)-beta-glucanase) as a feed additive for avian species, weaned piglets and minor weaned porcine species.

ENZY CARBOPLUS® is a feed additive that presents endo-1,4-beta-xylanase and endo-1,3(4)-beta-glucanase which is available in solid and liquid form. The enzymes present in the product are produced by two genetically modified strains of *Komagataella pastoris*. The species *K. pastoris* is considered by the European Food Safety Authority (EFSA) to be suitable for the qualified presumption of safety (QPS) approach to establishing safety for the target species, consumers and the environment. The production strains were identified and were considered safe. The genetic modifications raised no safety concerns. The strains and their recombinant DNA were not detected in the final product.

The results of the tolerance studies showed no negative effects in chickens and turkeys for fattening, laying hens and in weaned piglets. The Panel concluded that the additive is safe for chickens for fattening, turkeys for fattening and laying hens at the dose of 1,400 LXU and 120 LGU/kg feed and safe for weaned piglets at the dose of 700 LXU and 60 LGU/kg feed. The conclusion was extended to chickens reared for laying and turkeys raised for breeding at the level of 1,400 LXU and 120 LGU/kg feed. The safety of the additive was shown in major poultry and porcine species with a wide margin of safety. Therefore, the FEEDAP Panel extrapolated the conclusions to all avian species (1,400 LXU and 120 LGU/kg feed) and to minor porcine species for growing (700 LXU and 60 LGU/kg feed).

The toxicological studies are not required if the fermentation products are produced by a genetically modified microorganism that is considered by EFSA to qualify for the QPS approach to safety assessment and the genetic modification raises no concerns. The production strains belong to a species that is considered to qualify for the QPS approach to safety assessment provided that the identity is unambiguously established. The identity of the production strains was established and the genetic modifications raised no concerns. Therefore, the FEEDAP Panel concluded that the use of ENZY CARBOPLUS® as a feed additive raises no concern for consumers.

The additive in either form is not a skin or eye irritant nor a dermal sensitiser. Owing to the proteinaceous nature of the active substance, the additive is considered as a potential respiratory sensitiser.

Neither the production strains nor their recombinant DNA were detectable in the final product. Moreover, the active substances of the additive are proteins, and as such will be degraded/inactivated during passage through the digestive tract of animals or in the environment. Consequently, the additive does not raise concerns for the environment.

The results of the efficacy studies showed that the additive has a potential to be efficacious as a zootechnical additive in chickens for fattening at 4,000 LXU and 375 LGU per kg feed, in turkeys for fattening at 1,400 LXU and 120 LGU per kg feed, and in weaned piglets at 700 LXU and 60 LGU per kg feed. Owing to the lack of data, no conclusions could be drawn on the efficacy of the additive in laying hens. The Panel extended the conclusions reached in chickens and turkeys for fattening to chickens raised for laying and turkeys reared for breeding at the corresponding doses. Since the mode of action of xylanase and glucanase is well known and can be reasonably assumed to be the same within avian species or within porcine species, the Panel extrapolated the conclusions from turkeys for fattening to all avian species up to the point of lay and the conclusions from weaned piglets to all porcine species (in the category of weaned piglets).

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## 1. Introduction

### 1.1. Background and Terms of Reference

Regulation (EC) No 1831/2003<sup>1</sup> 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 Kaesler Nutrition GmbH<sup>2</sup> for authorisation of the product ENZY CARBOPLUS® (endo-1,4-beta-xylanase and endo-1,3(4)-beta-glucanase), when used as a feed additive for chickens for fattening and reared for laying, turkeys for fattening and reared for breeding, laying hens, weaned piglets, minor weaned porcine (e.g. warthogs), avian species (game birds, ducks, geese, pigeons, sporting and ornamental birds), including laying birds (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 27 August 2013.

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 ENZY CARBOPLUS® (endo-1,4-beta-xylanase and endo-1,3(4)-beta-glucanase), when used under the proposed conditions of use (see Section 3.1.5).

### 1.2. Additional information

ENZY CARBOPLUS® is an additive that presents endo-1,4-beta-xylanase and endo-1,3(4)-beta-glucanase activities that has not been authorised in the European Union (EU). The enzymes present in the product are produced by two genetically modified strains of *Komagataella pastoris* (DSM 25376 and DSM 26469). The species *K. pastoris* is considered by EFSA to be suitable for the qualified presumption of safety (QPS) approach to establishing safety for the target species, consumers and the environment (EFSA, 2007EFSA, 2008b; EFSA BIOHAZ Panel, 2017); this approach requires the identity of the strain to be established.

## 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<sup>3</sup> in support of the authorisation request for the use of ENZY CARBOPLUS® as a feed additive. The technical dossier was prepared following the provisions of Article 7 of Regulation (EC) No 1831/2003, Regulation (EC) No 429/2008<sup>4</sup> and the applicable EFSA guidance documents.

EFSA has verified the European Union Reference Laboratory report as it relates to the methods used for the control of the ENZY CARBOPLUS® in animal feed. The Executive Summary of the European Union Reference Laboratory report can be found in Annex A.<sup>5</sup>

### 2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of ENZY CARBOPLUS® is in line with the principles laid down in Regulation (EC) No 429/2008 and the relevant

<sup>1</sup> 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.

<sup>2</sup> Kaesler Nutrition GmbH, Zeppelinstrasse 3, 27472 Cuxhaven, Germany.

<sup>3</sup> FEED dossier reference: FAD-2013-0013.

<sup>4</sup> 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.

<sup>5</sup> The full report is available on the EURL website: [https://ec.europa.eu/jrc/sites/jrcsh/files/FinRep-FAD-2013-0013-EnzyCarboplus.doc\\_.pdf](https://ec.europa.eu/jrc/sites/jrcsh/files/FinRep-FAD-2013-0013-EnzyCarboplus.doc_.pdf)

guidance documents: Guidance on zootechnical additives (EFSA FEEDAP Panel, 2012a), Technical guidance: Tolerance and efficacy studies in target animals (EFSA FEEDAP Panel, 2011), Technical Guidance for assessing the safety of feed additives for the environment (EFSA, 2008a), Guidance for establishing the safety of additives for the consumer (EFSA FEEDAP Panel, 2012b), Guidance on studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012c), Technical Guidance: Extrapolation of data from major species to minor species regarding the assessment of additives for use in animal nutrition (EFSA, 2008b), Technical Guidance: Microbial Studies (EFSA, 2008c) and the Guidance on the risk assessment of genetically modified microorganisms and their products intended for food and feed use (EFSA GMO Panel, 2011).

### 3. Assessment

ENZY CARBOPLUS® is a feed additive that contains endo-1,4-beta-xylanase (Enzyme Commission number 3.2.1.8; xylanase) and endo-1,3(4)-beta-glucanase (Enzyme Commission number 3.2.1.6; glucanase) and is intended to be used as a zootechnical additive (functional group: digestibility enhancers) in chickens for fattening and reared for laying, turkeys for fattening and reared for breeding, laying hens, weaned piglets, minor weaned porcine and minor avian species.

#### 3.1. Characterisation

##### 3.1.1. Characterisation of the production organisms

The xylanase and the glucanase present in the additive are produced by two genetically modified strains of *K. pastoris* (formerly named as *Pichia pastoris*), deposited at the German Collection of Microorganisms and Cell Cultures (DSMZ) with the accession numbers DSM 25376 and DSM 26469, respectively.<sup>6</sup>

##### 3.1.1.1. Information related to production strain *Komagataella pastoris* DSM 25376

*Characteristics of the recipient or parental microorganism*<sup>7</sup>

The recipient strain is *K. pastoris* GS115. This species is considered by EFSA to be suitable for the QPS approach for safety assessment (EFSA, 2007; EFSA BIOHAZ Panel et al., 2017). Its genome has been fully sequenced (De Schutter et al., 2009). The identity of the strain was confirmed by analysis of the ITS sequences of the ribosomal operons.<sup>8</sup>

The synthetic *Xyn-CDBFV* gene, encoding xylanase,<sup>9</sup> is based on the xylanase gene of the fungus *Neocallimastix patriciarum*. The encoded amino acid sequence differs from the native one in seven amino acids<sup>10</sup> (Chen et al., 2001).

*Description of the genetic modification process*

The synthetic *Xyn-CDBFV* sequence, without the predicted signal peptide, was amplified by polymerase chain reaction (PCR), generating a product of circa 700 bp. This product was ligated into plasmid pPIC9 after digestion with the restriction enzymes *EcoR* I and *Not* I. Plasmid pPIC9 is a derivative of pBR322 and contains an ampicillin resistance gene. Plasmid pPIC9 also carries the  $\alpha$ -MF signal peptide for protein secretion, a selectable marker for growth of transformed *K. pastoris* on a histidine-deficient medium, and both extremes from the alcohol oxidase gene (AOX1) to enable the replacement of the original AOX gene with the *Xyn-CDBFV* xylanase gene by homologous recombination. The resulting plasmid pPIC9-xylanase was maintained in *Escherichia coli*.

Plasmid pPIC9-xylanase (8.7 kb) was purified from *E. coli* and digested with the restriction endonuclease *Bgl*II. A 6.3 kb fragment, containing the *Xyn-CDBFV* xylanase gene, the extremes of the AOX1 gene, and the *his4* gene used as a selectable marker, was gel-purified and introduced by electroporation into *K. pastoris* GS115. By means of homologous recombination (double crossover), the AOX1 construct of *K. pastoris* GS115 was replaced by the recombinant expression cassette, resulting in the production strain *K. pastoris* DSM 25376. The integrity of the recombinant DNA and its chromosomal insertion were confirmed by PCR.

<sup>6</sup> Technical dossier/Section II/Annex II.2.1.2.8.

<sup>7</sup> This section has been modified according to the confidentiality claim made by the applicant.

<sup>8</sup> Technical dossier/Section II/Annex II.2.1.2.1.

<sup>9</sup> Technical dossier/Section II/Annex II.2.1.2.2.

<sup>10</sup> Technical dossier/Section II/Annex II.2.1.2.3.

Southern analysis showed that *K. pastoris* DSM 25376 contains a single copy of the recombinant gene.<sup>11</sup> The absence of the ampicillin resistance gene from plasmid pPIC9 in the genomic DNA of the production strain was demonstrated by Southern analysis.<sup>12</sup> Genetic stability was confirmed by Southern analysis targeting the recombinant xylanase gene.<sup>12</sup>

*K. pastoris* DSM 25376 differs from its parental strain in the ability to produce xylanase and to grow in the absence of histidine in the culture medium. Microscopic analyses and various physiological tests, including response to different antimycotics, revealed no other difference between the recipient and the production strain.<sup>13</sup>

### 3.1.1.2. Information related to production strain *Komagataella pastoris* DSM 26469

This strain was also constructed from the recipient strain *K. pastoris* GS115 (see Section 3.1.1.1).

#### *Characteristics of the inserted sequences*

The synthetic glucanase gene was based on the gene present in *Paenibacillus* sp. F-40 (CGMCC 1775).<sup>6</sup>

#### *Description of the genetic modification process*

The glucanase sequence, without the predicted signal peptide, was amplified by PCR, generating a product of ca. 650 bp. This product was ligated into plasmid pPIC9, after digestion with the restriction enzymes *EcoR* I and *Not* I. The resulting plasmid pPIC9-glucanase was maintained in *E. coli* JM109.

Plasmid pPIC9-glucanase (ca 8.7 kb) was purified from *E. coli* JM109 and digested with the restriction endonuclease *Bgl*II. A ca. 6.3 kb fragment, containing the glucanase gene, both extremes of the AOX1 gene, and the *his4* gene used as a selectable marker, was gel-purified and introduced by electroporation into *K. pastoris* GS115. By means of homologous recombination (double crossover), the endogenous AOX1 construct of *K. pastoris* GS115 was replaced by the recombinant expression cassette, resulting in the production strain *K. pastoris* DSM 26469. The integrity of the recombinant DNA and its chromosomal insertion were confirmed by PCR.

Southern analysis showed that *K. pastoris* DSM 26469 contains a single copy of the recombinant gene.<sup>10</sup> The absence of the ampicillin resistance gene from plasmid pPIC9 in the genomic DNA of the production strain was demonstrated by Southern analysis.<sup>12</sup> Genetic stability was confirmed by Southern analysis targeting the recombinant glucanase gene.<sup>12</sup>

*K. pastoris* DSM 26469 differs from its parental strain in the ability to produce glucanase and to grow in the absence of histidine in the culture medium. Microscopic analyses and various physiological tests, including response to different antimycotics, revealed no other difference between the recipient and the production strain.<sup>13</sup>

### 3.1.2. Manufacturing process<sup>14</sup>

The two enzymes are produced separately following the same process. The enzymes are produced by submerged fermentation with the respective production strain. The fermentation broths are concentrated-purified afterwards and the resulting products are used to formulate the final formulations.

### 3.1.3. Characterisation of the additive

The additive is available in solid, ENZY CARBOPLUS®, and liquid form, ENZY CARBOPLUS® L. The two ensure a minimum of 25,000 xylanase units (LXU)<sup>15</sup> and 2,200 glucanase units (LGU)<sup>16</sup> per gram of product.

ENZY CARBOPLUS® contains the enzyme (1.5%) and 88.5% of (corn) starch and 10% moisture. The study of the batch-to-batch variation in five batches showed a mean value of xylanase and glucanase of 25,627 LXU and 2,301 LGU per gram, respectively, ranging from 25,100/2,243 (xylanase/

<sup>11</sup> Technical dossier/Section II/Annex II.2.1.2.4.

<sup>12</sup> Technical dossier/Supplementary information April 2014.

<sup>13</sup> Technical dossier/Section II/Annex II.1.4.1.2.

<sup>14</sup> This section has been amended following the provisions of Article 8(6) and Article 18 of Regulation (EC) No 1831/2003.

<sup>15</sup> LXU: one xylanase unit is the amount of enzyme that releases one  $\mu$ mole of reducing sugar equivalents per minute (as xylose from birch xylan) at pH 5.5 and 50°C.

<sup>16</sup> LGU: one glucanase unit is the amount of enzyme which liberates one  $\mu$ mole of reducing sugar equivalents per minute (as glucose from barley glucan) at pH 5.5 and 50°C.

glucanase) to 26,112/2,360 (coefficient of variation (CV) 1.6% and 1.7% for xylanase and glucanase, respectively).<sup>17</sup> The study of the particle distribution in three batches (laser diffraction) showed that particles below 100 µm amount 80% and particles below 10 µm amount 5%.<sup>18</sup> Dusting potential measured by the Stauber–Heubach test was 5.07 g/m<sup>3</sup>.<sup>19</sup>

ENZY CARBOPLUS® L contains the enzyme 1.5% (w/w), 15% of glycerol, 5% of sodium chloride, 0.30% of sodium benzoate, 0.15% potassium sorbate and water up to 100%. The study of the batch-to-batch variation in five batches showed a mean value of xylanase and glucanase of 25,912 LXU and 2,316 LGU per gram ranging from (xylanase/glucanase) 25,100/2,216 to 26,860/2,420 (CV 3.4% and 3.6% for xylanase and glucanase, respectively).<sup>20</sup> The specific weight of the formulation is 10.816–10.881 kN/m<sup>3</sup> and the viscosity ranges 3.79–4.17 mPa·s (at 25°C).<sup>21</sup>

Three batches of the final formulations were analysed for chemical and microbial contamination.<sup>22</sup> Chemical contamination included the analysis of lead (< 0.91 mg/kg in the solid and < 0.50 mg/kg in the liquid), cadmium (< 0.20 mg/kg), mercury (< 0.02 mg/kg) and arsenic (< 2.38 mg/kg in the solid and < 0.50 mg/kg in the liquid) and dioxins and dioxin-like PCBs (WHO-PCDD/F-PCB TEQ, < 0.32 ng/kg). The following mycotoxins were analysed: aflatoxins (B1, B2, G1 and G2, < 1.0 µg/kg), zearalenone (< 37.0 µg/kg in the solid and < 5.0 µg/kg in the liquid), ochratoxin A (< 0.20 µg/kg), HT-2 toxin and T2 toxin (< 10.0 µg/kg). The study of the microbial contamination included of *E. coli* (< 10 colony forming units/g) and *Salmonella spp.* (absence in 25 g).

The absence of antimycotic activity was proven in concentrated supernatant of culture of the production strains using *Candida albicans* EELA 188.<sup>13</sup> Antimicrobial activity was studied<sup>23</sup> in yeast broth cultures (10-fold concentrated) and in the liquid enzyme preparation (fivefold concentrated, three batches) by using five bacterial strains *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212, *E. coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 and *Bacillus subtilis* ATCC 6633 (EFSA, 2008c). The substances tested showed no antimicrobial activity.

No cultivable cells of the production strains were detected in three batches of 30 mL (liquid) or 1 g (solid) product, by incubation in liquid and solid non-selective media at 30°C for 96 h under conditions supporting the growth of *K. pastoris*.<sup>13</sup> No recombinant DNA was detected in 200 µL samples of three independent batches of the liquid form, by quantitative PCR, targeting DNA fragments of 79–80 bp of the xylanase gene and 91–92 bp of the glucanase gene.<sup>13</sup> Since the two formulations of the additive share a common manufacturing process, the results obtained in the liquid form apply also to the solid.

### 3.1.4. Stability and homogeneity

The batches of the additive used in the studies provided in this section had a different ratio xylanase:glucanase (range from 1 to 7) compared to the one specified for the additive (similar to 11). However, it is considered that this different ratio would not have an impact on the results of the stability of the enzymes contained in the product.

The shelf-life of the solid formulation was studied in three batches stored in closed containers at 25°C for 24 months or at 40°C for 6 months.<sup>24</sup> Recovery values for the two enzymes were > 80% in the two conditions tested. Another study was provided in three batches of the solid formulation stored at 20°C for one month. Recovery values were higher than 95%.<sup>25</sup>

The shelf-life of the liquid formulation was studied in three batches stored in closed containers at 5°C for 24 months or at 25°C for 6 months.<sup>24</sup> Recovery values were > 90% for xylanase at either temperature. For glucanase, the values after 24 months stored at 5°C showed a 65% recovery and 80% recovery after 6 months stored at 25°C.

The stability of three batches of the solid formulation was evaluated in a vitamin mineral premixture containing choline chloride stored at 25°C for 6 months under standard conditions in a small paper bag with an inside polyethylene coating.<sup>24</sup> The supplementation was of 45,000 LXU and 7,250 LGU/kg

<sup>17</sup> Technical dossier/Section II/Annex II.1.3.1.

<sup>18</sup> Technical dossier/Section II/Annex II.1.5.1.

<sup>19</sup> Technical dossier/Section II/Annex II.1.5.2.

<sup>20</sup> Technical dossier/Section II/Annex II.1.3.2.

<sup>21</sup> Technical dossier/Supplementary information April 2014/Annex II.1.5.4.

<sup>22</sup> Technical dossier/Supplementary information April 2014/Annex II.1.4.1.5.

<sup>23</sup> Technical dossier/Supplementary information April 2014/Annex II.1.4.1.6 and Supplementary information May 2015/Annex II.1.4.1.8.

<sup>24</sup> Technical dossier/Supplementary information March 2014/Annex II.4.1.1.

<sup>25</sup> Technical dossier/Supplementary information May 2017.



premixture. After the 6-month storage period, the recovery for xylanase was higher than 84% and for glucanase higher than 87%.

The stability in feed was studied for three batches of the solid formulation added to mash and pelleted feed and for three batches of the liquid formulation added to pelleted feed.<sup>24</sup> Samples of the feeds were stored for three months at 25°C. Recovery values were > 95% in all cases. The data also permitted to study the stability of the enzymes during pelleting which was of 90% for xylanase and of 70% for glucanase.

Homogeneity of the distribution of the enzyme activities was studied in the vitamin mineral premixture in the feeds used for the stability studies by analysing 10 subsamples of each premixture/feed. The coefficient of variation was below 8% in all cases.

### 3.1.5. Conditions of use

The applicant proposes the additive to be added to the feed for avian species at the recommended enzyme activity of 1,400 LXU and 120 LGU/kg feed (56 mg additive per kg feed) and in feed for weaned piglets (including minor species) at 700 LXU and 60 LGU/kg feed (28 mg additive per kg feed).

## 3.2. Safety

### 3.2.1. Safety of the genetic modification

The parental strain belongs to a species *K. pastoris*, which is considered by EFSA to be suitable for the QPS approach for safety assessment when used for enzyme production (EFSA BIOHAZ Panel, 2017). The production strains harbour one copy of the *Xyn-CDBFV* xylanase gene (*K. pastoris* DSM 25376), and one copy of a glucanase gene (*K. pastoris* DSM 26469), respectively. In addition, both strains carry a gene enabling growth in the absence of histidine. None of the introduced genes raises safety concern.

Neither the viable production strains nor their recombinant DNA were found in the final products. The product ENZY CARBOPLUS®, manufactured by fermentation with *K. pastoris* DSM 25376 and DSM 26469 strains, does not give rise to safety concern with regard to the genetic modification.

### 3.2.2. Safety for the target species

Tolerance trials in chickens and turkeys for fattening, laying hens and weaned piglets were submitted. The test item in the studies below reported was not the additive but a diluted form obtained by mixing the solid formulation of the additive with starch. The enzyme activities in the test items used were measured for each study and were close to 8,400 LXU and 775 LGU per gram (2.9 times dilution). The Panel considers this test item as acceptable and will refer to it as the additive. Some of the studies miss the information on the glucanase activity in the feeds. However, this can be estimated from the data on the xylanase activity in feed and the enzyme activities present in the test item used in each study.

In all the studies the significance level in the statistical evaluation was set at 0.05.

#### 3.2.2.1. Safety for chickens for fattening

A total of 432 one-day-old male chicks (Ross 308) were distributed randomly to 48 cages in groups of nine birds and allocated to three dietary treatments (representing 16 replicates per treatment).<sup>26</sup> Starter and grower diets based on wheat, rye and soya bean meal were either not supplemented (control) or supplemented with the additive to provide xylanase/glucanase at 2,100/180 (1.5× recommended dose) or 21,000/1,800 (15×) LXU/LGU per kg feed. Xylanase and glucanase were analysed (100/2,300, 4,120/2,683 and 30,300/4,739).<sup>27</sup> The feed was offered to the birds *ad libitum* in mash form for 35 days. Health of the animals was monitored daily and feed consumption and body weight were measured on days 1, 14 and 35 of the study. At the end of the study blood samples were obtained from 15 birds per treatment (sampled from 15 different cages) and analysed for biochemical<sup>28</sup> and haematological<sup>29</sup> parameters. An analysis of variance (ANOVA) was done with the data obtained (including treatment and block as effects), group means were compared with Duncan test.

<sup>26</sup> Technical dossier/Section III/Annex III.1.1.1 and Supplementary information April 2014.

<sup>27</sup> Technical dossier/Supplementary information April 2014/Annex II.4.1.2.

<sup>28</sup> Including: total protein, urea, cholesterol, glutamate dehydrogenase, aspartate aminotransferase, gamma glutamyl transpeptidase, bilirubin, calcium, inorganic P, beta-hydroxybutyrate.

<sup>29</sup> Including: erythrocytes, haematocrit, haemoglobin, mean corpuscular volume, mean corpuscular haemoglobin, red cell distribution width, mean corpuscular haemoglobin and white blood cells differential.

Mortality was low (< 3.5%) and not affected by the dietary treatments. Mean daily feed intake was 93 g, final body weight 2.2 kg and feed to gain ratio was 1.56. The final body weight was higher and the feed to gain ratio lower in the groups treated with the additive compared to the control (see efficacy section for details, trial 1). There were no significant differences between groups for the parameters evaluated in blood. Supplementation of the experimental diets with 15-fold the recommended dose (1,400 LXU and 120 LGU/kg feed) did not have negative effects on the birds.

### 3.2.2.2. Safety for turkeys for fattening

A total of 336 one-day-old male turkeys (Big 7) were distributed randomly in 48 cages in groups of seven birds and allocated to three dietary treatments (representing 16 replicates per treatment).<sup>30</sup> A basal diet, based on wheat and soya bean meal was either not supplemented (control) or supplemented with the additive to provide xylanase/glucanase at 2,100/180 (1.5× recommended dose) or 21,000/1,800 (15×) LXU/LGU per kg feed. Glucanase was not analysed, xylanase was measured in the feeds and found to be higher than the intended dosages (< 100, 3,900 and 44,533).<sup>12</sup> The feed was offered to the birds *ad libitum* as mash for 42 days. Health of the animals was monitored daily and feed consumption and body weight were measured on days 1 and 42 of the study. Blood samples were obtained from 15 birds per treatment (one per cage with the exception of one cage) and analysed for biochemical<sup>31</sup> and haematological<sup>32</sup> parameters. An ANOVA was performed with the data obtained).

Mortality was low (< 2%) and not affected by the dietary treatments. No significant differences were observed in the performance of the birds. Mean daily feed intake was 76 g/bird, final body weight 1.89 kg, average daily gain was 44 g and feed to gain ratio 1.76. Regarding the blood parameters, the groups receiving the additive had lower blood urea (4.9 vs 3.6 mg/dL for control and supplemented groups, respectively) and inorganic phosphate (11.0 vs 9.8 mg/dL) compared to the control. These differences in blood urea and inorganic phosphate are considered of little biological relevance. Overall, the supplementation of the experimental diets with 15-fold the recommended dose did not have negative effects on the turkeys.

### 3.2.2.3. Safety for laying hens

Two studies in laying hens were submitted.

In the first study, a total of 222 18-week-old Hy-Line hens were individually caged and allocated to three dietary treatments (representing 74 individual hens per treatment).<sup>33</sup> A basal diet based on triticale, rye, wheat and soya bean meal was either not supplemented (control) or supplemented with the additive to provide (xylanase/glucanase), 2,100/180 (1.5×) or 21,000/1,800 (15×) LXU/LGU per kg feed (confirmed by analysis for xylanase 115, 2,450 and 22,100 LXU/kg). Glucanase was not analysed.<sup>12</sup> Feed was offered *ad libitum* and in mash form for 70 days (1–10 week laying period). Body weight was measured at the beginning and at the end of the study. Health, feed intake and laying performance were monitored/recorded throughout the study. Eggs were weighed every two weeks and egg quality parameters were measured on 15 eggs per treatment on weeks 4, 8 and 10 of study. At the end of the experiment, blood samples from 15 hens per treatment, randomly selected, were collected in order to analyse them for biochemical<sup>34</sup> and haematological<sup>35</sup> parameters. An ANOVA was done with the data.

No hens died and no differences were noted on the body weight of the hens or in their feed intake (mean value 118 g/day per hen). Total number of eggs (55.8, 58.6, 55.9 for 0/0, 1.5× and 15×, respectively), egg mass produced (3.37 kg, 3.53 and 3.33) and feed to egg mass (2.51, 2.42 and 2.61) were better in the 1.5× group compared to 15× group. The results in the 15× group did not differ to those of the control. Egg quality parameters showed no modifications. No significant differences were identified in the blood parameters measured.

<sup>30</sup> Technical dossier/Section III/Annex III.1.1.5.

<sup>31</sup> Including: total protein, urea, cholesterol, glutamate dehydrogenase, aspartate aminotransferase, gamma glutamyl transpeptidase, bilirubin, calcium, inorganic P.

<sup>32</sup> Including: erythrocytes, haematocrit, haemoglobin, leucocytes, thrombocytes, mean corpuscular volume, mean corpuscular haemoglobin, red cell distribution width, white blood cells differential.

<sup>33</sup> Technical dossier/Section III/Annex III.1.1.2 and Supplementary information April 2014.

<sup>34</sup> Total protein, blood urea, cholesterol, glutamate dehydrogenase, aspartate aminotransferase, gamma-glutamyl-transpeptidase, bilirubin, calcium and inorganic phosphate.

<sup>35</sup> Red blood cells, haemoglobin, haematocrit, leucocytes, segmented neutrophils, lymphocytes, monocytes, eosinophils, basophils, thrombocytes, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration and red cell distribution width.

In the second study, a total of 90 25-week-old Lohmann-Brown hens were penned in groups of five hens and allocated to three dietary treatments (representing six replicates per treatment).<sup>36</sup> A basal diet based on maize, rye and soya bean meal was either not supplemented (control) or supplemented with the additive to provide (xylanase/glucanase) 2,100/180 (1.5×) or 21,000/1,800 (15×) LXU/LGU per kg feed. The results of the analysis of xylanase < 200, 1,360 and 10,625 LXU/kg, showed that the 1.5 and 15× dosage were not reached, in fact they represented 1x and 8x. Glucanase was not measured.<sup>12</sup> Feed was offered in mash form for 56 days. Body weight was measured at the beginning and at the end of the study. Health, feed intake and laying performance were monitored/recorded throughout the study. At the end of the experiment, blood samples from 10 hens (one or two hens per replicate) were collected in order to analyse them for biochemical<sup>37</sup> and haematological<sup>38</sup> parameters. An ANOVA was applied to the data.

No hen died and no significant differences were identified on the body weight, feed intake (mean value 109 g/day), total number of eggs (54.5) nor in the egg mass produced (3.3 kg). The feed to egg mass ratio (1.88, 1.83, 1.81 for 0/0, 1.5x and 15x) was better in the 15× group as compared to control. No differences were found in the blood parameters measured with the exception of sodium which was significantly higher in the treated hens compared to control but the increase can be considered of no biological relevance (149 vs 153 mmol/L).

The results of these two studies showed that the supplementation of the experimental diets up to 15-fold the recommended dose did not have negative effects on the laying hens.

#### 3.2.2.4. Safety for weaned piglets

The applicant submitted two trials, in the first, a total of 1,487 male and female weaned piglets (PIC, 6.5 kg body weight, 24 days old) were distributed in a total of 50 floor pens in groups of 26–34 piglets and allocated to five dietary treatments (10 replicates per treatment).<sup>39</sup> Prestarter and starter diets, based on maize, wheat, barley and soybean meal, were supplemented with the additive to provide xylanase/glucanase at 0/0, 700/60 (1× recommended dose), 1,400/120, 2,100/180, 21,000/1,800 (30×) kg feed. Enzyme activities were confirmed by analysis<sup>40</sup> (87/1028, 599/1,273, 1,033/1,330, 1,763/1,523, 18,325/4,476). The feed was offered in mash (prestarter) or pelleted (starter) form and *ad libitum* for 42 days. Body weight and feed intake were measured on day 14 and day 42 of the study (body weight was also measured at the beginning). On day 42, blood was collected from 15 animals of each experimental group for biochemical measurements.<sup>41</sup> No haematological parameters were analysed on the blood samples. An ANOVA was done on the data obtained and mean groups were compared with Duncan test.

Mortality was low (2.5%) and not related to the treatments. Final weight (23.8 kg), daily weight gain (413 g/day) and feed to gain ratio (1.52) were significantly improved in groups receiving the additive compared to control animals (for details, see efficacy trial 2). Blood chemistry parameters showed no differences between treatments. This trial was done considering 30-fold the recommended dose and lacked the measurements on the haematological parameters. Therefore, the applicant submitted a second trial.

In the second trial,<sup>42</sup> a total of 60 female and castrated male weaned piglets (Large White × Landrace, initial body weight of 7.5 kg, 25 days old) were penned in groups of two and allocated to three dietary treatments (representing 10 replicates per treatment). Starter and grower diets basal diets, based on wheat, barley, rye and soya bean meal were either not supplemented (control) or supplemented with the additive to provide xylanase/glucanase at 2,500/220 (3.5× recommended dose) or 25,000/2,200 (35×) LXU/LGU per kg feed. Xylanase and glucanase were analysed in the feeds (128/153, 1,275/863 and 17,175/5,200). The feed was offered to the piglets *ad libitum* in mash form for 42 days. Health of the animals was monitored twice daily, feed consumption and body weight were measured weekly and feed to gain ratio was calculated. At the

<sup>36</sup> Technical dossier/Section III/Annex III.1.1.3 and Supplementary information April 2014.

<sup>37</sup> Sodium, potassium, chloride, calcium and phosphorus, total cholesterol, triglycerides, bilirubin, uric acid, glucose, albumin, total protein, alanine amino transferase, aspartate amino transferase and alkaline phosphatase.

<sup>38</sup> Red blood cells, haemoglobin, haematocrit, leucocytes, neutrophils, lymphocytes, monocytes, eosinophils, basophils, mean corpuscular volume, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration.

<sup>39</sup> Technical dossier/Section III/Annex III.1.1.4 and Supplementary information April 2014/Annex II.4.1.2.

<sup>40</sup> Glucanase was measured only in the starter diet.

<sup>41</sup> Including: total protein, blood urea, cholesterol, glutamate dehydrogenase, non-esterified fatty acids, aspartate aminotransferase, gamma-glutamyl transferase, bilirubin, calcium, phosphorus and lactate dehydrogenase.

<sup>42</sup> Technical dossier/Supplementary information April 2015/Annex III.1.1.7.

end of the study, blood samples were obtained from 10 pigs per treatment (one per pen) and analysed for biochemical<sup>43</sup> and haematological<sup>44</sup> parameters. An ANOVA was done with the data obtained.

No animals died. Total feed intake per pig was not different between the treatments (30 kg/pig). Final body weight of the piglets was 28.4, 29.4 and 29.9 kg for 0, 3.5× and 35×, respectively, and the corresponding figures for feed to gain ratio was 1.48, 1.39 and 1.35. The final body weight in the 35-fold group was higher than in the control. Regarding the blood parameters there were no significant differences between groups except a significant increase in the mean corpuscular haemoglobin concentration in the animals receiving the 35-fold group compared to the other groups (29 vs 32 g/dL). This modification although significant is considered of little biological relevance.

Overall, the supplementation of the experimental diets up to 35-fold the recommended dose did not have negative effects on the weaned piglets.

### 3.2.2.5. Conclusions on safety for the target species

The FEEDAP Panel concludes that the additive is safe for chickens for fattening, turkeys for fattening, laying hens and weaned piglets at the corresponding recommended levels (1,400 LXU and 120 LGU/kg feed for chickens and turkeys for fattening and laying hens and 700 LXU and 60 LGU/kg feed for piglets). The conclusion on the safety in chickens and turkeys for fattening can be extended to chickens reared for laying and turkeys reared for breeding, respectively.

The safety of the additive has been shown in major poultry species/categories and weaned piglets with a margin of safety higher than ten-fold the recommended dose. Considering that the additive is proposed to be used at the same levels in other avian and porcine species the safety for the latest can be presumed without the need for specific studies. Therefore, the FEEDAP Panel concludes that the conclusions regarding the safety can be extrapolated to all avian species at 1,400 LXU and 120 LGU/kg and to minor porcine species (growing stages) at 700 LXU and 60 LGU/kg feed.

### 3.2.3. Safety for the consumer

Toxicological studies are not required if the fermentation products are produced by a genetically modified microorganism for which the recipient strain is considered by EFSA to qualify for the QPS approach to safety assessment and for which the genetic modification does not give raise to concerns. The enzymes present in the additive are produced by two genetically modified strains of *K. pastoris*; this species is considered to qualify for the QPS approach to safety assessment for enzyme production provided that the identity is unambiguously established. The identity of the production strains was established. Moreover, the genetic modifications to which the strains were subject raised no concerns. Therefore, the fermentation products obtained from these two strains are considered safe for the consumers.

The applicant provided various toxicity studies conducted with the final liquid formulation to support the safety of the product: three genotoxicity studies and a subchronic oral toxicity study. The results of a bacterial reverse mutation assay,<sup>45</sup> an *in vitro* mammalian cell micronucleus test<sup>46</sup> and an *in vivo* comet assay in the rat<sup>47</sup> showed no evidence for genotoxic potential. In the subchronic oral toxicity study in rats,<sup>48</sup> no treatment-related effects were reported. These studies can be considered as added evidence of the safety of the product.

Considering the above and the composition of the additive, the FEEDAP Panel concludes that the use of ENZY CARBOPLUS® as a feed additive raises no concern for consumers.

### 3.2.4. Safety for the user

No specific tests were provided to study the effects on the respiratory system. Owing to the proteinaceous nature of the active substance, the additive is considered as a potential respiratory

<sup>43</sup> Including: sodium, potassium chloride, calcium, inorganic phosphate, alanine amino transferase, aspartate amino transferase, alkaline phosphatase, total cholesterol, triglycerides, urea, bilirubin, glucose, albumin and total protein.

<sup>44</sup> Including: erythrocytes, leukocytes, thrombocytes, white blood cells differential, haemoglobin, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration.

<sup>45</sup> Technical dossier/Section III/Annex III.2.2.2.1.

<sup>46</sup> Technical dossier/Section III/Annex III.2.2.2.2.

<sup>47</sup> Technical dossier/Section III/Annex III.2.2.2.3.

<sup>48</sup> Technical dossier/Section III/Annex III.2.2.3.

sensitiser. Considering the high dusting potential of the solid formulation (5.07 g/m<sup>3</sup>) and the percentage of particle below 10 µm (5%), there is a potential for exposure by inhalation.

The effects on the skin and eyes were investigated with the two final formulations. The data included acute dermal<sup>49</sup> and eye<sup>50</sup> irritation studies in the rabbit which were performed according to OECD Guideline 404 or OECD Guideline 405, respectively. Results showed that the additive in either form is not irritant to skin or eye. Moreover, skin sensitisation studies in the guinea pig were performed according to OECD Guideline 406.<sup>51</sup> Results showed that the additive in either form is not a dermal sensitiser.

#### 3.2.4.1. Conclusions on safety for the user

The additive in either form is not a skin or eye irritant nor a dermal sensitiser. Owing to the proteinaceous nature of the active substance, the additive is considered as a potential respiratory sensitiser.

#### 3.2.5. Safety for the environment

The production strains and their recombinant DNA were not detected in the final product. The active substances of the additive are proteins, and as such will be degraded/inactivated during passage through the digestive tract of animals or in the environment. Therefore, the additive does not pose concerns to the environment.

### 3.3. Efficacy

The test item in the studies below reported was not the additive but a diluted form obtained by mixing the solid formulation of the additive with starch. The enzyme activities in the test items used were measured for each study and was close to 8,900 LXU and 750 LGU per gram (2.9 times dilution). The Panel considers this test item as acceptable and will refer to it as the additive. Some of the studies miss the information on the glucanase activity in the feeds. However, this can be estimated from the data on the xylanase activity in feed and the enzyme activities present in the test item used in each study. In all the studies the significance level in the statistical evaluation was set at 0.05.

#### 3.3.1. Efficacy for chickens for fattening

Four long-term trials were considered (three efficacy trials and the tolerance trial). In one of the efficacy trials,<sup>52</sup> the birds were caged in groups of two animals until day 21 of life. At that point, one of the animals per cage was removed from the study. This fact leaves as the only reliable parameter the body weight of the birds that remained under study, further to this fact, the Panel considers that the removal of the animals may bias the outcome of the study. Consequently, this trial was not considered further.

For the other trials, the details on the study design are provided in Table 1 and the main results in Table 2.

In all trials, 1-day-old male birds were used and the birds were fed either a non-supplemented diet (control) or a diet supplemented with the additive to provide different enzymes concentrations. Xylanase was analysed in all studies, glucanase was measured only in trial 1. The diets were administered from day 1 of life until day 35 of life. 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 was analysed with an ANOVA, using the pen as the experimental unit. Group means were compared with Duncan test in trial 1 and 3 and Student–Newman–Keuls test in trial 2.

In trial 1, the body weight and the feed to gain ratio were significantly improved at the dose of 2,100 LXU and 180 LGU per kg feed. The analysis of the diets revealed a higher enzyme activity in the feed than the intended one (dose of 3,600 LXU and 410 LGU per kg feed). In the other two trials, birds fed the dose of 700 LXU and 60 LGU, or above, showed a significantly better feed to gain ratio (in trial 2) or a higher final body weight in trial 3.

<sup>49</sup> Technical dossier/Section III/Annexes III.3.1.2.3 and III.3.1.2.4.

<sup>50</sup> Technical dossier/Section III/Annexes III.3.1.2.1 and III.3.1.2.2.

<sup>51</sup> Technical dossier/Section III/Annexes III.3.1.2.5 and III.3.1.2.6.

<sup>52</sup> Technical dossier/Section IV/Annex IV.3.1 Supplementary information April 2014/Annex IV.3.1 and Annex II.4.1.2 and Supplementary information April 2015 IV.3.1.addendum.

**Table 1:** Trial design and dosages 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 activity (LXU/LGU per kg feed)	
				Intended	Analysed <sup>(a)</sup>
1 <sup>(b)</sup>	432 (9) 16	Ross 308 Males (35 days)	Wheat, rye soya bean meal (pellets)	0/0 2,100/180 21,000/1,800	–/– 4,011/373 30,195/2,429
2 <sup>(c)</sup>	769 (12–13) 16	Ross 308 Males (35 days)	Wheat, rye, soya bean meal (pellets)	0/0 700/60 1,400/120 2,100/180	< 200/– 847/– 1,715/– 2,882/–
3 <sup>(d)</sup>	3,600 (60) 60	Ross 308 Males (35 days)	Wheat, rye, soya bean meal (crumble/pellets)	0/0 700/60 1,400/120 2,100/180	< 200/– 532/– 1,214/– 2,156/–

(a): The enzyme activity present in the control diet was subtracted from the supplemented feeds.

(b): Technical dossier/Section III/Annex III.1.1.1.

(c): Technical dossier/Section IV/Annex IV.3.2 and supplementary information April 2014/Annex II.4.1.2.

(d): Technical dossier/Section IV/Annex IV.3.3 and supplementary information April 2014/Annex II.4.1.2.

**Table 2:** Effects of ENZY CARBOPLUS® on the performance of chickens for fattening

Trial	Enzyme activity (LXU/LGU per kg feed)	Daily feed intake (g/bird)	Final body weight (g)	Feed to gain ratio	Mortality and culling (%)
1	0/0	93	2,110 <sup>b</sup>	1.58 <sup>a</sup>	3.5
	2,100/180	95	2,223 <sup>a</sup>	1.52 <sup>b</sup>	1.4
	21,000/1,800	91	2,178 <sup>a</sup>	1.50 <sup>b</sup>	2.8
2	0/0	98 <sup>a</sup>	1,943	1.76 <sup>a</sup>	1.02
	700/60	93 <sup>b</sup>	1,955	1.67 <sup>b</sup>	2.58
	1,400/120	93 <sup>b</sup>	1,957	1.66 <sup>b</sup>	0.51
	2,100/180	93 <sup>b</sup>	1,974	1.64 <sup>b</sup>	2.05
3	0/0	101 <sup>b</sup>	2,038 <sup>b</sup>	1.77	0.89
	700/60	106 <sup>a</sup>	2,160 <sup>a</sup>	1.74	0.55
	1,400/120	106 <sup>a</sup>	2,167 <sup>a</sup>	1.73	0.89
	2,100/180	106 <sup>a</sup>	2,165 <sup>a</sup>	1.73	0.78

<sup>a,b</sup>: Mean values within a trial and within a column with a different superscript are significantly different  $p < 0.05$ .

Therefore, three studies showed an improvement of the performance of the birds, two at the level of 700 LXU and 60 LGU per kg feed and a third study at an analysed level of 4,011 LXU and 374 LGU per kg feed. Therefore, the Panel considers that the additive has a potential to be efficacious at a level similar to 4,000 LXU and 375 LGU per kg feed (delivered by ca 165 mg of additive per kg feed).

The Panel notes that the efficacious enzyme activities in chickens for fattening are higher than the recommended by the applicant. The conclusions on the tolerance were drawn based on the recommendation made by the applicant, however, the enzyme activities tested, parameters measured and the results obtained in the tolerance trial allow the Panel to consider that 4,000 LXU and 375 LGU per kg feed is safe for chickens for fattening.

### 3.3.2. Efficacy for turkeys for fattening

Two short-term trials and three long-term trials were submitted.

#### 3.3.2.1. Short-term trials

Two balance trials were provided. In the first, a total of 72 one-day-old male turkeys (BUT Big 6) were kept in groups of six birds and allocated to three dietary treatments from day 0 to 28 of life.<sup>53</sup>

<sup>53</sup> Technical dossier/Supplementary information April 2014/Annex IV.3.10 and supplementary information April 2015.

On day 28, the birds were transferred to individual balance cages (24 replicates per treatment). In the second,<sup>54</sup> a total of 192 six-day-old male turkeys (Hybrid Converter) were caged in groups of four and allocated to three dietary treatments (representing 16 replicates per treatment). In the two trials, the basal diets based on wheat, rye and soya bean meal were either not supplemented (control) or supplemented with the additive to provide xylanase/glucanase at 700/60 or 1,400/120 LXU/LGU per kg feed. In the first trial, only xylanase was analysed and showed enzyme activities higher than the intended (70, 1,500 and 4,700 LXU per kg feed). In the second, the analysis confirmed the intended dosages for the two enzymes (130/< 40, 945/42 and 1,675/86 LXU/LGU per kg feed). A balance study was performed from days 28 to 36 of life in the first balance trial and on days 22–25 in the second. Total collection of excreta was done. Feed and excreta were analysed for energy content. An ANOVA was done with the data and group means were compared with Tukey test (trial 1) or Duncan (trial 2).

In the first trial, the metabolisable energy content of the diets in turkeys fed the highest dose (2,100/180 LXU/LGU per kg feed) was significantly higher compared to the control group (12.0 vs 11.7 MJ/kg) but not at the lower tested dose (700/60 LXU/LGU per kg feed; 11.8 MJ/kg feed). In the second trial, the metabolisable energy content of the diets was higher in the birds receiving the additive at either dose compared to the control (10.08, 10.45 and 10.87 MJ per kg feed for control, 700/60 and 2,100/180 groups, respectively).

### 3.3.2.2. Long-term trials

The details on the study design of the three long-term trials submitted are provided in Table 3 and the main results in Table 4. In all trials, one-day-old male birds were used and the birds were fed either with non-supplemented diet (control) or a diet supplemented with the additive to provide different enzymes concentrations. All studies included a group with 1,400 LXU and 120 LGU per kg feed. Xylanase and glucanase were analysed in feeds. The diets were administered for 84 days. 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 was analysed with an ANOVA, using the pen as the experimental unit and group means were compared with Tukey test.

**Table 3:** Trial design and dosages of the efficacy trials performed in turkeys for fattening

Trial	Total no of animals (animals × replicate) replicates × treatment	Breed sex (duration)	Composition feed (form)	Enzyme activity (LXU/LGU per kg feed)	
				Intended	Analysed <sup>(a)</sup>
1 <sup>(b)</sup>	900 (75) 4	Big 6 Male (84 days)	Wheat, rye, soya bean meal (pellets)	0/0	–/–
				700/60	372/140
				1,400/120	789/251
2 <sup>(c)</sup>	1,280 (20) 16	BUT 8 Male (84 days)	Wheat, soya bean meal (mash)	0/0	–/–
				700/60	705/90
				1,400/120	1,800/121
				2,100/180	2,710/247
3 <sup>(d)</sup>	945 (33–34) 7	Big 6 Male (84 days)	Wheat, rye, soya bean meal (crumble/pellets)	0/0	–/–
				700/60	550/41
				1,400/120	1,301/101
				2,100/180	2,360/140

(a): The enzyme activity present in the control diet was subtracted from the supplemented feeds.

(b): Technical dossier/Section IV/Annex IV.3.6 and Supplementary information April 2014/Annex IV.3.6 and Annex II.4.1.2.

(c): Technical dossier/Section IV/Annex IV.3.7 and Supplementary information April 2014/Annex II.4.1.2.

(d): Technical dossier/Section IV/Annex IV.3.8 and Supplementary information April 2014/Annex II.4.1.2.

The results in trial 1 showed no differences in any of the parameters studied between the different groups. In trial 2, a higher body weight and a better feed to gain ratio were found in the birds receiving 1,400 LXU and 120 LGU per kg feed. In trial 3, a better feed to gain ratio of the birds was found at the same level.

<sup>54</sup> Technical dossier/Supplementary information April 2015/Annex IV.3.11.

**Table 4:** Effects of ENZY CARBOPLUS® on the performance of turkeys for fattening

Trial	Enzyme activity (LXU/LGU per kg feed)	Feed intake <sup>(1)</sup> (kg)	Final body weight (kg)	Feed to gain ratio	Mortality (%)
1	0/0	0.224	8.17	2.33	4.0
	700/60	0.226	8.40	2.28	4.7
	1,400/120	0.227	8.49	2.27	4.0
2	0/0	18.5	8.37 <sup>c</sup>	2.21 <sup>a</sup>	5.0
	700/60	18.6	8.45 <sup>bc</sup>	2.20 <sup>ab</sup>	2.8
	1,400/120	18.6	8.48 <sup>b</sup>	2.19 <sup>bc</sup>	5.3
	2,100/180	18.7	8.59 <sup>a</sup>	2.17 <sup>c</sup>	3.8
3	0/0	0.252	10.35	2.03 <sup>a</sup>	2.9
	700/60	0.252	10.30	2.02 <sup>a</sup>	1.3
	1,400/120	0.248	10.41	1.97 <sup>b</sup>	2.1
	2,100/180	0.244	10.36	1.98 <sup>b</sup>	0.4

(1): For trial 1 and 3, the feed intake is daily feed intake per bird and for trial 2 it is total feed intake per bird.

<sup>a,b,c</sup>: Mean values within a trial and within a column with a different superscript are significantly different  $p < 0.05$ .

Based on the results of one short-term trial (second balance trial) and of two long-term trials (second and third), the FEEDAP Panel concludes that the additive has a potential to be efficacious as a zootechnical additive in turkeys for fattening at 1,400 LXU and 120 LGU per kg feed.

### 3.3.3. Efficacy for laying hens

Four short-term trials were submitted, three of them were not considered further due to the nature of the parameters measured.<sup>55</sup> In these three digestibility studies, several parameters were measured including laying performance, egg quality parameters, digestibility (ash, crude protein, crude fat, crude fibre) but the effect of the additive on the metabolisable energy content of the diets was not studied. Consequently, these studies cannot be considered to support the efficacy of the additive.

The fourth short-term trial<sup>56</sup> was conducted with 60 Lohmann-Brown layers 24 weeks old were caged in groups of two and distributed to three dietary treatments (10 replicates per treatment). A basal diet (Gross energy content ~ 17 MJ/kg feed) based on rye and soya bean meal was either not supplemented (control) or supplemented with the additive to provide xylanase/glucanase 700/60 or 1,400/120 LXU/LGU per kg feed. Xylanase and glucanase were analysed: 605/293 and 1,700/600 (unsupplemented diet values subtracted). The feed was offered *ad libitum* from week 24 to 28 in mash form and contained titanium dioxide. Laying performance of the hens was measured and on the last week samples of ileal and excreta were collected. A balance study was performed on the last three days of the study. Feed and excreta samples were analysed for different parameters including the energy. An ANOVA was done with the data and the mean groups were compared with Tukey test.

The results of the balance trial showed higher energy retention in the groups fed the enzymes compared to control (84% vs 79%), leading to a higher metabolisable energy content of the diets (13.2 MJ/kg feed in the control and 14.9 and 14.4 MJ/kg feed in the 700/60 and 1,400/120 LXU/LGU per kg feed groups, respectively). Laying performance was not different between the groups.

Evidence of efficacy was provided in one trial, therefore the Panel considers that the information provided is insufficient to conclude on the efficacy of the additive in laying hens.

### 3.3.4. Efficacy for piglets

A total of four long-term trials were submitted. One trial<sup>57</sup> was not considered further because of the high mortality (8.7%) and culling (5.6%).

For the three studies considered, the details on the study design are provided in Table 5 and the main results in Table 6. In the three trials, the treatment groups included a group with 700 LXU and 60 LGU per kg feed. The two enzyme activities in feed were analysed in trials 1 and 2 (glucanase only in the starter diet, not in prestarter), in trial 3 only xylanase was measured. The piglets received the feeds for 42 days. Feed intake and body weight of the piglets was measured and feed to gain ratio

<sup>55</sup> Technical dossier/Section IV/Annexes IV.2.1, IV.2.2 and IV.2.4.

<sup>56</sup> Technical dossier/Section IV/Annex IV.2.3 and supplementary information April 2014/Annex IV.2.3\_amendment.

<sup>57</sup> Technical dossier/Section IV/Annex IV.3.4 and Supplementary information April 2014/Annex II.4.1.2.



was calculated. The data was analysed with an ANOVA, using the pen as the experimental unit and group means were compared with Tukey test in trials 1 and 3 and with Duncan test in trial 2.

**Table 5:** Trial design and dosages of the efficacy trials performed in weaned piglets

Trial	Total no of animals (animals × replicate) replicates × treatment	Age (days) at start (sex)	Composition feed (form)	Enzyme activity (LXU/LGU per kg feed)	
				Intended	Analysed <sup>(a)</sup>
1 <sup>(b)</sup>	120 (2) 15	28 (Females and castrated males)	Wheat, barley, rye and soya bean meal (pellet)	0/0 700/60 1,400/120 2,100/180	-/- 466/8 1,061/38 1,664/69
2 <sup>(c)</sup>	1,487 (26–34) 10	24 (Females and castrated male)	Maize, wheat and barley (pelleted/mash)	0/0 700/60 1,400/120 2,100/300 21,000/3,000	-/- 513/122 947/151 1,677/248 17,726/3,203
3 <sup>(d)</sup>	144 (6) 8	25 (females and males)	Wheat, barley, rye and soya bean meal (mash)	0/0 700/60 1,400/120	-/- 690/- 1,450/-

(a): The enzyme activity present in the control diet was subtracted from the supplemented feeds.

(b): Technical dossier/Section IV/Annex IV.3.5 and Supplementary information April 2014/Annex II.4.1.2.

(c): Technical dossier/Section IV/Annex III.1.1.4 and Supplementary information April 2014/Annex II.4.1.2.

(d): Technical dossier/Supplementary information April 2014/Annex IV.3.9.

**Table 6:** Effects of ENZY CARBOPLUS® on the performance of weaned piglets

Trial	Enzyme activity (LXU/LGU per kg feed)	Feed intake <sup>(1)</sup>	Body weight (kg)		Feed to gain ratio	Mortality (%)
			Initial	Final		
1	0/0	544	8.5	22.4	1.64 <sup>a</sup>	0
	700/60	548	8.5	23.7	1.52 <sup>b</sup>	0
	1,400/120	550	8.5	23.4	1.55 <sup>b</sup>	0
	2,100/180	540	8.5	23.2	1.56 <sup>b</sup>	0
2	0/0	616	6.5	22.7 <sup>c</sup>	1.62 <sup>a</sup>	3.5
	700/60	612	6.6	23.6 <sup>bc</sup>	1.51 <sup>b</sup>	1.6
	1,400/120	629	6.4	24.3 <sup>ab</sup>	1.49 <sup>b</sup>	1.8
	2,100/300	645	6.4	25.0 <sup>a</sup>	1.47 <sup>b</sup>	2.5
	21,000/3,000	609	6.3	23.4 <sup>bc</sup>	1.52 <sup>b</sup>	3.0
3	0/0	30.8	6.7	26.4	1.57 <sup>a</sup>	0
	700/60	31.0	6.7	27.7	1.48 <sup>b</sup>	0
	1,400/120	30.8	6.6	27.7	1.46 <sup>b</sup>	0

(1): For trial 1 and 2, the feed intake is daily feed intake per piglet (g) and for trial 3 it is total feed intake per piglet (kg).

<sup>a,b,c</sup>: Mean values within a trial and within a column with a different superscript are significantly different  $p < 0.05$ .

The three long-term trials considered showed no or a low mortality rate. In all three trials, the feed to gain ratio was significantly better in the piglets receiving the enzymes from 700 LXU and 60 LGU per kg feed and onwards. Therefore, the additive has a potential to be efficacious as a zootechnical additive in weaned piglets at 700 LXU and 60 LGU per kg feed.

### 3.3.5. Conclusions on efficacy for the target species

The additive has a potential to be efficacious as a zootechnical additive in chickens for fattening at 4,000 LXU and 375 LGU per kg feed, in turkeys for fattening at 1,400 LXU and 120 LGU per kg feed and in weaned piglets at 700 LXU and 60 LGU per kg feed. The conclusions reached in chickens for fattening and turkeys for fattening can be extended to chickens raised for laying and turkeys reared for breeding at the corresponding levels.

The Panels notes that the efficacious level in chickens for fattening is higher than the one recommended by the applicant. The conclusions on the tolerance were drawn based on the recommended level, however, the enzyme activities tested, parameters measured and the results

obtained in the tolerance trial allow the Panel to consider that 4,000 LXU and 375 LGU per kg feed is safe for chickens for fattening.

No conclusions can be drawn on the efficacy of the additive in laying hens due to the lack of data.

Since the mode of action of xylanase and glucanase is well known and can be reasonably assumed to be the same within avian species or within porcine species, the Panel extrapolates the conclusions drawn on the efficacy in turkeys for fattening to all avian species up to the point of lay and the conclusions drawn in weaned piglets to all porcine species (in the category of weaned piglets). No conclusions can be drawn on the efficacy of the product in minor poultry species for laying.

### 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<sup>58</sup> and Good Manufacturing Practice.

## 4. Conclusions

The production strains are considered safe and the genetic modifications raise no safety concerns. The strains and their recombinant DNA were not detected in the final product.

The additive is safe for chickens for fattening, turkeys for fattening, laying hens and weaned piglets at the levels proposed by the applicant (1,400 LXU and 120 LGU/kg feed for avian species and 700 LXU and 60 LGU/kg feed for pigs). The conclusion can be extended to chickens reared for laying and turkeys for fattening at the same dose. The conclusions from the major species can be extrapolated to all avian species at the dose of 1,400 LXU and 120 LGU/kg and to minor porcine species (weaned piglets) at the dose of 700 LXU and 60 LGU/kg feed.

The use of ENZY CARBOPLUS® as a feed additive raises no concerns for consumers of food products derived from animals fed with the additive.

The additive in either form is not a skin or eye irritant nor a dermal sensitiser but is considered as a potential respiratory sensitiser.

The use of ENZY CARBOPLUS® as a feed additive is of no concern for the environment.

The additive has a potential to be efficacious as a zootechnical additive in chickens for fattening at 4,000 LXU and 375 LGU per kg feed, in turkeys for fattening at 1,400 LXU and 120 LGU per kg feed, and in weaned piglets at 700 LXU and 60 LGU per kg feed. The conclusions reached in chickens for fattening and turkeys for fattening can be extended to chickens raised for laying and turkeys reared for breeding at the corresponding levels. The conclusions on the efficacy from turkeys for fattening can be extrapolated to all avian species up to the point of lay and the conclusions from weaned piglets to all porcine species (weaned piglets). The Panel cannot conclude on the efficacy of the additive in laying hens or minor poultry species for laying.

## Documentation provided to EFSA

- 1) ENZY CARBOPLUS® for avian species and weaned piglets. June 2013. Submitted by Kaesler Nutrition GmbH.
- 2) ENZY CARBOPLUS® for avian species and weaned piglets. Supplementary information. April 2014. Submitted by Kaesler Nutrition GmbH.
- 3) ENZY CARBOPLUS® for avian species and weaned piglets. Supplementary information. May 2014. Submitted by Kaesler Nutrition GmbH.
- 4) ENZY CARBOPLUS® for avian species and weaned piglets. Supplementary information. April 2015. Submitted by Kaesler Nutrition GmbH.
- 5) ENZY CARBOPLUS® for avian species and weaned piglets. Supplementary information. May 2017. Submitted by Kaesler Nutrition GmbH.
- 6) Evaluation report of the European Union Reference Laboratory for Feed Additives on the Method(s) of Analysis for ENZY CARBOPLUS®.
- 7) Comments from Member States.

<sup>58</sup> Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 laying down requirements for feed hygiene. OJ L 35, 8.2.2005, p. 1.

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## Abbreviations

ANOVA	analysis of variance
AOX	alcohol oxidase gene
CV	coefficient of variation
DSMZ	German Collection of Microorganisms and Cell Cultures
FEEDAP	EFSA Panel on Additives and Products or Substances used in Animal Feed
PCB	polychlorinated biphenyl
PCDD	polychlorinated dibenzo-p-dioxin
PCR	polymerase chain reaction
QPS	qualified presumption of safety
TEQ	toxic equivalents

## Annex A – Executive Summary of the Evaluation Report of the European Union Reference Laboratory for Feed Additives on the Methods of Analysis for ENZY CARBOPLUS®

In the current application, authorisation is sought under article 4(1) for ENZY CARBOPLUS® and ENZY CARBOPLUS® L, under the category/functional 4(a) 'zootechnical additives'/digestibility enhancers' according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought for the use of the feed additive for chickens and turkeys for fattening, chickens reared for laying, turkeys reared for breeding, laying hens, minor avian species including laying birds (game birds, geese, pigeons, sporting and ornamental birds), weaned pigs, and minor weaned porcine (e.g. warthogs).

According to the Applicant, the active agents of the ENZY CARBOPLUS® products are  $\beta$ -xylanase (EC 3.2.1.8) and  $\beta$ -glucanase (EC 3.2.1.6) produced by fermentation of two genetically modified yeast strains *Pichia pastoris* SUNHY 002 (DSM 25376) and SUNHY 004 (DSM 26469). The xylanase and  $\beta$ -glucanase enzymatic activities are expressed by the Applicant in LXU and LGU units, respectively. One LXU or LGU unit is the amount of enzyme which releases one micromole of reducing sugars equivalents (as xylose or glucose) from birch xylan or barley glucan per minute at pH 5.5 and 50°C.

The product is intended to be marketed in a powder and liquid formulations having a guaranteed minimum xylanase and  $\beta$ -glucanase activities of 25,000 (LXU/g or LXU/mL) and 2,200 (LGU/g or LGU/mL). The carrier in the solid formulation is starch, while glycerol, sodium chloride and sodium benzoate are used for the liquid formulation. The feed additive is intended to be included into premixtures and/or feedingstuffs with a minimum xylanase activity of 700 or 1,400 LXU/kg and a minimum  $\beta$ -glucanase activity of 60 or 120 LGU/kg depending on the target specie.

For the determination of xylanase and  $\beta$ -glucanase activities in the feed additive, premixtures and feedingstuffs, the Applicant submitted two single-laboratory validated and further verified colorimetric methods. Both methods are based on the quantification of water soluble dyed fragments produced by the action of the respective enzymes on the appropriate commercially available (Megazyme) cross-linked substrate. Based on the acceptable performance characteristics, the EURL recommends for official control the validated and further verified colorimetric method for the determination of xylanase and  $\beta$ -glucanase activities 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.