European Food Safety Authority

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# SCIENTIFIC OPINION

# Safety and efficacy of AveMix® XG 10 (endo-1,4-beta-xylanase and endo-1,3(4)-beta-glucanase) for use as feed additive for chickens for fattening[1,2]

## Scientific Opinion of the Panel on Additives and Products or Substances used in Animal Feed

**(Question No EFSA-Q-2008-308)**

## Adopted on 13 May 2009

**PANEL MEMBERS**

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**SUMMARY**

Following a request from the European Commission, the European Food Safety Authority (EFSA) was asked to deliver a scientific opinion on the safety and efficacy of AveMix® XG 10 when used as a feed additive for chickens for fattening.

The additive AveMix® XG 10 is a preparation of endo-1,3(4)-β-glucanase (E.C. 3.2.1.6) and endo-1,4-β-xylanase (E.C. 3.2.1.8) produced by two strains of *Trichoderma reesei*. The additive is produced in solid and liquid forms, and is intended for use as feed additive for chickens for fattening at a recommended dose of 4000 XU and 660 BGU $kg^{-1}$ complete feedingstuffs.

The solid and the liquid forms of the product are considered to be essentially equivalent in terms of safety for the target animals, consumers and the environment, and efficacy when used in diets for chickens for fattening at the same dose.

The FEEDAP Panel notes that the ratio of the two enzymes in the additive used for animal studies differed from that specified by the applicant. The following conclusions are based on the formulation as analysed and the corresponding increased enzyme concentrations in complete feedingstuffs.

[1] For citation purposes: Scientific Opinion of the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) on a request from the European Commission on the safety and efficacy of AveMix® XG 10 (endo-1,4-beta-xylanase and endo-1,3(4)-beta-glucanase) as feed additive for chickens for fattening. *The EFSA Journal* (2009) 1094, 1-17

[2] This Scientific Opinion has been edited following the provisions of Article 8(6) and Article 18 of Regulation (EC) No 1831/2003. The modified setions are indicated in the text.

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On the basis of the results from three efficacy studies made with chickens for fattening, in which significant increases in body weight gain were observed, the FEEDAP Panel concludes that there is evidence to support the efficacy of AveMix® XG 10 at the dose of 4000 XU $kg^{-1}$ and 900 BGU $kg^{-1}$ complete feedingstuffs.

A tolerance study performed with a 100-fold overdose of the product did not produce any adverse effects. Consequently, the FEEDAP Panel concludes that enzyme concentrations resulting from the supplementation of AveMix® XG 10 at 100 mg $kg^{-1}$ feedingstuffs are safe for chickens for fattening.

The results of the studies on genotoxicity and the 90-day study do not indicate any reason for concern regarding consumer safety from the use of AveMix® XG 10 as a feed additive.

From the results of tests on the individual enzyme concentrates contained in the additive, AveMix® XG 10 is not considered to be a dermal or eye irritant. However, it is considered to be both a dermal and respiratory sensitiser and should be handled accordingly.

The active substances of AveMix® XG 10 are proteins and as such will be degraded/inactivated during the passage through the digestive tract of animals. Therefore, no risk for the environment is expected.

**Key words:** zootechnical additive, digestibility enhancer, endo-1,4-β-xylanase, endo-1,3(4)-β-glucanase, chickens for fattening, safety, efficacy

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## BACKGROUND

Regulation (EC) No 1831/2003[3] 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 the company AVEVE NV[4] for authorisation of the product AveMix® XG 10, enzyme preparation containing endo-1,4-β-xylanase (E.C. 3.2.1.8) and endo-1,3(4)-β-glucanase (E.C. 3.2.1.6), to be used as a feed additive for chickens for fattening (category: zootechnical; functional group: digestibility enhancers) under the conditions mentioned in Table 1.

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.[5] According to Article 8 of that Regulation, 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. The particulars and documents in support of the application were considered valid by EFSA as of 31 July 2008.

The additive AveMix® XG 10 is a preparation of endo-1,3(4)-β-glucanase produced by *Trichoderma reesei* (MUCL 49754) and endo-1,4-β-xylanase produced by *Trichoderma reesei* (MUCL 49755). This product has not been previously authorised in the Community.

## TERMS OF REFERENCE

According to Article 8 of Regulation (EC) No 1831/2003, EFSA shall determine whether the feed additive complies with the conditions laid down in Article 5. EFSA shall deliver an opinion on the efficacy and the safety for the target animal(s), user and consumer and the environment of the product AveMix® XG 10, containing endo-1,3(4)-β-glucanase produced by *Trichoderma reesei* (MUCL 49754) and endo-1,4-β-xylanase produced by *Trichoderma reesei* (MUCL 49755), when used under the conditions described in Table 1.

## ACKNOWLEDGEMENTS

The European Food Safety Authority wishes to thank the Working Group on Enzymes as well as Friedrich Schöne for the preparation of this opinion.

[3] OJ L 268, 18.10.2003, p.29
[4] Aveve NV, Minderbroedersstraat 8, 3000 Leuven, Belgium
[5] Dossier reference: FAD-2008-0011

Table 1. **Register entry as proposed by the applicant**

| | |
|---|---|
| **Additive** | endo-1,4-β-xylanase (EC 3.2.1.8) and endo-1,3(4)-β-glucanase (EC 3.2.1.6); AveMix® XG 10 |
| **Registration number/EC No/No (if appropriate)** | endo-1,4-β-xylanase : EC 3.2.1.8<br>endo-1,3(4)-β-glucanase: EC 3.2.1.6 |
| **Category of additive** | Zootechnical Additive |
| **Functional group of additive** | Digestibility enhancer |

| **Description** | | | |
|---|---|---|---|
| **Composition, description** | **Chemical formula** | **Purity criteria (if appropriate)** | **Method of analysis (if appropriate)** |
| Enzyme preparation containing endo-1,4- β -xylanase (EC 3.2.1.8) produced by *Trichoderma reesei* and endo-1,3(4)- β -glucanase (EC 3.2.1.6) produced by *Trichoderma reesei* Solid or Liquid having a minimum activity of endo-1,4- β -xylanase (EC 3.2.1.8): 40000 XU[6] $g^{-1}$ and of endo-1,3(4)- β –glucanase (EC 3.2.1.6): 6600 BGU[7] $g^{-1}$ | Not applicable | Complies with JECFA guidelines on microbial quality, heavy metals, toxins and undesirable substances | Assay based on colorimetric reaction of dinitrosalicylic acid on reducing sugar yield produced by action of endo-1,4- β -xylanase on a xylan containing substrate or endo-1,3(4)- β -glucan containing substrate |

| | |
|---|---|
| **Trade name (if appropriate)** | AveMix® XG 10 |
| **Name of the holder of authorisation (if appropriate)** | Aveve Biochem |

| **Conditions of use** | | | | |
|---|---|---|---|---|
| **Species or category of animal** | **Maximum Age** | **Minimum content** | **Maximum content** | **Withdrawal period (if appropriate)** |
| | | **Units of activity $kg^{-1}$ of complete feedingstuffs** | | |
| Chickens for fattening | Slaughter age | Endo-1,4-β-xylanase: 4000 XU<br>Endo-1,3(4)-β-glucanase: 660 BGU | | Not relevant |

| **Other provisions and additional requirements for the labelling** | |
|---|---|
| **Specific conditions or restrictions for use (if appropriate)** | In the directions for use of the additive and premixture, indicate the storage temperature, storage life, and stability to pelleting |
| **Specific conditions or restrictions for handling (if appropriate)** | - |
| **Post market monitoring (if appropriate)** | - |

[6] XU is the amount of enzyme which releases 1 µmol of reducing sugar per minute from xylan of oat spelt under defined test conditions (pH 5; 50 °C)

[7] BGU is the amount of enzyme which releases 1 µmol of reducing sugar per minute from β-glucan of barley under defined test conditions (pH 4.8; 50 °C)

| | |
|---|---|
| **Specific conditions for use in complementary feedingstuffs (if appropriate)** | Recommended dosages per kilogram of complete feedingstuff:<br>Endo-1,4- β -xylanase: 4000 XU<br>Endo-1,3(4)- β -glucanase: 660 BGU<br>For use in compound feed Rich in non-starch polysaccharides (mainly arabinoxylans and betaglucans) |

| **Maximum Residue Limit (MRL) (if appropriate)** | | | |
|---|---|---|---|
| **Marker residue** | **Species or category of animal** | **Target tissue(s) or food products** | **Maximum content in tissues** |
| - | - | - | - |

**ASSESSMENT**

## 1. Introduction

The additive AveMix® XG 10 is an enzyme preparation of endo-1,3(4)-β-glucanase (E.C. 3.2.1.6) and endo-1,4-β-xylanase (E.C. 3.2.1.8), produced by two strains of *Trichoderma reesei*. This product is intended for use as feed additive for chickens for fattening at a recommended dose of 4000 XU and 660 BGU $kg^{-1}$ complete feedingstuffs (100 mg AveMix® XG 10 $kg^{-1}$). AveMix® XG 10 has not been previously authorised as feed additive in the European Community.

The applicant is seeking the authorisation of the product as a zootechnical additive, under the functional group of digestibility enhancers.

## 2. Characterisation[8]

### 2.1. Characterisation of the product

AveMix® XG 10 is an enzyme preparation of endo-1,3(4)-β-glucanase and endo-1,4-β-xylanase produced by two non-genetically modified strains of *Trichoderma reesei* (MUCL 49754 and MUCL 49755). Both strains are deposited at the Belgian Coordinated Collections of Micro-organisms.[9]

The enzymes are produced separately by submerged fermentation of the production strains. The final product is made by mixing of the separate enzyme preparations with the appropriate carrier/preservative.

#### 2.1.1. Qualitative and quantitative composition

The product is presented in two different formulations, solid and liquid, both of which ensure a minimum activity of endo-1,4-β-xylanase of 40000 XU $g^{-1}$ and of endo-1,3(4)-β-glucanase of 6600 BGU $g^{-1}$.

The batch to batch variation of the enzyme activity is, in the solid formulation, 11.9 % and 3.6 % for endo-1,4-β-xylanase and endo-1,3(4)-β-glucanase activities, respectively (three batches). For the liquid formulation, the batch to batch variation is 0.3 % and 6.1 % for endo-1,4-β-xylanase and endo-1,3(4)-β-glucanase, respectively (three batches).

AveMix® XG 10 solid is a brownish powder composed of 27–32 % of the dry enzyme concentrate and 68–73 % of the carrier (soybean meal). Particle size analysis of AveMix® XG 10 solid shows that 48.2 % of the particles are below 100 μm, 26.5 % below 50 μm and 0.2 % below 10 μm, with a dusting potential of 0.3 % as measured by Heubach test.[10] The product has a bulk density of 0.6 g $mL^{-1}$.

AveMix® XG 10 liquid is a brown liquid preparation composed of 77–82 % of the liquid enzyme concentrate diluted in 18–23 % of a mixture of water and sorbitol. The liquid form of the product has a density of 1.18 kg $L^{-1}$ and a viscosity of 12 cP.[11]

Both preparations conform to the general specifications for enzyme preparations used in food processing as proposed by the Joint FAO/WHO Expert Committee on Food Additives

[8] This section has been edited following the provisions of Article 8(6) and Article 18 of Regulation (EC) No 1831/2003.
[9] Technical Dossier/Section II/Annex II.1.3.1
[10] Technical Dossier/Section II/Annex II.1.5.4
[11] Technical Dossier/Section II/Annex II.1.5.2

(JECFA). The product is regularly monitored for heavy metals (Pb, Cd, Hg), F and As, biological contaminants (*E. coli*, *Salmonella sp.*), antimicrobial activity, absence of the production strains and mycotoxins (aflatoxin B1, ochratoxin A, T-2 Toxin, deoxynivalenol and zearalenone) to ensure that the maximum levels are not exceeded. Compliance was confirmed by the analysis of three batches of each formulation of the final product.[12, 13, 14,15]

## 2.2. Stability and homogeneity

The applicant proposes a shelf life for both formulations of six months. The shelf life of the solid formulation of AveMix® XG 10 was assessed in three batches[16] stored at two different conditions: 25 °C/65 % RH and 30 °C/65 % RH for 6, 10 or 13 months (one batch for each of the durations). Enzyme recovery for both enzymes was approximately 100 % after six months of storage, when kept at 25 °C; it was 89 % and 81 % for endo-1,3(4)-β-glucanase and endo-1,4-β-xylanase after 13 months, respectively. Similar recoveries were obtained when the additive was kept at 30 °C.

The shelf life of the liquid formulation of AveMix® XG 10 was assessed in three batches[17] stored for six months under two different conditions: 25 °C and 30 °C. Mean enzyme recoveries after six months, when kept at 25 °C, were 75 % and 94 % for endo-1,3(4)-β-glucanase and endo-1,4-β-xylanase, respectively, and when kept at 30 °C recoveries were 66 % and 85 % for endo-1,3(4)-β-glucanase and endo-1,4-β-xylanase, respectively.

The enzymes activities of AveMix® XG 10 (three batches), when added to a premix containing vitamins and minerals (trace elements) and maintained at 25 °C for three months, were 110 % for the endo-1,4-β-xylanase and 99 % for the endo-1,3(4)-β-glucanase activity, respectively.[18] No data was available for storage periods up to six months.

The stability of AveMix® XG 10 (three batches, solid formulation) in feedingstuffs was tested[19] in cereal-based complete feed at the recommended dosage. The feedingstuffs were kept at 25 °C and for a period of three months, after which the enzymes activities were 107 % for the endo-1,4-β-xylanase and 83 % for the endo-1,3(4)-β-glucanase. No stability data for the liquid formulation in feedingstuffs have been provided by the applicant.

The enzyme activities of AveMix® XG 10 (one batch, solid form) during the conditioning and pelleting was tested at a temperature of pelleting of 75 °C.[20] The results showed that the enzymes activities were after the preconditioning 79 % for the endo-1,4-β-xylanase and 86 % for the endo-1,3(4)-β-glucanase, and after the pelleting 84 % for the endo-1,4-β-xylanase and 85 % for the endo-1,3(4)-β-glucanase.

The homogeneity of both formulations was tested in ten samples each.[21] The results showed a coefficient of variation of approximately 5 % for the solid formulation in a cereal-based diet. Homogeneity of the liquid formulation determined using post-pelleting application showed a coefficient of variation of about 14 %.

[12] Technical Dossier/Section II/Annex II.1.4.3
[13] Technical Dossier/Section II/Annex II.5.1.7
[14] Technical Dossier/Section II/Annex II.2.6.1
[15] Technical Dossier/Section II/Annex II.2.7.3 and II.2.7.4
[16] Technical Dossier/Section II/Annex II.3.1.1, II.3.1.2, II.3.1.3
[17] Technical Dossier/Section II/Annex II.3.1.4, II.3.1.5, II.3.1.6
[18] Technical Dossier/Section II/Annex II.3.1.8
[19] Technical Dossier/Section II/Annex II.3.1.9
[20] Technical Dossier/Section II/Annex II.3.1.7
[21] Technical Dossier/Section II/Annex II.3.2.3, II.3.2.4

## 2.3. Conditions of use

AveMix® XG 10 is intended to be used as feed additive for chickens for fattening at a recommended dose of 100 mg $kg^{-1}$ complete feedingstuffs, delivering 4000 XU (endo-1,4-β-xylanase) and 660 BGU (endo-1,3(4)-β-glucanase) $kg^{-1}$.

## 2.4. Evaluation of the analytical methods by the Community Reference Laboratory (CRL)

EFSA has verified the CRL report as it relates to the methods used for the control of the active substances in animal feed. The Executive Summary of the CRL report can be found in the Appendix.

## 3. Efficacy for chickens for fattening

The applicant provided three performance trials conducted with chickens for fattening in two different locations to prove the efficacy of AveMix® XG 10 (solid formulation).

*First trial*

The first performance trial[22] was carried out with 160 one-day-old male chickens (Ross 308), which were kept in cages of eight birds, with ten replicates per treatment. A basal diet based on maize and soybean meal was supplemented with AveMix® XG 10 at 0 or 100 mg $kg^{-1}$ (4976 XU and 890 BGU $kg^{-1}$). The mash diets were offered *ad libitum* to the birds for 42 days (starter and grower phases), during which the performance in terms of body weight gain and feed consumption was measured weekly and the feed to gain ratio was calculated.

The results (Table 2) showed a higher body weight gain in chickens fed AveMix® XG 10 compared to those fed the negative control. However, no effects on feed intake or in the feed conversion were observed. The mortality in both groups was within the normal range.

Table 2. **Performance of chickens fed AveMix® XG 10 for 42 days**

| AveMix® XG 10 (mg $kg^{-1}$) | Feed intake (kg $bird^{-1}$) | Body weight gain (kg $bird^{-1}$) | Feed/gain (kg $kg^{-1}$) |
|---|---|---|---|
| 0 | 3.98 | 2.30[a] | 1.73 |
| 100 | 4.03 | 2.39[b] | 1.69 |

[a, b]: values within one column with different superscripts are different ($P \leq 0.05$)

*Second trial*

The second performance trial[23] was carried out with 160 one-day old male chickens (Ross 308), which were allocated in pens of eight birds, with ten replicates per treatment. A basal diet based on wheat, maize and soybean meal was supplemented with AveMix® XG 10 at 0 or 100 mg $kg^{-1}$ (4560 XU and 900 BGU $kg^{-1}$). The mash diets were offered *ad libitum* to the birds for 42 days (starter and grower phases), during which the performance in terms of body weight gain and feed consumption was measured weekly and the feed conversion ratio was calculated.

The results (Table 3) showed a higher feed intake and body weight gain in chickens fed AveMix® XG 10 compared to those fed the negative control. No mortalities occurred.

[22] Technical Dossier/Section III/Annex III.2.1.1
[23] Technical Dossier/Section III/Annex III.2.1.2

Table 3. **Performance of chickens fed AveMix® XG 10 for 42 days**

| AveMix® XG 10 (mg kg$^{-1}$) | Feed intake (kg bird$^{-1}$) | Body weight gain (kg bird$^{-1}$) | Feed/gain (kg kg$^{-1}$) |
|---|---|---|---|
| 0 | 3.86[a] | 2.34[a] | 1.65 |
| 100 | 4.22[b] | 2.61[b] | 1.62 |

[a, b]: values within one column with different superscripts are different ($P \leq 0.05$)

*Third trial*

The third performance trial[24] was carried out with 736 one-day-old chickens (Ross) separated by sex, which were allocated in floor pens of 46 birds, with eight replicates per treatment (four replicates for each sex). A basal diet based on wheat, soy protein concentrate (hipro soya) and full fat soya, considered as the negative control, was supplemented with AveMix® XG 10 at 0 or 100 mg kg$^{-1}$ (4365 XU and 918 BGU kg$^{-1}$). The crumb/pelleted diets were offered *ad libitum* to the birds for 42 days (starter, grower and finisher phases). During the experimental period, the feed intake was measured weekly, the body weight of the animals was assessed at the end of each phase (starter, grower and finisher) and mortality was monitored throughout the experiment. Litter condition was evaluated on days 21 and 42 (scoring from 1 (good) to 5 (poor)) and the score of hock lesions was assessed in 20 birds per treatment at the end of the study.

The results showed no interactions between the two main factors considered (sex x diet); therefore, the results presented in Table 4 are the mean values for the dietary treatments. The results showed an increase in the body weight gain of the birds fed AveMix® XG 10, compared to those fed the control diet. There were no significant changes in feed intake or feed conversion rate. Hock lesion score was lower for the birds fed AveMix® XG 10 compared to those fed the negative control diet. Mortality and culling, and the overall litter score, were not affected by the dietary treatments.

Table 4. **Performance of chickens fed AveMix® XG 10 for 42 days**

| AveMix® XG 10 (mg kg$^{-1}$) | Feed intake (kg bird$^{-1}$) | Body weight gain (kg bird$^{-1}$) | Feed/gain (kg kg$^{-1}$) | Mortality and culling (%) |
|---|---|---|---|---|
| 0 | 4.28 | 2.45[a] | 1.71 | 5.7 |
| 100 | 4.35 | 2.61[b] | 1.63 | 3.0 |

[a, b]: values within one column with different superscripts are different ($P \leq 0.05$)

## Conclusions on efficacy for chickens for fattening

Analyses of AveMix® XG 10 used in animal studies showed values for the two enzymes of approximately 50000 XU and 10000 BGU g$^{-1}$, which differs from the specification provided by the applicant. Therefore, all the conclusions drawn are based on the actual formulation used in the experiments and the resulting enzyme activities in complete feed.

Three performance trials demonstrated an increased final body weight of chickens for fattening after the supplementation with the solid formulation of AveMix® XG 10 at 100 mg kg$^{-1}$ complete feed. No differences between solid and liquid formulation in terms of efficacy should be expected. Therefore, the FEEDAP Panel concludes that there is evidence to support the efficacy of AveMix® XG 10 at the minimum dose of 4000 XU kg$^{-1}$ and 900 BGU kg$^{-1}$.

[24] Technical Dossier/Section III/Annex III.2.1.3

# 4. Safety

## 4.1. Safety for chickens for fattening

One tolerance trial[25] was carried out with 160 one-day-old male chickens (Ross 308), which were allocated in pens of eight birds, with ten pens per treatment. The basal diet based on wheat, corn and soybean meal was supplemented with AveMix® XG 10 (solid formulation) at 0 or 10 g $kg^{-1}$ feed (100 times the recommended dose; 499241 XU and 107712 BGU $kg^{-1}$). The diets in mash form were offered *ad libitum* to the birds for 42 days (starter and grower phases), during which the performance in terms of body weight gain and feed consumption was measured weekly and the feed to gain ratio was calculated. Mortality and health status were monitored throughout the experiment.

The results showed a better feed to gain ratio in the birds fed AveMix® XG 10 solid compared to those fed the negative control diet, but no differences were found in the feed intake or in the body weight gain after 42 days. There were no negative effects on animal health status and mortality was not affected by the dietary treatments.

**Conclusions on the safety for chickens for fattening**

The tolerance trial provided showed no negative effects on the performance of chickens for fattening when AveMix® XG 10 was fed at 100 times the proposed dose. The FEEDAP Panel concludes that enzyme concentrations resulting from the supplementation of complete feedingstuffs with AveMix® XG 10 at 100 mg $kg^{-1}$ are safe for chickens for fattening.

## 4.2. Safety for the consumer

The assessment of the consumer safety was performed with two mutagenicity/clastogenicity studies *in vitro* and a 13-week repeated oral dose study in rats carried out with each of the two fermentation products used to formulate AveMix® XG 10.

### 4.2.1. Bacterial reverse mutation assays

Two bacterial reverse mutation tests were separately conducted to evaluate the mutagenic activity of the endo-1,4-β-xylanase[26] and endo-1,3(4)-β-glucanase.[27]

Both tests were conducted according to OECD guideline 471. The mutagenic activity was tested in four strains of *Salmonella typhimurium*: TA1535, TA1537, TA98 and TA100, and in the *Escherichia coli* WP2*uvrA*, both in the absence and the presence of metabolic activation (S9-mix). Five enzyme concentrations were tested in each study from 62 to 5000 μg $plate^{-1}$ and negative and positive controls were included. The results showed no dose-related or more than two-fold increase in the mean number of revertant colonies as compared to the reversion rate observed with the negative control. It is thus concluded that neither product is mutagenic in this assay.

### 4.2.2. Chromosomal aberration tests

Two chromosomal aberration tests in cultured Chinese hamster ovary (CHO) cells were separately conducted to evaluate the clastogenic activity of the endo-1,4-β-xylanase[28] and

[25] Technical Dossier/Section IV/Annex IV.2.1
[26] Technical Dossier/Section IV/Annex IV.3.1.1
[27] Technical Dossier/Section IV/Annex IV.3.1.2
[28] Technical Dossier/Section IV/Annex IV.3.1.3

endo-1,3(4)-β-glucanase.[29] The clastogenicity tests carried out separately for each of the two enzymes followed OECD guideline 473. For each enzyme, two trials were carried out. In both cases, the first trial, was conducted both in the absence and the presence of a metabolic activation system (S9-mix), the treatment of the CHO cells lasted for four hours and the harvesting was carried out at 18 hours; the concentrations evaluated were 250, 1000, and 1500 μg $mL^{-1}$. For the second trial, in the presence of S9-mix, the treatment lasted for four hours and the harvesting was carried out at 18 hours; the concentrations tested were 400, 1000 and 1500 μg $mL^{-1}$. In the absence of S9-mix, the treatment lasted for 18 hours and the harvesting was carried out at 18 hours; the concentrations tested were 200, 400 and 600 μg $mL^{-1}$. Positive and negative controls were included in both trials.

The enzymes did not cause any increase in the number of aberrant cells at any of the concentrations and time points studied, when compared to the negative control; thus, they are not considered clastogenic under the conditions of the test.

### 4.2.3. Subchronic oral toxicity study

The study[30] was conducted in accordance with OECD guideline 408 and Annex 5D of Directive 2001/59/EC.[31] The trial was carried out over 13 weeks in groups of ten Wistar WU rats of each sex, caged in groups of five. There was a total of seven experimental groups, with one negative control, three groups given diets containing endo-1,4-β-xylanase at 500, 1500 and 4000 mg $kg^{-1}$, and three more groups given diets containing endo-1,3(4)-β-glucanase at 500, 1500 and 4000 mg $kg^{-1}$. Throughout the experimental period, the animals were fed *ad libitum* and observed at least once daily. The body weight of the rats was recorded weekly throughout the study and food intake measured continuously. Water consumption was measured over three five-day periods, during weeks 1, 6 and 11 of the study. Ophthalmologic observations were carried out before the start and in the last week of the study for all the animals in the control and the high-dose groups. Neurobehavioural studies were carried out on males at week 12 and on females at week 13. During the last week of the study, urinalysis was conducted on all the rats. All rats were necropsied and blood samples were taken for haematological and biochemical analyses, and organ weights were recorded. Samples of a wide range of tissues and organs from all animals were preserved and processed for histopathological examination; microscopic examination was confined to the control and high-dose groups.

The enzyme activity of the experimental diets was confirmed by analysis.

There were no significant adverse effects of treatment on any of the parameters observed during or at the end of the experimental period.

### 4.2.4. Conclusion on the safety for the consumer

The results of the studies on genotoxicity and the 90-day study do not indicate any reason for concern regarding consumer safety from the use of AveMix® XG 10 as a feed additive.

## 4.3. Safety for the user

For each fermentation product, eye and dermal irritancy and skin sensitisation tests were carried out.

[29] Technical Dossier/Section IV/Annex IV.3.1.4
[30] Technical Dossier/Section IV/Annex IV.3.2
[31] OJ L 225, 21.08.2001, p. 150

### 4.3.1. Irritancy tests

*Acute dermal irritation*

An acute dermal irritation test was carried out with three albino rabbits, according to Directive 92/69/EC (method B.4) and OECD guideline 404.

The tests with the two enzymes (endo-1,4-β-xylanase[32] and endo-1,3(4)-β-glucanase[33]) were performed separately and were carried out with specific pathogen-free bred New Zealand white albino rabbits. Three males were used in each test. Twenty-four hours after the acute exposure to endo-1,4-β-xylanase, one rabbit showed slight erythema, while in the case of the endo-1,3(4)-β-glucanase all the rabbits showed slight erythema. After 48 hours, there were no further signs of skin irritation, thus both products are considered not to be irritant to the skin.

*Acute eye irritation test*

An acute eye irritation test was carried out with three albino rabbits, according to Directive 92/69/EC (method B.5) and OECD guideline 405.

The tests with the two enzymes (endo-1,4-β-xylanase[34] and endo-1,3(4)-β-glucanase[35]) were performed separately and were carried out with specific pathogen-free bred New Zealand white albino rabbits. Three males were used in each test. In both cases, one hour after the exposition to the test substances, slight redness and slight swelling of the conjunctivae as well as slight ocular discharge were observed in the rabbits; and in one case, slight iritis was presented. After 48 hours, there was no further sign of eye irritations. From these results, the products are not considered to be irritant to the eye.

### 4.3.2. Skin sensitisation

The assessments of the skin sensitising potential of endo-1,4-β-xylanase[36] and endo-1,3(4)-β-glucanase[37] were performed separately in a local lymph node assay in mouse. These tests followed OECD guideline 429, Directive 2004/73/EC (method B.42) and EPA guideline OPPTS 870.2600.

The tests were carried out for each enzyme separately with specific pathogen-free bred female mice (CBA/CaOlaHsd). In each trial, there was a total of five experimental groups, with five females each. The treatments included one negative control, three groups with either endo-1,4-β-xylanase or endo-1,3(4)-β-glucanase from *Trichoderma reesei* at dose levels of 10 %, 24 % and 50 %, and one positive control. For three consecutive days, 25 µl of the test substances were injected at the dorsum of each ear to all animals. On day 5, the animals received $^{3}$H-methyl thymidine; five hours later, the animals were sacrified and the auricular lymph nodes excised in order to determine the $^{3}$H-methyl thymidine incorporation.

No signs of irritancy were found with either enzyme. The results of the $^{3}$H-methyl thymidine incorporation determined that it was significantly enhanced by the presence of both enzymes, indicating that they should be regarded as skin sensitisers.

[32] Technical dossier/safety/Annex IV. 4.1.3
[33] Technical dossier/safety/Annex IV 4.1.4
[34] Technical dossier/safety/Annex IV.4.1.1
[35] Technical dossier/safety/Annex IV.4.1.2
[36] Technical dossier/safety/Annex IV.4.2.1
[37] Technical dossier/safety/Annex IV. 4.2.2

### 4.3.3. Toxic effects on the respiratory system

The applicant recognises that enzyme additives are regarded as respiratory sensitisers (R42) and classifies the product accordingly.

### 4.3.4. Conclusions on the safety for user

From the results of tests conducted with the component enzymes, AveMix® XG 10 is not considered to be a dermal or eye irritant. However, it is considered to be both a dermal and respiratory sensitiser and should be handled accordingly.

## 4.4. Safety for the environment

The active substances of AveMix® XG 10 are proteins and as such will be degraded/inactivated during the passage through the digestive tract of animals. Therefore, no risks for the environment are expected and no further environmental risk assessment is required.

## 5. Post-market monitoring

No risks associated with the use of the product are foreseen. It is considered that there is no need for specific requirements for a post-market monitoring plan other than those established in the Feed Hygiene Regulation[38] and Good Manufacturing Practice.

## CONCLUSIONS AND RECOMMENDATIONS

### CONCLUSIONS

The FEEDAP Panel notes that the ratio of the two enzymes in the additive used for animal studies differed from that specified by the applicant. The following conclusions are based on the formulation as analysed and the corresponding increased enzyme concentrations in complete feedingstuffs.

The solid and the liquid forms of the product are considered to be essentially equivalent in terms of safety for the target animals, consumers and the environment, and efficacy when used in diets for chickens for fattening at the same dose.

The FEEDAP Panel concludes that there is evidence to support the efficacy of AveMix® XG 10 at a dose of 4000 XU $kg^{-1}$ and 900 BGU $kg^{-1}$.

The FEEDAP Panel concludes that enzyme concentrations resulting from the supplementation of AveMix® XG 10 at 100 mg $kg^{-1}$ feedingstuffs are safe for chickens for fattening.

The results of the studies on genotoxicity and the 90-day study do not indicate any reason for concern regarding consumer safety from the use of AveMix® XG 10 as a feed additive.

From the results of tests conducted with the individual enzyme concentrates contained in the additive, AveMix® XG 10 is not considered to be a dermal or eye irritant. However, it is considered to be both a dermal and respiratory sensitiser and should be handled accordingly.

The active substances of AveMix® XG 10 are proteins and as such will be degraded/inactivated during the passage through the digestive tract of animals. Therefore, no risk for the environment is expected.

[38] OJ L 35, 8.2.2005, p.1

## RECOMMENDATIONS

The FEEDAP Panel recommends that the concentration of the two enzymes in the complete feedingstuffs should be 4000 XU and 900 BGU $kg^{-1}$, for endo-1,4-β-xylanase and endo-1,3(4)-β-glucanase respectively, reflecting the dose used in efficacy and safety studies, and that the specification for AveMix® XG 10 should reflect this ratio.

## DOCUMENTATION PROVIDED TO EFSA

1. AveMix® XG 10 for chickens for fattening. January 2008. Submitted by AVEVE NV.
2. Supplementary information for AveMix® XG 10 for chickens for fattening. October 2008. Submitted by AVEVE NV.
3. Supplementary information for AveMix® XG 10 for chickens for fattening. January 2009. Submitted by AVEVE NV.
4. Evaluation report of the Community Reference Laboratory for Feed Additives on the methods of analysis for endo-1,4-β-xylanase (E.C. 3.2.1.8) and endo-1,3(4)-β-glucanase (E.C. 3.2.1.6) from AveMix® XG .
5. Comments from Member States received through the ScienceNet.

## APPENDIX

### Executive Summary of the Evaluation Report of the Community Reference Laboratory for Feed Additives on the Method(s) of Analysis for endo-1,4-β-xylanase (E.C. 3.2.1.8) and endo-1,3(4)-β-glucanase (E.C. 3.2.1.6) from AveMix® XG.

The current application authorisation is sought for *AveMix XG 10* under the category 'zootechnical additives', group 4(a), according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought to use *AveMix XG 10* as a digestibility enhancer for chickens for fattening. The product is intended to be marketed as solid and liquid formulations.

The active agents of *AveMix XG 10* are (1) endo-1,4-β-*xylanase* and (2) endo-1,3(4)- β-*glucanase* produced by the strain (MUCL 49755) and the strain (MUCL 49754)) of *Trichoderma reesei*, respectively. The enzymatic activities are expressed in *xylanase* unit (XU) and *betaglucanase* unit (BGU). According to the applicant one XU-unit is the amount of enzyme which releases 1 µmol of reducing sugar (xylose equivalent) per minute from xylan of oat spelt at 50°C, pH = 4.8, whereas one BGU-unit is the amount of enzyme which releases 1 µmol of reducing sugar (cellobiose equivalent) per minute from betaglucan of barley at 50$^{o}$C, pH = 5.0.

Both solid and liquid formulations of *Avemix XG10* have an endo-1,4-β-*xylanase* activity of 40 000 XU/g and an endo-1,3(4)-β-*glucanase* activity of 6 600 BGU/g. The product is intended to be mixed into *premixtures* and/or *feedingstuffs* to provide an endo-1,4-β-*xylanase* activity of 4 000 XU/kg *feedingstuffs* and an endo-1,3(4)-β-*glucanase* activity of 660 BGU/kg *feedingstuffs*.

For the determination of the activity of endo-1,4-β-*xylanase* in the *feed additives* and *premixtures* a colorimetric method based on the formation of reducing sugar reacting with Dinitrosalicylic acid (DNS) is proposed. The method is in-house validated only for the *feed additives*, and the following performance characteristics were reported: -a recovery rate of 104 %, - a relative standard deviation for repeatability ($RSD_r$) of 4%, - a relative standard deviation for intermediate precision ($RSD_R$) of 5 %, - a limit of detection (LOD) and a limit of quantification (LOQ) of 722 and 820 XU per gram of the *product*. No sufficient validation parameters have been provided for the determination of the activity of endo-1,4-β-*xylanase* in the *premixtures*. For the determination of the activity of endo-1,4-β-*xylanase* in the *feedingstuffs* the applicant provided upon request from the CRL an in-house validated method based on the measurement of the rate of release of water soluble dyed fragments by endo-1,4-ß-*xylanase* from the dye cross-linked wheat arabinoxylan in a form of "Xylazyme AX tablet". The following performance characteristics were reported: - a recovery of 104 %, - a $RSD_r$ of 4 %, - a $RSD_R$ of 4 %, - an LOD and LOQ of 939 and 1878 XU/kg feedingstuffs.

For the determination of the activity of endo-1,3(4)-β-*glucanase* in the *feed additives* and *premixtures* a colorimetric method based on the formation of reducing sugar reacted with DNS is proposed. The method is in-house validated only for the *feed additives*, and the following performance characteristics were reported: -a recovery rate of 107 %, - a $RSD_r$ of 2 %, - a $RSD_R$ of 4 %, - an LOD and LOQ of 100 and 116 BGU/g *product*. No sufficient validation parameters have been provided for the determination of the activity of endo-1,3(4)-β-*glucanase* in the *premixtures*. For the determination of the activity of endo-1,3(4)-β-*glucanase* in the *feedingstuffs* the applicant provided upon request from the CRL an in-house validated method based on the measurement of the rate of release of water soluble dyed fragments by endo-1,3(4)-β-*glucanase* from the dye cross-linked barley glucan. The following performance characteristics were reported: - a recovery of 109 %, - a $RSD_r$ of 6 %, - a $RSD_R$ of 5 %, -an LOD and LOQ of 111 and 222 BGU/kg *feedingstuffs*.

Based on acceptable performance characteristics, the proposed methods are considered suitable for determination of endo-1,4-ß-*xylanase* and endo-1,3(4)-β-*glucanase* activities in *feed additives* and *feedingstuffs* (not in *premixtures*) for official control purposes in the frame of authorisation.

Further testing or validation is not considered necessary.