European Food Safety Authority

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SCIENTIFIC OPINION

Safety and efficacy of Avizyme 1505 (endo-1,4-β-xylanase, α-amylase, subtilisin) as a feed additive for chickens and ducks for fattening[1]

Scientific Opinion of the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) and of the Panel on Genetically Modified Organisms (GMO)

(Question No EFSA-Q-2007-020)

Adopted on 17 June 2009 by the FEEDAP Panel and on 29 October 2008 by the GMO Panel

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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) and the Panel on Genetically Modified Organisms (GMO) were asked to issue a scientific opinion on the safety and efficacy of Avizyme 1505 as a feed additive for chickens and ducks for fattening.

Avizyme 1505 is an enzyme preparation with three declared activities, i.e. endo-1,4-β-xylanase, α-amylase and alkaline protease (subtilisin). These three enzymes are produced by three genetically modified micro-organisms: endo-1,4-β-xylanase by *Trichoderma reesei*,

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α-amylase by *Bacillus amyloliquefaciens* and alkaline protease by *Bacillus subtilis*. In the *T. reesei* strain, no DNA sequences causing concern have been introduced. In the *B. amyloliquefaciens* strain, resistance genes for neomycin and bleomycin are present in a plasmid which has been made non-mobile. In the *B. subtilis* strain, a chloramphenicol resistance gene has been inserted into the chromosome. The production micro-organisms are removed after fermentation and are not detected in the final enzyme preparations. The recombinant DNA is below the limit of detection in the final enzyme preparations. With respect to the genetic modification, there are no environmental safety concerns in relation to the final product.

Avizyme 1505 is intended to be used in diets for chickens and ducks for fattening at a dose range of 50–200 mg kg^{-1} complete feed.

Based on the tolerance study provided, it is concluded that Avizyme 1505 is safe for chickens and ducks for fattening at the recommended dose range.

The studies conducted on the individual components of Avizyme 1505 are considered an adequate investigation of the safety of this product for the consumer. On the basis of the results of both genotoxicity tests and 90-day studies, it is not expected that there would be any risk to the consumer from the use of Avizyme 1505 in animal feed.

There are no concerns for the safety of users provided that the MSDS recommendations are followed and appropriate measures are taken to avoid dermal and inhalation exposure.

The active components of Avizyme 1505 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.

The efficacy of Avizyme 1505 has been demonstrated in chickens for fattening at the dose of 125 mg kg^{-1} complete feed (equivalent to 187.5 U β-xylanase kg^{-1}, 250 U α-amylase kg^{-1} and 2500 U protease kg^{-1}). The efficacy of Avizyme 1505 in ducks for fattening as minor species has been demonstrated at a dose of 50 mg kg^{-1}.

The FEEDAP Panel notes that the methods of analysis for the enzymes present in Avizyme 1505 are not considered by the CRL as fit for the purpose of official control methods.

Key words: zootechnical additive, digestibility enhancer, enzyme, xylanase, protease, α-amylase, chickens for fattening, ducks for fattening, safety, efficacy, genetically modified micro-organisms

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BACKGROUND

Regulation (EC) No 1831/2003[2] 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 an 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 Danisco Animal Nutrition[3] for authorisation of the product Avizyme 1505 to be used as a feed additive for chickens and ducks for fattening (category: zootechnical additives; functional group: digestibility enhancers) under the conditions described 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.[4] 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 10 July 2007.

The additive Avizyme 1505 is a preparation of endo-1,4-β-xylanase produced by the genetically modified micro-organism (GMM) *Trichoderma reesei* (ATCC PTA 5588), α-amylase produced by the GMM *Bacillus amyloliquefaciens* (ATCC 3978) and subtilisin (protease) produced by the GMM *Bacillus subtilis* (ATCC 2107). 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. Therefore, EFSA shall deliver an opinion on the efficacy and the safety for the target animals, the consumer, user and the environment of the product Avizyme 1505 which is a preparation of endo-1,4-β-xylanase produced by *Trichoderma reesei* (ATCC PTA 5588), α-amylase produced by *Bacillus amyloliquefaciens* (ATCC 3978) and subtilisin (protease) produced by *Bacillus subtilis* (ATCC 2107) when used under the conditions described in Table 1.

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[2] OJ L 268, 18.10.2003, p.29
[3] Danisco Animal Nutrition, legal entity Finnfeeds International Limited, PO Box 777, Marlborough, Wiltshire, SN8 1XN, United Kingdom
[4] Dossier reference: FAD-2006-0039

Table 1. Description and conditions of use of the additive as proposed by the applicant

Additive	Avizyme 1505
Registration number/EC No/No (if appropriate)	Endo-1,4-beta-xylanase EC 3.2.1.8 Subtilisin EC 3.4.21.62 Alpha-amylase E.C. 3.2.1.1
Category of additive	Zootechnical additives
Functional group of additive	Digestibility enhancer

Description			
Composition, description	Chemical formula	Purity criteria (if appropriate)	Method of analysis (if appropriate)
Preparation of Endo-1,4-beta-xylanase EC 3.2.1.8 produced by *Trichoderma reesei* ATCC PTA 5588), alpha-amylase produced by *Bacillus amyloliquefaciens* (ATCC 3978) and subtilisin produced by *Bacillus subtilis* (ATCC 2107), with a minimum activity of: - Dry form : Endo-1,4- beta-xylanase: 1500 U g^{-1} Subtilisin: 20000 U g^{-1} Alpha-amylase: 2000 U g^{-1}	N/A	Guaranteed minimum activity of Endo-1,4- beta-xylanase: 1500 U g^{-1} Subtilisin: 20000 U g^{-1} Alpha-amylase: 2000 U g^{-1}	Xylanase: 1 U is the amount of enzyme which liberates 0.5 µmol of reducing sugar (expressed as xylose equivalents) from a cross-linked oat spelt xylan substrate at pH 5.3 and 50ºC in one minute. Subtilisin: 1 U is the amount of enzyme which liberates 1 µmol of phenolic compound (tyrosine equivalents) from a casein substrate per minute at pH 7.5 and 40ºC. Amylase: 1 U is the amount of enzyme which liberates 1 µmol of glucosidic linkages from a water insoluble cross-linked starch polymer substrate per minute at pH 6.5 and 37ºC.

Trade name (if appropriate)	AVIZYME 1505
Name of the holder of authorisation (if appropriate)	Danisco Animal Nutrition (legal entity Finnfeeds International Limited)

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		Endo-1,4-beta-xylanase: 75 Subtilisin: 1000 Alpha-amylase: 100	Endo-1,4-beta-xylanase: 300 Subtilisin: 4000 Alpha-amylase: 400	
Ducks for fattening		Endo-1,4-beta-xylanase: 75 Subtilisin: 1000	Endo-1,4-beta-xylanase: 300 Subtilisin: 4000	

		Alpha-amylase: 100	Alpha-amylase: 400	

Other provisions and additional requirements for the labelling	
Specific conditions or restrictions for use (if appropriate)	In the directions for use of the additive, indicate the storage temperature, storage life and stability to pelleting. For use in compound feed rich in starch and non-starch polysaccharides (mainly arabynoxylans and beta-glucans), e.g. containing more than 40% maize.
Specific conditions or restrictions for handling (if appropriate)	Harmful. Irritating to respiratory system and skin. Risk of serious damage to the eyes. Ay cause sensitization by inhalation. Do not breathe dust. Avoid contact with skin. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. Wear suitable clothing gloves and eyes/face protection.
Post-market monitoring (if appropriate)	All batches of Avizyme 1505 are shipped in closed containers and the label information includes the name and address of the producer, a batch number and a bar code. All costumers are supplied with copies of the Material Safety Datasheet for the product that includes an emergency contact number. This enables any user to contact the company and provide batch specific information on the use of a product. In addition batch traceability and complaints systems are in place so as to enable rapid investigation and resolution of any negative reports of usage or complaints. The traceability system ensures that any person to whom we have supplied the product can be rapidly identified
Specific conditions for use in complementary feedingstuffs (if appropriate)	Not applicable

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

Avizyme 1505 is a triple enzyme mixture containing endo-1,4-β-xylanase produced by a strain of the genetically modified micro-organism (GMM) *Trichoderma reesei* (ATCC PTA 5588), α-amylase produced by a strain of the GMM *Bacillus amyloliquefaciens* (ATCC 3978) and subtilisin (protease) produced by a strain of the GMM *Bacillus subtilis* (ATCC 2107). The active substances are obtained by submerged fermentation of the above mentioned genetically modified micro-organisms. The additive is intended for use in chickens and ducks for fattening feed rich in starch and non-starch-polysaccharides.

2. Characterisation

2.1. Characterisation of the product

Avizyme 1505 is marketed in dry powder form containing endo-1,4-β-xylanase 1500 U g^{-1}, protease 20000 U g^{-1} and α-amylase 2000 U g^{-1}. The active agents represent approximately 23 % of the additive, the remaining components being wheat flour (approximately 75 %) and calcium propionate (0.2 %).

Avizyme 1505 conforms to the 'General specifications and considerations for enzymes used in food processing' as recommended by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). The product is routinely monitored for heavy metals (Cd, Hg, Pb), arsenic and biological contaminants (total mesophiles, total coliforms, *Salmonella*). The product is monitored for mycotoxins (aflatoxin B1, ochratoxin and zearalenone) and antimicrobial activity. Appropriate maximum levels are set. Compliance with these limits was confirmed in three batches of the product.[5]

Data from three batches showed that approximately 9 % of the particles in the product have a diameter below 100 µm.[6]

2.2. Characterisation of the production organism

2.2.1. Information relating to the genetically modified micro-organism

2.2.1.1. Characteristics of the recipient or parental micro-organism

Avizyme 1505 is a mixture of three enzyme preparations with the following declared activities: endo-1,4-β-xylanase, α-amylase and alkaline protease (subtilisin).

Parent for the endo-1,4-β-xylanase production strain

The recipient strain is *Trichoderma reesei* (teleomorph *Hypocrea jecorina*, Kuhls *et al.*, 1996) RL-P37, a fungus that overproduces cellulase as a result of several classical mutagenesis steps starting from the wild–type strain QM6a. *T. reesei* is a mesophilic deuteromycete that naturally secretes cellulase and xylanase enzymes. The strain QM6a has been used widely in industry for more than 40 years; *T. reesei* RL-P37 has been used for years to produce commercial cellulase preparations on a large scale. *T. reesei* is non-pathogenic and non-toxic based on several

[5] Technical dossier/Section II/Reference B1
[6] Technical dossier/Section II/Reference B3

pathogenicity and toxicological studies (Nevalainen *et al*. 1994). The strain RL-P37 used was found not to produce mycotoxins and to have no antibiotic activity. Sequence analysis of the genome of *T. reesei* has recently been determined (Martinez *et al.*, 2008).

Parent for the α-amylase production strain

Bacillus amyloliquefaciens has been designated QPS status by EFSA (EFSA, 2007). *B. amyloliquefaciens* has been used for many years for the production of extracellular enzymes. The recipient *B. amyloliquefaciens* EBA-1 is an asporogeneous strain obtained as a result of conventional mutation and selection methods from the strain BZ53. No production of enterotoxins or emetic toxins was detected in the parental *B. amyloliquefaciens* strain EBA-1 and in the final production strain as determined by the CHO/MTT cytotoxicity assay.

Parent for the alkaline protease production strain

Bacillus subtilis has been designated QPS status by EFSA (EFSA, 2007). The recipient strain is *B. subtilis* BG125, derived from the *B. subtilis* type strain 168. *B. subtilis* BG125 has been used for large-scale production purposes without any adverse effects on health. For *B. subtilis* strain 2310, also derived from the parental strain 168, an oral administration study in mice did not reveal pathogenicity.

The ability of *B. subtilis* ATCC SD 2107 to produce toxins was already assessed by the FEEDAP Panel (EFSA, 2003). In that opinion it was concluded that this was not a concern.

2.2.1.2. Characteristics of the donor organism

Donor of the gene encoding endo-1,4-β-xylanase

The donor *T. reesei* RL-P37 strain was the same as the recipient strain. The donor strain produces a thermosensitive endo-1,4-β-xylanase enzyme. The corresponding gene was modified to encode a thermotolerant enzyme (Xylanase Y5) that differs from the original one by four amino acids (three changed and one added) before its reintroduction into the donor/recipient strain.

Donor of the gene encoding α-amylase

The gene encoding α-amylase was isolated from the donor strain *B. amyloliquefaciens* BZ53, which is the sporogeneous parent of the asporogeneous recipient strain. It was cloned into plasmid pUB110, initially derived from *Staphylococcus aureus,* carrying the antibiotic resistance genes for neomycin and bleomycin. This plasmid has been well characterised and is widely used as a vector for the production of enzymes.

Donor of the gene encoding alkaline protease

The *apr* gene encoding alkaline protease (subtilisin) was isolated from the donor strain *B. amyloliquefaciens* ATCC 23844. The gene was modified to encode a protein with a change in one amino acid, and placed under the control of expression signals derived from *B. subtilis* gene encoding subtilisin. The chloramphenicol resistance marker gene was derived from *S. aureus*.

2.2.1.3. Description of the genetic modification process

Development of endo-1,4 β-xylanase production strain

The *T. reesei* RL-P37 strain was selected for deficiency in orotidine-5'-monophosphate decarboxylase encoded by the *pyr4* gene. In four subsequent rounds of gene replacement

recombination, the coding sequences of the cellobiohydrolases 1 and 2 (*cbh1* and *cbh2*) and part of the endoglucanase 1 and 2 (*egl1* and *egl2*) coding sequences were deleted.

The Xylanase Y5 coding sequence was cloned into an expression vector. The expression cassette consists of the *cbh1* promoter, the coding sequence of Xylanase Y5, the 5' *cbh1* terminator region, the *pyr4* gene and the 3' *cbh1* terminator region; all originated from *T. reesei* with the exception of some short remnants of multicloning sites of less than 15 bp each. The expression cassette was cloned in an *Agrobacterium tumefaciens* vector between the left and right border sequences and transferred to the recipient *T. reesei* strain by conjugation. By Southern blotting and sequence analysis of the left and right junction sequences it was confirmed that a total of four copies of the expression cassette were integrated. One of these copies was inserted within the *pyr4* region of one of the other copies. Besides some nucleotides of the border sequences, no other vector sequences were inserted.

Development of α-amylase production strain

The α-amylase encoding gene of *B. amyloliquefaciens* BZ53 was cloned into the *mob* gene of the plasmid pUB110 rendering this plasmid incapable of mobilisation by conjugation. The recombinant plasmid including resistance genes for neomycin and bleomycin was transferred to *B. amyloliquefaciens* EBA-1 by protoplast transformation and was stably maintained during production.

Development of alkaline protease production strain

The recipient strain *B. subtilis* BG125 was mutated by recombinant technology at several genes affecting protease production, extracellular enzyme production, sporulation and amino acid auxotrophy.

The alkaline protease encoding gene (*apr*) of *B. amyloliquefaciens* ATCC 23844 was modified by site-directed mutagenesis at four different bases; two were silent mutations and two mutations resulted in one amino acid change. The modified gene was placed under the control of the expression signals of the *B. subtilis* gene encoding subtilisin. The codon that resulted in one amino acid replacement exists also in the wild-type *B. licheniformis* gene encoding subtilisin. The expression cassette was cloned in a non-replicative plasmid together with the chloramphenicol resistance marker gene and introduced in the *B. subtilis* recipient strain by transformation of competent cells. The plasmid sequences were stably integrated in the chromosome in the final production strain by recombination at the *aprE* promoter.

2.2.2. Information relating to the production process

The Xylanase Y5, the α-amylase and the alkaline protease (subtilisin) are produced separately, each by submerged, aerobic and pure culture fermentation of the production strain. The manufacturing process involves three sequential steps: laboratory-scale inoculum preparation, fermentation of the inoculum and main fermentation. Conventional process controls are in place.

2.2.3. Information relating to the product purification process

The Xylanase Y5, the α-amylase and the alkaline protease (subtilisin) are recovered from the fermentation broth by biomass separation involving a series of filtration steps including ultrafiltration. The production process is expected to generate an enzyme product in which the production micro-organism has been removed. The production micro-organisms were not detected in the enzyme preparations, i.e. there was less than 1 CFU g^{-1}. The amount of recombinant DNA was below the limit of detection (5 ng of target sequence mL^{-1}) in the

endo-1,4-β-xylanase, the α-amylase and the alkaline protease preparations when tested with a PCR that amplified DNA fragments of 506 bp, 400 bp and 500 bp, respectively.

2.3. Stability and homogeneity

The enzyme activities of Avizyme 1505 (three batches) were measured during storage at 20 or 35 ºC.[7] Enzyme activity was approximately 100 % after 16 months at 20 ºC (endo-1,4-β-xylanase: 112 %, α-amylase: 102 %, protease: 93 %), and >90 % after 12 months at 35ºC (endo-1,4-β-xylanase: 91 %, α-amylase: 100 %, protease: 91 %).

The stability of Avizyme 1505 in premixtures (three batches each) was studied at 20 and 35 ºC.[8] After six months of storage at 20 ºC the enzyme activities were 106 %, 125 % and 100 % for endo-1,4-β-xylanase, α-amylase and protease, respectively, and after three months at 35 ºC these figures were 86 %, 112 % and 89 %.

Stability of the product in mash complete feed (maize and soybean meal based) was tested when supplemented at a low dose (50 mg kg^{-1}) and a high dose (200 mg kg^{-1}) in separate tests.[9] Feed was kept for up to six months at two temperatures 20 ºC and 35 ºC. When supplemented at a low dose the enzyme recoveries after three months were higher than 100 % when kept at both temperatures. After six months enzyme recoveries were 78 %, 144 % and 121 % for endo-1,4-β-xylanase, α-amylase and protease respectively, when kept at 20 ºC, and 56 %, 92 % and 98 % for endo-1,4-β-xylanase, α-amylase and protease when kept at 30 ºC. When supplemented at a high dose the enzyme recoveries after three months were 70 %, 80 % and 95 %, when kept at 20 ºC and 96 %, 79 % and 92 % when kept at 35 ºC for endo-1,4-β-xylanase, α-amylase and protease respectively. After six months enzymes recoveries were 96 % 79 % and 92 % when kept at 20 ºC and 84 %, 84 % and 88 % when kept at 35 ºC for endo-1,4-β-xylanase, α-amylase and protease respectively.

Avizyme 1505 retained 98 %, 74 % and 97 % for endo-1,4-β-xylanase, α-amylase and protease activities, respectively during pelleting at 80-85 ºC.[10]

Homogeneity of Avizyme 1505 in vitamin/mineral premixtures (containing trace elements) was tested and showed an average coefficient of variation below 10 %.[11] Avizyme 1505 showed uniform distribution in the feed (mash and pellets), with an average coefficient of variation of 14 %.[12]

2.4. Conditions of use

The minimum dose for inclusion in complete feed for chickens for fattening and ducks for fattening are 75 U endo-1,4-β-xylanase kg^{-1}, 100 U α-amylase kg^{-1} and 1000 U protease kg^{-1} , and the maximum levels as proposed by the applicant for both categories are 300 U endo-1,4-β-xylanase kg^{-1} , 400 U α-amylase kg^{-1} and 4000 U protease kg^{-1}, delivered by 50–200 mg of the additive kg^{-1} complete feed.

[7] Technical dossier/Section II/Reference B9
[8] Technical dossier/Section II/Reference B20
[9] Technical dossier/Section II/Reference B17 and B18
[10] Technical dossier/Section II/Reference B19
[11] Technical dossier/Section II/Reference B20
[12] Technical dossier/Section II/Reference B17

2.5. 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 substance in animal feeds. The Executive Summary of the CRL report can be found in the Appendix.

The FEEDAP Panel notes that the methods of analysis for the enzymes present in Avizyme 1505 are not considered by the CRL as fit for the purpose of official control methods. The FEEDAP Panel recommends that more sensitive methods should be developed.

3. Safety

3.1. The safety aspects of the genetic modification

3.1.1. Information relating to the GMM and comparison of the GMM with its conventional counterpart

a) Description of the genetic trait(s) or phenotypic characteristics and any new trait which can be expressed or no longer expressed

Endo-1,4-β-xylanase

The recipient strain *T. reesei* RL-P37 contains a deficient orotidine-5'-monophosphate decarboxylase (*pyr4*) gene and deletions in the cellobiohydrolases 1 and 2 (*cbh1* and *cbh2*) genes and of the endoglucanase 1 and 2 (*egl1* and *egl2*) genes, rendering the strain incapable of using cellulose as carbon source.

The production strain *T. reesei* ATCC PTA 5588 expresses the gene encoding thermostable Xylanase Y5 from *T. reesei.* It contains four copies of the xylanase expression cassette integrated into the chromosome.

α-amylase

The production strain *B. amyloliquefaciens* ATCC SD-3978 contains a pUB110 derived plasmid with resistance genes for neomycin and bleomycin from *Staphylococcus aureus* and the α-amylase gene, originated from *B. amyloliquefaciens*, inserted in the *mob* gene which it renders inactive.

Alkaline protease

The production strain *B. subtilis* ATCC SD-2107 contains mutations in several genes affecting protease production, extracellular enzyme production, sporulation and amino acid auxotrophy. The alkaline protease gene of *B. amyloliquefaciens*, modified at four bases, and the chloramphenicol resistance marker gene were stably inserted at the *aprE* promoter site of the recipient strain.

b) Structure and amount of any vector and/or donor nucleic acid remaining in the final construction of the modified micro-organism

T. reesei ATCC PTA 5588

Apart from short cloning remnants and *Agrobacterium tumefaciens* border nucleotides, all inserted DNA was derived from the *T. reesei* recipient strain, as was confirmed by Southern analysis and by sequence determination of the junction DNA.

B. amyloliquefaciens ATCC SD-3978

The production strain contains a plasmid derived from pUB110, originally isolated from *S. aureus*, with resistance genes for neomycin and bleomycin. The α-amylase gene originated from the *B. amyloliquefaciens* donor strain and is inserted in the *mob* gene of this plasmid, rendering it incapable of mobilisation by conjugation.

B. subtilis ATCC SD-2107

An alkaline protease encoding gene of *B. amyloliquefaciens*, modified at four bases, and a chloramphenicol resistance marker, originally derived from an *S. aureus* strain, were stably inserted at the *aprE* promoter site of the recipient strain.

3.1.2. Conclusion regarding the genetic modification

Endo-1,4-β-xylanase producing strain *T. reesei* ATCC PTA 5588

The strain contains, apart from short cloning remnants and *Agrobacterium tumefaciens* border nucleotides, only sequences derived from the *T. reesei* host strain, which raise no safety concerns.

α-amylase producing strain *B. amyloliquefaciens* ATCC SD-3978

The strain contains a plasmid derived from pUB110, originally isolated from *S. aureus*, with resistance genes for neomycin and bleomycin. The plasmid sequences are fully characterised and widely used in other strains for the production of industrially important enzymes. In this plasmid the α-amylase gene, originated from the *B. amyloliquefaciens* donor strain is inserted in the *mob* gene rendering the plasmid incapable of mobilisation by conjugation. However, there are other DNA transfer mechanisms and thus the presence of genes encoding antibiotic resistance is a potential risk (addressed in section 3.5).

Alkaline protease producing strain *B. subtilis* ATCC SD-2107

The strain was mutated by recombinant technology at several genes affecting protease production, extracellular enzyme production, sporulation and amino acid auxotrophy. As a result several deletions and point mutations were introduced, causing no particular safety concern. An alkaline protease gene of *B. amyloliquefaciens*, modified at four bases, and a chloramphenicol resistance marker, originally derived from an *S. aureus* strain, were stably inserted at the *aprE* promoter site on the chromosome of the recipient strain. The presence of a gene encoding antibiotic resistance is a potential risk (addressed in section 3.5).

3.2. Safety for the target species

3.2.1. Tolerance for chickens for fattening

A total of 960 one-day-old male Ross PM3 chickens for fattening were distributed into 24 pens with 40 birds per pen and distributed into one of the three experimental diets (eight replicates per treatment).[13] The basal diets (starter and grower) based on maize and soybean meal were supplemented at 0, 200 (1X) or 3000 (15X) mg kg^{-1} (enzyme activities confirmed by analyses). The pelleted diets were offered *ad libitum* for 35 days.

During the experimental period (1-36 days of age), mortality, feed intake and body weight of the birds were monitored and feed to gain ratio calculated. At the end of the study, blood

[13] Supplementary information/ Appendix 1

samples from 10 animals per treatment were collected and were analysed for haematology (total erythrocyte count, total leukocyte count, haemoglobin concentration, mean cell volume, platelet count and differential leukocyte count), and biochemical parameters (fibrinogen, glucose, urea, total protein, albumin, ALKP, ALAT, LDH, ASAT, GGT, albumin/globulin ration, creatinine, calcium, phosphorus, chloride, total bilirubin, sodium, potassium).

Mortality was generally low and not affected by the treatments (1.5 %, 1.3 % and 2.8 %). Feed intake (3469, 3577, 3657 g animal^{-1}), final body weight (2344, 2328, 2392 g animal^{-1}) and feed to gain ratio (1.52, 1.57 and 1.57 g g^{-1}) were not modified by the treatments. Of all the blood parameters measured, only mean cell volume and phosphorus content were modified by the experimental treatments. Mean cell volume was lower in the supplemented groups compared to the control (116 μm^3 vs 120 μm^3) and the phosphorus concentration was highest in the 15X group compared to the other groups (1.9 mmol L^{-1} vs 2.3 mmol L^{-1}).

3.2.2. Conclusions on safety for the target species

The tolerance study provided for chickens for fattening showed no negative effects of the supplementation with 15-fold overdose of Avizyme 1505. The safety of the product for ducks for fattening is assumed from the results obtained in the tolerance study in chickens for fattening.

The FEEDAP Panel concludes that Avizyme 1505 is safe under the conditions of use for chickens and for ducks for fattening.

3.3. Safety for the consumer

Consumer safety assessment studies were performed both *in vitro* (mutagenicity/clastogenicity studies) and *in vivo* (90-day repeated dose oral toxicity study in rats). The studies were conducted with each enzyme component separately; a liquid enzyme preparation identified as Y5-Xylanase, protease concentrate and amylase identified as GBR 50016.

3.3.1. Genotoxicity studies including mutagenicity

Genotoxicity was tested using a bacterial reverse mutation assay and a chromosome aberration assay in cultured human lymphocytes separately for each of the three components of Avizyme 1505.

3.3.1.1. Bacterial reverse mutation assay – endo-1,4-β-xylanase

The enzyme preparation containing xylanase was tested[14] at doses of up to 5000 μg plate^{-1} according to OECD guideline 471, in four strains of *Salmonella typhimurium* (TA98, TA100, TA1535 and TA1537) and in *Escherichia coli* WP2 *uvrA* pKM101, both with and without microsomal enzyme activation. Results of the studies showed no evidence of mutagenic effect either with or without activation.

3.3.1.2. Bacterial reverse mutation assay – protease

The enzyme preparation containing subtilisin and the formulation ingredients MP- STAB were tested[15] at doses of up to 5000 μg plate^{-1} in four strains of *Salmonella typhimurium* (TA98,

[14] Technical dossier/Section IV/Reference D3
[15] Technical dossier/Section IV/Reference D5

TA100, TA1535 and TA1537) and in *Escherichia coli* WP2 *uvrA*, both with and without microsomal enzyme activation. Results of the studies showed no evidence of mutagenic effect of the product or its ingredients either with or without activation.

3.3.1.3. Bacterial reverse mutation assay – α-amylase

The enzyme preparation containing α-amylase and the formulation ingredients MP- STAB were tested[16] at concentrations up to 25 μg mL^{-1} according to a protocol based on OECD guideline 471, in four strains of *Salmonella typhimurium* (TA98, TA100, TA1535 and TA1537), both with and without microsomal enzyme activation. Results of the studies showed no evidence of any mutagenic effect of the product either with or without activation.

3.3.1.4. Chromosome aberration assay – endo-1,4-β-xylanase

The enzyme preparation containing xylanase was tested[17] for chromosome aberrations in cultured human peripheral lymphocytes according to a protocol based on OECD guideline 473, with and without microsomal enzyme activation. Exposure to concentrations up to the equivalent of approximately 50000 μg bulk enzyme preparation mL^{-1} lasted for 4-5, 24 or 48 hours and cells were harvested at 24 or 48 hours. While there was a clear response in positive controls, there was no evidence for genotoxic effects in lymphocytes exposed to the test article with or without metabolic activation.

3.3.1.5. Chromosome aberration assay – protease

The enzyme preparation containing subtilisin was tested[18] for chromosome aberrations in cultured human peripheral lymphocytes, both with and without microsomal enzyme activation. Exposure to concentrations up to 20 μL mL^{-1} lasted for 19 or 43 hours and cells were harvested at 24 or 48 hours. While there was a clear response in positive controls, there was no evidence for chromosome aberrations in lymphocytes exposed to the test article with or without metabolic activation.

3.3.1.6. Chromosome aberration assay – α-amylase

The enzyme preparation containing α-amylase was tested[19] for chromosome aberrations in cultured human peripheral lymphocytes in compliance with GLP and according to a protocol based on OECD guideline 473, with and without microsomal enzyme activation. Exposure to concentrations up to 5000 μg mL^{-1} lasted for 21 or 42 hours and cells were harvested at 24 or 48 hours. While there was a clear response in positive controls, there was no evidence for genotoxic effects in lymphocytes exposed to the test article with or without metabolic activation.

3.3.2. Oral toxicity studies

Three 90-day oral toxicity studies were conducted in rats with the three enzyme components of Avizyme 1505.

[16] Technical dossier/Section IV/Reference D7
[17] Technical dossier/Section IV/Reference D4
[18] Technical dossier/Section IV/Reference D6
[19] Technical dossier/Section IV/Reference D8

3.3.2.1. 90-day study – endo-1,4-β-xylanase

Groups of ten Sprague-Dawley rats of each sex caged singly were administered xylanase by gavage at 0, 30000, 75000 or 225000 U kg body weight^{-1} day^{-1} for at least 90 consecutive days.[20] The study was conducted in accordance with OECD guideline 408. Clinical condition, body weight and feed intake were monitored throughout the study. Opthalmoscopy was carried out at the start on all animals and on controls and high dose only at the end of the study. Behavioural and functional tests were conducted at the end of the study. Clinical chemistry and haematological examination was made on blood collected at the end of the study. Urine was also collected and examined at the end of the study. All animals were subject to a necropsy examination at which the macroscopic appearance of tissues and the weight of a range of organs were recorded. A comprehensive range of tissues was preserved for histopathological examination. Tissues from all control and high dose animals were processed and examined microscopically.

One low dose male died on day 88 with a renal mass diagnosed as nephroblastoma which was not related to treatment. There were no treatment-related adverse effects in any of the end-points measured.

3.3.2.2. 90-day study – protease

Groups of 20 Sprague-Dawley rats of each sex caged in groups of five were fed diets containing 0, 5000, 15000 or 50000 mg kg^{-1} subtilisin for at least 90 consecutive days.[21] A further group of 20 rats of each sex received the vehicle (Propylene Glycol: Water, 40:60). The study was conducted in compliance with GLP. Clinical condition, body weight and feed intake were monitored throughout the study. Opthalmoscopy was carried out at the start and end of the study. Clinical chemistry and haematological examination was made on blood collected from ten rats of each sex from each group at 30 days and at the end of the study. Urine was also collected and examined from 10 rats of each sex from each group at the end of the study. All animals were subjected to a necropsy examination at which the macroscopic appearance of tissues and the weight of a range of organs were recorded. A comprehensive range of tissues was preserved for histopathological examination. Tissues from all control and high dose animals were processed and examined microscopically, in addition the salivary glands of the remaining groups were examined.

At the highest dose group effects were seen on body weight and in the highest dose group and these were related to the palatability of the test diets thus was also reflected in reduced feed intake during the first weeks of the study.

One control male was found dead on day 14 but the cause of death could not be determined. Enlargement of salivary glands was noted for the highest two doses at necropsy. The histological changes in the submandibular salivary glands were described as cell hypertrophy. These changes are considered in the study report to be more likely to be secondary effects due to the unpalatability of the diet at these concentrations rather than a direct toxic effect. Such changes have been previously reported as a physiological response to other dietary proteases in rats and are therefore considered of no relevance to consumers of food products derived from animals fed diets containing the enzyme.

[20] Technical dossier/Section IV/Reference D9
[21] Technical dossier/Section IV/Reference D10

3.3.2.3. 90-day study – α-amylase

Groups of ten rats of each sex caged in groups of five were fed diets containing 0, 1000, 5000 or 15000 mg kg^{-1} of α-amylase for at least 90 consecutive days.[22] The study was conducted in accordance with OECD guideline 408. Clinical condition, body weight and food intake were monitored throughout the study. Opthalmoscopy was carried out at the start and end of the study. Clinical chemistry and haematological examination was made on blood collected at the end of the study. All animals were subjected to a necropsy examination at which the macroscopic appearance of tissues and the weight of a range of organs were recorded. A comprehensive range of tissues was preserved for histopathological examination. Major organs from all animals were processed and examined microscopically.

At the highest dose effects were seen on body weight and in the submandibular salivary glands which were described as cell hypertrophy and degeneration of granular convoluted ducts. A few animals from the intermediate dose group showed milder but similar effects. These changes were probably a result of treatment but are considered likely to be specific to the rat as a response to dietary composition, and therefore not considered of relevance to consumers of food products derived from animals fed diets containing the enzyme.

The body weight and food intake measurements at the highest dose show a pattern typically associated with unpalatability.

3.3.3. Conclusions on safety for the consumer

It is concluded that the studies conducted on the individual components of Avizyme 1505 are adequate investigation of the safety of this product and on the basis of the results of both genotoxicity tests and 90-day studies it is not expected that there would be any risk to the consumer from use of Avizyme 1505 in animal feed.

3.4. Safety for the user

3.4.1. Dermal Irritation

The complete product Avizyme 1505 was tested for dermal irritancy in a study with three rabbits conducted according to OECD guideline 404.[23] A sample of 0.5 g of the product was moistened with 0.5 mL of water and applied to the skin. Based on very slight erythema which persisted for 48 h in two animals the substance is classified as a mild irritant to rabbit skin.

3.4.2. Eye Irritation

The complete product Avizyme 1505 was tested for dermal irritancy in a study with three rabbits conducted in compliance with GLP and according to OECD guideline 405.[24] A sample of 0.1 mL of the dry product (approximately 70 mg) was introduced in to the conjunctival sac. Based conjunctival and iridial effects which persisted for 48 h the substance is classified as a mild irritant to the rabbit eye.

[22] Technical dossier/Section IV/Reference D11
[23] Technical dossier/Section IV/Reference D12
[24] Technical dossier/Section IV/Reference D13

3.4.3. Skin sensitisation

The complete product Avizyme 1505 was tested for skin sensitisation Local Lymph Node Assay (LLNA) conducted according to OECD guideline 429.[25] Under the conditions of the test the product is considered to be a non-sensitiser.

3.4.4. Acute Inhalation

Three acute inhalation studies have been conducted, one on each component of Avizyme 1505.

3.4.4.1. Acute inhalation - endo-1,4-β-xylanase

A group of ten Sprague-Dawley rats were exposed to an atmosphere containing 4.96 mg xylanase L^{-1} for four hours.[26] The study was conducted according to OECD guideline 403. No deaths or abnormalities were observed during or following exposure.

3.4.4.2. Acute inhalation - protease

Groups of five Sprague-Dawley rats of each sex were exposed to atmospheres containing 2.0, 0.17, 0.83, 1.5 or 1.8 mg subtilisin L^{-1} for four hours.[27] All animals exposed to 2.0 mg subtilisin L^{-1} died either during the exposure or soon afterwards. Some deaths occurred in all groups apart from that exposed to 0.17 mg subtilisin L^{-1}. Generally animals that died showed signs of haemorrhage and congestion in lungs and nasal tissues.

3.4.4.3. Acute inhalation - α-amylase

Groups of five rats of each sex were exposed to an atmosphere containing 0 or 3.9 mg alpha-amylase L^{-1} for four hours. The study was conducted according to OECD guideline 403.[28] During exposure and for several days afterwards there was some evidence of stress in the group exposed to alpha-amylase, indicated by rapid respiration, piloerection, lack of grooming and weight loss, but no deaths occurred.

3.4.5. Dusting potential

Since approximately up to 10 % of Avizyme 1505 particles are inhalable and the acute inhalation effects of the protease component have been identified, appropriate warnings and precautions are required.

3.4.6. Conclusion on safety for the user

Risk prevention measures are available in the Avizyme 1505 Material Safety Data Sheets, including safety recommendations relating to avoidance of inhalation exposure due to the possibility that the product may cause sensitisation by inhalation. It is concluded that there is no concern for the safety of users provided that the MSDS recommendations are followed and appropriate measures are taken to avoid dermal and inhalation exposure.

[25] Technical dossier/Section IV/Reference D14
[26] Technical dossier/Section IV/Reference D15
[27] Technical dossier/Section IV/Reference D16
[28] Technical dossier/Section IV/Reference D17

3.5. Safety for the environment

The production micro-organisms are removed from the products prior to mixing and were not detected in the enzyme preparations. The amount of recombinant DNA was below the limit of detection (5 ng of target sequence mL^{-1}) in the endo-1,4-β-xylanase, the α-amylase and the alkaline protease preparations when tested with a PCR that amplified DNA fragments of 506 bp, 400 bp and 500 bp, respectively. With respect to the genetic modification, there are no environmental safety concerns in relation to the final product because of the absence of viable production microorganisms and recombinant DNA in the final product.

The active components of Avizyme 1505 are proteins and largely degraded in the upper digestive tract of animals and the environment and no further environmental risk assessment is required.

4. Efficacy

4.1. Efficacy for chickens for fattening

Four efficacy trials with male chickens for fattening have been carried out over the 42-day growing period in three different locations.

Trial 1

A total of 480 one-day-old Ross chickens for fattening were randomly distributed into four treatments with 15 replicates of eight birds per treatment.[29] The maize-based diet was offered *ad libitum* and supplemented with an enzyme preparation providing doses equivalent to 0, 100, 125 and 200 mg Avizyme 1505 kg^{-1} complete feed. The enzyme activity in feed was confirmed by analysis, except subtilisin which was calculated. Feed intake and body weight gain were measured and feed to gain ratio calculated. Data were analysed using the GLM procedure using the pen as the experimental unit.

All animals were in good health during the trial and the overall mortality was 1.2 % and not affected by treatment. The performance data are presented in Table 2.

Table 2. **Effect of Avizyme 1505 on performance of chickens for fattening (42 days)**

Avizyme 1505 equivalents (mg kg^{-1})	Xylanase/amylase/ protease (U kg^{-1} feed)	Feed intake (g $bird^{-1}$)	Body weight gain (g)	Feed/gain (kg kg^{-1})
0	0/0/0	4795[a]	2661	1.80[a]
100	150/200/2000	4769[a]	2679	1.78[a]
125	187.5/250/2500	4747[a]	2694	1.76[ab]
200	300/400/4000	4570[b]	2670	1.71[b]

[a, b]: Means in a column with different superscripts are statistically different (P<0.05)

Supplementation with Avizyme 1505 at any level did not affect body weight gain. However, supplementation with 200 mg kg^{-1} significantly reduced feed intake and consequently improved the feed to gain ratio.

Trial 2

A total of 1536 one-day-old Ross chickens for fattening were randomly distributed into four treatments with 12 replicates of 32 birds per treatment.[30] The maize-based diet was offered *ad*

[29] Technical dossier/Section III/Reference C1
[30] Technical dossier/Section III/Reference C2

libitum, and supplemented with an enzyme preparation providing doses equivalent to 0, 50, 100 and 200 mg Avizyme 1505 kg^{-1} complete feed. The enzyme activities in feed were confirmed by analyses within the limitations of the methods used. Feed intake, body weight and feed to gain ratio were measured and calculated. Data were analysed using the GLM procedure using the pen as the experimental unit.

All animals were in good health during the trial and the overall mortality was 4.7 % and not affected by treatment. The performance data are presented in Table 3.

Table 3. **Effect of Avizyme 1505 on performance of chickens for fattening (42 days)**

Avizyme 1505 equivalents (mg kg^{-1})	Xylanase/amylase/protease (U kg^{-1} feed)	Feed intake (g $bird^{-1}$ day^{-1})	Body weight (g)	Feed/gain (kg kg^{-1})
0	0/0/0	111.3^{b}	2699^{c}	1.79^{a}
50	75/100/1000	111.3^{b}	2745^{bc}	1.74^{b}
100	150/200/2000	118.3^{a}	2858^{a}	1.80^{a}
200	300/400/4000	117.0^{a}	2796^{ab}	1.80^{a}

[a, b, c]: Means in a column with different superscripts are statistically different (P<0.05)

Feed to gain ratio was improved by the supplementation with Avizyme 1505 at 50 mg kg^{-1}. Higher doses of Avizyme 1505 increased both final body weight and feed intake, without affecting feed conversion, in comparison to the control.

Trial 3

A total of 480 one-day-old Ross chickens for fattening were randomly distributed into four treatments with 15 replicates of eight birds per treatment.[31] The maize-based diet was offered *ad libitum* and supplemented with an enzyme preparation providing doses equivalent to 0, 100, 125 and 200 mg Avizyme 1505 kg^{-1} complete feed. The enzyme activities in feed were confirmed by analyses within the limitations of the methods used. Feed intake, body weight gain and feed conversion ratio were measured and calculated. Data were analysed using the GLM procedure using the pen as the experimental unit.

All animals were in good health during the trial and the overall mortality was very low (0.6 %) and not affected by treatment. The performance data are presented in Table 4.

Supplementation with Avizyme 1505 at 125 mg kg^{-1} or higher, significantly increased body weight gain and improved the feed to gain ratio, without affecting feed intake.

Table 4. **Effect of Avizyme 1505 on performance of chickens for fattening (42 days)**

Avizyme 1505 equivalents (mg kg^{-1})	Xylanase/amylase/ protease (U kg^{-1} feed)	Feed intake (g $bird^{-1}$)	Body weight gain (g)	Feed/gain (kg kg^{-1})
0	0/0/0	4343	2186^{b}	1.99^{a}
100	150/200/2000	4394	2275^{ab}	1.93^{ab}
125	187.5/250/2500	4302	2281^{a}	1.89^{bc}
200	300/400/4000	4323	2323^{a}	1.86^{c}

[a, b, c]: Means in a column with different superscripts are statistically different (P<0.05)

[31] Technical dossier/Section III/Reference C3

Trial 4

A total of 400 day-old Ross chickens for fattening were randomly distributed into two treatments with eight replicates of 25 birds per treatment.[32] The maize-based diet was offered *ad libitum* and supplemented with an enzyme preparation at doses equivalent to 0 and 100 mg Avizyme 1505 kg^{-1} complete feed. The enzyme activities in feed were confirmed by analyses within the limitations of the methods used. Feed intake, body weight gain and feed to gain ratio were measured and calculated. Data were analysed using the GLM procedure using the pen as the experimental unit.

All animals were in good health during the trial and the overall mortality was low (0 and 1.5 % in control and Avizyme 1505 groups). The performance data are presented in Table 5.

Table 5. **Effect of Avizyme 1505 on performance of chickens for fattening (42 days)**

Avizyme 1505 equivalents (mg kg^{-1})	Xylanase/amylase/ protease (U kg^{-1} feed)	Feed intake (g $bird^{-1}$)	Body weight gain (g)	Feed/gain (kg kg^{-1})
0	0/0/0	3421[b]	1995[b]	1.74
100	150/200/2000	3608[a]	2096[a]	1.73

[a, b, c]: Means in a column with different superscripts are statistically different (P<0.05)

Supplementation with Avizyme 1505 at 100 mg kg^{-1} significantly increased feed intake and body weight gain without affecting feed to gain ratio.

4.2. Efficacy for ducks for fattening

Three efficacy trials with ducks for fattening have been carried out over the 42-day growing period in two different locations.

Trial 1

A total of 574 one-day-old SM3 ducks were randomly divided into seven treatments with ten replicates of 8/9 birds per treatment.[33] The maize-rye-based diet was offered *ad libitum* and Avizyme 1505 was added to starter and to finisher diets at 0, 50, 150, 200, 250 and 300 mg kg^{-1}. The enzyme activities in feed were confirmed by analyses within the limitations of the methods used. Average daily feed intake, final body weight and feed to gain ratio were measured and calculated. Data were analysed using the GLM procedure using the pen as the experimental unit.

All animals were in good health during the trial and the overall mortality was approximately 7 % and not affected by treatment. The performance data are presented in Table 6.

Supplementation of the diet with Avizyme 1505 significantly increased feed intake at the dose levels of 50 and 300 mg kg^{-1}, and final body weight at all dose levels, except at 150 mg kg^{-1} without affecting feed to gain ratio.

[32] Supplementary information
[33] Technical dossier/Section III/Reference C5

Table 6. **Effect of Avizyme 1505 on performance of ducks for fattening (42 days)**

Avizyme 1505 (mg kg^{-1})	Xylanase/amylase/ protease (U kg^{-1} feed)	Feed intake (g $bird^{-1}$ day^{-1})	Final body weight (g)	Feed/gain (kg kg^{-1})
0	0/0/0	130.8[a]	2777[a]	2.03
50	75/100/1000	137.5[b]	2887[b]	2.08
100	150/200/2000	134.4[ab]	2884[b]	2.00
150	225/300/3000	131.4[a]	2836[ab]	2.04
200	300/400/4000	136.1[ab]	2928[b]	2.00
250	375/500/5000	136.1[ab]	2908[b]	2.03
300	450/600/6000	139.4[b]	2976[b]	2.03

[a, b, c, d]: Means in a column with different superscripts are statistically different (P<0.05)

Trial 2

A total of 240 one-day-old male Pekin ducks were randomly divided into two treatments with ten replicates of 12 birds per treatment.[34] The maize-wheat-based diet was offered *ad libitum*, and Avizyme 1505 was added to starter and finisher diets at 0 or 200 mg kg^{-1}. The enzyme activities in feed were confirmed by analyses within the limitations of the methods used. Feed intake, body weight gain and feed conversion ratio were measured and calculated. Data were analysed using the GLM procedure using the pen as the experimental unit.

All animals were in good health during the trial and the overall mortality was low (2.5%) and not affected by treatment. The performance data are presented in Table 7.

Table 7. **Effect of Avizyme 1505 on performance of ducks for fattening (42 days)**

Avizyme 1505 (mg kg^{-1})	Xylanase/amylase/protease (U kg^{-1} feed)	Feed intake (g $bird^{-1}$)	Body weight gain (g)	Feed/gain (kg kg^{-1})
0	0/0/0	7236	3655[b]	1.98
200	300/400/4000	7360	3776[a]	1.95

[a, b]: Means in a column with different superscripts are statistically different (P<0.05)

Supplementation with 200 mg kg^{-1} Avizyme 1505 significantly increased body weight gain, without affecting feed intake or feed to gain ratio.

Trial 3

A total of 240 one-day-old male Pekin ducks were randomly divided into two treatments with ten replicates of 12 birds per treatment.[35] The maize-rye-based diet was fed offered *ad libitum*, and Avizyme 1505 was added to starter and finisher diets at 0 or 200 mg kg^{-1}. The enzyme activities in feed were confirmed by analyses within the limitations of the methods used. Feed intake, body weight gain and feed conversion ratio were measured and calculated. Data were analysed using the GLM procedure using the pen as an experimental unit.

All animals were in good health during the trial and the overall mortality was very low and not affected by treatment. The performance data are presented in Table 8.

Table 8. **Effect of Avizyme 1505 on performance of ducks for fattening (42 days)**

Avizyme 1505 (mg kg^{-1})	Xylanase/amylase/protease (U kg^{-1} feed)	Feed intake (g $bird^{-1}$)	Body weight gain (g)	Feed/gain (kg kg^{-1})
0	0/0/0	7266	3632[b]	2.00
200	300/400/4000	7301	3753[a]	1.95

[a, b]: Means in a column with different superscripts are statistically different (P<0.05)

[34] Technical dossier/Section III/Reference C6
[35] Technical dossier/Section III/Reference C7

Supplementation with 200 mg kg^{-1} Avizyme 1505 significantly increased body weight gain without affecting feed intake or feed conversion ratio.

4.3. Conclusions on efficacy

In all four trials presented with chickens for fattening, significant effects were observed in animal performance. Feed to gain was improved at 50 mg kg^{-1} in one trial (Trial 2) and at 125 mg kg^{-1} in another trial (Trial 3) while body weight gain was improved with supplementation at 100 mg kg^{-1} in another trial (Trial 4). Therefore it is concluded that efficacy of Avizyme 1505 in chickens for fattening is demonstrated at a dose of 125 mg kg^{-1}(equivalent to 187.5 U endo-1,4 β-xylanase kg^{-1}, 250 U α-amylase kg^{-1} and 2500 U subtilisin kg^{-1}). Insufficient evidence has been provided to support the efficacy at the lower doses.

In all three trials with ducks for fattening significant effects in animal performance were observed. Two of the studies (Trial 2 and 3) were performed at the single dose of 200 mg kg^{-1}. In a dose titration study, final body weight was significantly improved with the supplementation with 50 mg kg^{-1} (Trial 1). Taking into consideration the dose-titration study, it is concluded that there is evidence of the potential efficacy of Avizyme 1505 in ducks at a dose of 50 mg kg^{-1} (equivalent to 75 U endo-1,4 β-xylanase kg^{-1}, 100 U α-amylase kg^{-1} and 1000 U subtilisin kg^{-1}).

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[36] and Good Manufacturing Practice.

CONCLUSIONS

Based on the tolerance study provided, it is concluded that Avizyme 1505 is safe for chickens and ducks for fattening at the recommended dose range.

The studies conducted on the individual components of Avizyme 1505 are considered an adequate investigation of the safety of this product for the consumer. On the basis of the results of both genotoxicity tests and 90-day studies, it is not expected that there would be any risk to the consumer from the use of Avizyme 1505 in animal feed.

There are no concerns for the safety of users provided that the MSDS recommendations are followed and appropriate measures are taken to avoid dermal and inhalation exposure.

The active components of Avizyme 1505 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.

The efficacy of Avizyme 1505 has been demonstrated in chickens for fattening at the dose of 125 mg kg^{-1} complete feed (equivalent to 187.5 U endo-1,4-β-xylanase kg^{-1}, 250 U α-amylase kg^{-1} and 2500 U protease kg^{-1}). Efficacy of Avizyme 1505 in ducks for fattening as minor species has been demonstrated at a dose of 50 mg kg^{-1} (equivalent to 75 U endo-1,4-β-xylanase kg^{-1}, 100 U α-amylase kg^{-1} and 1000 U subtilisin kg^{-1}).

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

DOCUMENTATION PROVIDED TO EFSA

1. Dossier on Avizyme 1505. Multiple species: for chickens and ducks for fattening. December 2006. Submitted by Danisco Animal Nutrition.
2. Dossier on Avizyme 1505. Multiple species: for chickens and ducks for fattening. Reply to issues raised in letter from EFSA dated 17 July 2007. May 2008. Submitted by Danisco Animal Nutrition.
3. Dossier on Avizyme 1505. Multiple species: for chickens and ducks for fattening. Reply to issues raised in letter from EFSA dated 13 June 2008. June 2008. Submitted by Danisco Animal Nutrition.
4. Dossier on Avizyme 1505. Multiple species: for chickens and ducks for fattening. Reply to issues raised in letter from EFSA dated 4 July 2008. September 2008. Submitted by Danisco Animal Nutrition.
5. Dossier on Avizyme 1505. Multiple species: for chickens and ducks for fattening. Reply to issues raised in letter from EFSA dated 27 October 2008. June 2009. Submitted by Danisco Animal Nutrition.
6. Evaluation report of the Community Reference Laboratory feed additives authorisation on the methods(s) of analysis for Avizyme 1505 for chickens and ducks for fattening.
7. Comments from Member States received through the ScienceNet.

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APPENDIX

Executive Summary of the Evaluation Report of the Community Reference Laboratory Feed Additives Authorisation on the Method(s) of Analysis for Avizyme 1505 for chickens and ducks for fattening.

In the current application authorisation is sought for *Avizyme 1505* 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 *Avizyme 1505* as a digestibility enhancer for chickens for fattening and ducks for fattening. The product is intended to be marketed as a granular powder formulation.

The active agents of Avizyme 1505 are 1) endo-1,4-β-xylanase, produced by a strain of *Trichoderma reesei* (ATCC PTA 5588), 2) α-amylase, produced by a strain of *Bacillus amyloliquefaciens* (ATCC 3978) and 3) subtilisin, produced by a strain of *Bacillus subtilis* (ATCC 2107). Enzymatic activity of the active agents is expressed in units (U):

- One U of endo-1,4-β-xylanase is the amount of enzyme that liberates 0.5 µmol of reducing sugar (xylose equivalents) per minute from a cross-linked oat spelt xylan at pH 5.3 and 50°C;
- One U of α-amylase is the amount of enzyme that liberates 1 µmol of glucosidic linkages per minute from a water insoluble cross-linked starch polymer substrate at pH 6.5 and 37°C;
- One U of subtilisin is the amount of enzyme that liberates 1 µmol of phenolic compound (tyrosine equivalents) per minute from a casein substrate at pH 7.5 and 40°C.

The product has a target activity of 1500 U endo-1,4-β-xylanase g^{-1}, 2000 U α-amylase g^{-1} and 20000 U subtilisin g^{-1}. *Avizyme 1505* is intended to be mixed into *premixtures* and/or *feedingstuffs* to obtain an enzyme activity level of 75 to 300 U endo-1,4-β-xylanase kg^{-1}, 100 to 400 U α-amylase kg^{-1} and 1000 to 4000 U subtilisin kg^{-1} in *feedingstuffs.*

In general, the methods proposed for the determination of the activity of the active agents in different matrices are based on quantification of dyed compounds produced by enzymatic action of commercially available substrates. Enzymatic activity of the samples is calculated using reference enzyme standards, available from the applicant upon request, of which the activity is obtained applying the conditions described by the definitions of units. When analysing *feedingstuffs,* calibration is performed on standards prepared from identical blank feed samples fortified with exact amounts of the reference enzymes. In the case that identical blank feed samples are *not* available, a standard addition technique is employed. The applicant introduced some adaptations to the protocols provided by the suppliers of substrates. All modified methods have been single-laboratory validated and showed acceptable performance characteristics such as limit of detection, limit of quantification and relative standard deviation for repeatability.

For the determination of the activity of endo-1,4-β-xylanase in the *feed additive*, *premixtures* and *feedingstuffs*, the applicant proposes a method based on the quantification (λ = 590 nm) of water soluble dyed fragments produced by the action of endo-1,4-β-xylanase on cross-linked wheat xylan substrates. Enzymatic activity is calculated using a reference enzyme standard, of which the activity is measured at pH 5.3 and 50°C on a cross-linked oat spelt xylan. Analyses are carried out at pH 4.0 and 40°C (*feed additive*), at pH 5.3 and 40°C (*premixtures*) and at pH 4.2 and 50°C (*feedingstuffs*).

For the determination of the activity of α-amylase in the *feed additive,* the applicant proposes a method based on the quantification (λ = 405 nm) of free *p*-nitrophenol produced by the action of α-amylase on blocked *p*-nitrophenyl maltoheptaoside at pH 5.6 and 37°C. Enzymatic activity is calculated using a reference enzyme standard, of which the activity is measured at pH 6.5 and 37°C. For the analysis of the activity of α-amylase in *premixtures* and *feedingstuffs,* quantification (λ = 620 nm) of dyed oligomers produced by the action of α-amylase on azurine-crosslinked starch at pH 6.4 and 37°C is proposed.

For the determination of the activity of subtilisin in the *feed additive, premixtures* and *feedingstuffs,* the applicant proposes a method based on the quantification (λ = 590 nm) of *dyed oligomers* produced by the action of subtilisin on azurine-cross linked casein. Enzymatic activity is calculated using a reference enzyme standard, of which the activity is measured by quantification of *phenolic compounds* released from casein at pH 7.5 and 40°C. Analyses are carried out at pH 10 and 50°C (*feed additive* and *feedingstuffs*) and at pH 8.0 and 40°C (*premixtures*).

Though the methods proposed by the applicant are based on well known principles and show acceptable performance characteristics, the CRL is concerned that the suggested approach of measuring the enzymatic activity at *different* conditions in various matrices compared to the conditions described by the definitions of units and to the conditions of the determination of the activity of reference enzymes, introduces additional uncertainty into the measurements. Therefore, for consistent analytical results, the CRL recommends:

- that the enzymatic activity in the *feed additive*, in *premixtures* and in *feedingstuffs* is determined at identical conditions;
- that the harmonised analytical conditions are identical with conditions described by the definitions of units;
- that the minimum activity of endo-1,4-β-xylanase, specified in the register entry (75 U kg^{-1}) is replaced by the limit of quantification of the method, which is 500 U/kg;
- that the minimum activity of α-amylase, specified in the register entry (100 U kg^{-1}) is replaced by the limit of quantification of the method, which is 160 U kg^{-1};
- and that the minimum activity of subtilisin, specified in the register entry (1000 U kg^{-1}) is replaced by the limit of quantification of the method, which is 2000 U kg^{-1}.

In the case that the analytical conditions remain *different* for determination of enzymatic activity in various matrices and *different* from those as described by the definitions of units, the CRL cannot evaluate the proposed methods for their suitability for official controls.

Further testing or validation is not considered necessary.