European Food Safety Authority

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Safety and efficacy of Natugrain® TS (endo-1,4-ß-xylanase and endo-1,4-ß-glucanase) as a feed additive for piglets (weaned), chickens for fattening, laying hens, turkeys for fattening and ducks[1]

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

(Question No EFSA-Q-2008-013)

Adopted on 9 December 2008 by the FEEDAP Panel and on 3 December 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 deliver a scientific opinion on the safety and efficacy of Natugrain® TS (endo-1,4-ß-xylanase and endo-1,4-ß-glucanase) as a feed additive within the category of zootechnical additives, functional group digestibility enhancer, for chickens, turkeys and ducks for fattening, laying hens and piglets.

The additive Natugrain® TS is produced in two formulations, i.e. Natugrain® TS L (liquid) and Natugrain® TS (solid). Both formulations contain thermostable endo-1,4-β–xylanase and

[1] For citation purposes: 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) on a request from the European Commission on the safety and efficacy of Natugrain® TS (endo-1,4-ß-xylanase and endo-1,4-ß-glucanase) as a feed additive for piglets (weaned), chickens for fattening, laying hens, turkeys for fattening and ducks. *The EFSA Journal* (2008) 914, 1-21

* One member of the Panel did not participate in the discussion on the subject referred to above because of possible conflicts of interest.

thermostable endo-1,4-β-glucanase, both enzymes being produced by genetically modified strains of *Aspergillus niger*. The genes encoding these enzymes were each derived from a thermotolerant ascomycete fungus, *Talaromyces emersonii* and were cloned in multiple copies into production strains of *A. niger* to increase enzyme yield. The enzyme mixture contains no cultivable production organisms and the level of recombinant DNA is below the limit of detection of 100 ng DNA g^{-1} of the solid enzyme product and 17 ng DNA mL^{-1} of the liquid product.

The efficacy of Natugrain® TS and Natugrain® TS L has been demonstrated at the minimum dose proposed in chickens for fattening (50 mg kg^{-1}), laying hens (100 mg kg^{-1}), turkeys for fattening (100 mg kg^{-1}) and piglets (100 mg kg^{-1}). Efficacy in ducks for fattening (50 mg kg^{-1}) can be extrapolated based on the efficacy for chickens and turkeys for fattening.

Based on the tolerance studies provided by the applicant, Natugrain® TS and Natugrain® TS L are considered to be safe for the target species at the respective maximum proposed doses.

Considering the lack of mutagenicity in two assays and the absence of any relevant effects in a 90-day study, it is concluded that the use of Natugrain® TS as an additive in animal feed would pose no risk for the consumer.

The solid form of the product is non-irritant for the eye and skin. It is presumed to be a potential skin and respiratory sensitiser. Although no study is provided on dermal sensitisation or on the effects of inhalation exposure, any precaution appropriate to protecting the user from the respiratory sensitising properties would be sufficient to protect against any other adverse effects.

The active ingredients of Natugrain® TS 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 envisaged.

Key words: zootechnical additive, digestibility enhancer, endo-1,4-ß-xylanase, endo-1,4-ß-glucanase, genetically modified micro-organisms, *Aspergillus niger*, chickens for fattening, turkeys for fattening, ducks for fattening, laying hens, piglets, efficacy, safety

TABLE OF CONTENTS

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 lies 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 BASF Aktiengesellschaft[3] for authorisation of the product Natugrain® TS, to be used as a feed additive for piglets, chickens for fattening, turkeys for fattening, laying hens and ducks (category: zootechnical additives; 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.[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 16 May 2008.

The additive Natugrain® TS is a preparation of endo-1,4-ß-xylanase produced by the genetically modified micro-organism *Aspergillus niger* (CBS 109.713) and endo-1,4-ß-glucanase produced by the genetically modified micro-organism *Aspergillus niger* (DSM 18404). 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), consumer, user and the environment of the product Natugrain® TS, which is a preparation of endo-1,4-ß-xylanase produced by the genetically modified micro-organism *Aspergillus niger* (CBS 109.713) and endo-1,4-ß-glucanase produced by the genetically modified micro-organism *Aspergillus niger* (DSM 18404), when used under the conditions described in Table 1.

ACKNOWLEDGEMENTS

The European Food Safety Authority wishes to thank Mike Gasson, John Heritage and Friedrich Schöne for their contribution to the preparation of this opinion.

[2] OJ L 268, 18.10.2003, p.29
[3] BASF Aktiengesellschaft, 67056 Ludwigshafen/Rhein, Germany
[4] Dossier reference: FAD-2007-0044

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

Additive	Endo-1,4-ß-xylanase; endo-1,4-ß-glucanase
Registration number/EC No/No (if appropriate)	-
Category of additive	Zootechnical additive
Functional group of additive	Digestibility enhancers

Description			
Additive	**Chemical formula, description**	**Purity criteria (if appropriate)**	**Method of analysis (if appropriate)**
Endo-1,4-beta-xylanase (EC 3.2.1.8) endo-1,4-ß-glucanase (EC 3.2.1.4)	Preparation of endo-1,4-ß-xylanase produced by *Aspergillus niger* (CBS 109.713) and endo-1,4-ß-glucanase produced by *Aspergillus niger* (DSM 18404) having a minimum activity of: Solid form: 5600 TXU[(1)] and 2500 TGU[(2)]/g Liquid form: 5600 TXU and 2500 TGU/g	-	[(1)] 1 TXU is the amount of enzyme which liberates 5 micromole of reducing sugars (xylose equivalents) from wheat arabino-xylan per minute at ph 3,5 and 40°C. [(2)] 1 TGU is the amount of enzyme which liberates 1 micromole reducing sugars (glucose equivalents) from barley ß-glucan per minute at pH 3,5 and 40°C.

Trade name (if appropriate)	Natugrain® TS
Name of the holder of authorisation (if appropriate)	BASF Aktiengesellschaft, 67056 Ludwigshafen/Germany

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		
Piglets (weaned)	-	560 TXU/250 TGU	-	-
Chicken for fattening	-	280 TXU/125TGU		
Laying hens	-	560 TXU/250 TGU	-	-
Turkeys for fattening	-	560 TXU/250 TGU	-	-
Ducks		280 TXU/125TGU	-	-

Other provisions and additional requirements for the labelling	
Specific conditions or restrictions for use (if appropriate)	In the directions of use of the additive and premixture, indicate the storage temperature, storage life and stability to pelleting. Recommended dose per kilogram of complete feedingstuffs: Piglets (weaned): 560 - 840 TXU / 250-375 TGU Chickens for fattening: 280 - 840 TXU / 125-375 TGU Laying hens: 560 - 840 TXU /250-375 TGU Turkeys for fattening: 560 - 840 TXU /250-375 TGU Ducks 280 - 840 TXU /125-375 TGU For use in compound feed rich in non-starch polysaccharides (mainly beta-glucans and arabinoxylans), e. g. containing more than 30 % wheat, barley, rye and/or triticale

Specific conditions or restrictions for handling (if appropriate)	Not appropriate
Post-market monitoring (if appropriate)	BASF has a general traceability system and a complaint procedure in place. An emergency telephone number is printed on each label.
Specific conditions for use in complementary feedingstuffs (if appropriate)	Not appropriate.

Maximum Residue Limit (MRL) (if appropriate)			
Marker residue	**Species or category of animal**	**Target tissue(s) or food products**	**Maximum content in tissues**
Not appropriate	-	-	-

ASSESSMENT

1. Introduction

Natugrain® TS is a feed additive based on endo-1,4-β-xylanase and endo-1,4-β-glucanase, produced by the genetically modified micro-organisms *Aspergillus niger* (CBS 109.713) and *Aspergillus niger* (DSM 18404), respectively, and is presented in solid (TS) and liquid (TS L) forms.[5] The active substances are obtained by submerged fermentation of the micro-organisms. The additive is intended for use in feed rich in non-starch polysaccharides (arabinoxylans and β-glucans), such as wheat, barley, rye and triticale for weaned piglets, chickens for fattening, turkeys for fattening, ducks for fattening and laying hens.

2. Characterisation

2.1. Characterisation of the product

Natugrain® TS is a yellowish-brown powder with a bulk density of 0.35 g mL^{-1} with less than 10 % of the particles with a size below 150 µm and no particles below 75 µm. Natugrain® TS consists of active enzymes concentrates of endo-1,4-β-xylanase (E.C.3.2.1.8) (1-5 %), endo-1,4-β-glucanase (E.C. 3.2.1.6) (0.1-2 %), vegetable oil (≤ 1 %) and the carrier wheat middlings. Natugrain® TS L, a light brown to brown liquid with a density of 1.1-1.3 g mL^{-1}, consists of active enzyme concentrate (endo-1,4-β-xylanase: 5-10 %; endo-1,4-β-glucanase: 0.1-5 %), sorbitol (21 %), glycerol (26 %), sodium-benzoate (≤ 0.2 %) and water (37.8-47.7 %).[6]

The guaranteed minimum activity levels in both solid and liquid forms are 5600 TXU g^{-1} endo-1,4-β-xylanase and 2500 TGU g^{-1} endo-1,4-β-glucanase.

Both preparations conform to the Food Chemical Codex (FCC) and 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 products are regularly monitored for heavy metals (Pb, Cd and Hg) and arsenic, biological contaminants (total aerobic plate count, coliforms, *Escherichia coli*, *Salmonella sp.*, *Staphylococcus aureus*, *Clostridium perfringens*, *Pseudomonas aeruginosa*), antimicrobial activity, absence of the production strain and for mycotoxins (aflatoxin B1, ochratoxin, T-2 toxin and zearalenone) to ensure that they do not exceed the recommended maximum levels.[7] Data of three analysed batches are given.

2.2. Characterisation of the production organisms

2.2.1. Information relating to the genetically modified micro-organisms

Natugrain® TS contains two enzymes, an endo 1, 4-β-xylanase and an endo-1, 4-β–glucanase, each produced by separate genetically modified derivatives of *Aspergillus niger*.

2.2.1.1. Characteristics of the recipient or parental micro-organism

The original parent strain is *A. niger* NRRL3122. This was subjected to several rounds of classical mutagenesis to yield *A. niger* GAM-53 (DS3045), a strain isolated because of its enhanced production of glucoamylase. This strain has an established history of safe use, having

[5] Technical dossier/Section II. 2.1.1
[6] Technical dossier/Section II. 2.1.3
[7] Technical dossier/Section II. 2.1.3

been used for more than fifteen years in the commercial production of glucoamylase, an enzyme widely used in the industrial processing of starch. The strain was found not to produce known mycotoxins or antibiotics under the fermentation conditions that were used.[8]

The host strain, *A. niger* ISO-502, was derived from *A. niger* GAM-53 (DS3045) using recombinant DNA technology. The primary modification in *A. niger* ISO-502 was to create deletions in each of the seven copies of the *gla*A locus, which encodes glucoamylase in the parent strain, and to introduce a different restriction endonuclease site into each Δ*gla*A locus. An additional modification removed the *pep*A gene, encoding pepsin, thus reducing the proteolytic capacity of strain *A. niger* ISO-502. This strain was further modified to generate the final producer strains, *A. niger* XEA64 (DS38163) and *A. niger* LU13118 (as described below).[9]

2.2.1.2. Characteristics of the donor micro-organism

The source of *xem*A, the gene encoding endo-1, 4-xylanase and of *cea*, the gene encoding endo 1, 4-β-glucanase, is the thermotolerant ascomycete fungus *Talaromyces emersonii*, strain FBG1. This fungus is currently used for the production of enzymes used in brewing.[9]

2.2.1.3. Description of the genetic modification process

Thermostable endo 1,4-β-xylanase

The *xem*A gene, which encodes the thermostable endo 1, 4- β-xylanase, was isolated by growing the donor fungus under conditions favourable for the production of xylanase and then isolating mRNA from the culture. This was used to generate a cDNA expression library in *Escherichia coli* using a vector containing the 5' and 3' transcription initiator and terminator signals, respectively of the *gla*A gene from the host *A. niger* GAM 53. Restriction endonuclease digestion was used to release the cDNA expression cassettes, which were then used to transform *A. niger* ISO-502. Transformants were screened for over-production of endo 1, 4-β–xylanase and the expression unit from one such strain was cloned into a standard *A. niger* expression vector, pGBTOP. A second vector was engineered from pGBTOP, this one containing the *amd*S gene from *Aspergillus nidulans*. This gene encodes acetamidase and is used as a selectable marker. The presence of the *amd*S gene allows this fungus to grow normally on acetamide agar as the sole source of nitrogen and carbon. To remove the vector backbone and increase the efficiency of transformation, the expression cassette was excised from both of the vectors. Restriction endonuclease fragments were separated by electrophoresis and used to co-transform protoplasts of *A. niger* ISO-502. An excess of DNA carrying the *xem*A gene was used and transformants were recovered on agar containing acetamide. One of these transformants, designated XEA702-1, was found to contain several in tandem integrated copies of the *xem*A expression units flanked by one *amd*S expression unit in one of the Δ*gla*A loci. Counterselection to remove the *amd*S marker was achieved by growing transformants on agar containing fluoroacetamide; cells harbouring the *amd*S gene metabolise this substrate to ammonia and fluoroacetate, which are toxic. The strain contains no additional *E. coli* or *amd*S DNA as was confirmed by Southern analysis. Three rounds of classical selection filled three of the empty Δ*gla*A loci resulting in strain XEA64 containing multiple copies of the *xem*A gene.[9]

Thermostable endo-1,4-β-glucanase

The *cea* gene, which encodes the thermostable endo-1, 4-β-glucanase, was isolated by growing the donor fungus under conditions favourable for the production of β-glucanase and then

[8] Technical dossier/Section II. 2.2.5
[9] Technical dossier/Section II. 2.2.3

isolating mRNA from the culture. This was used to generate a cDNA library in *Escherichia coli* using a vector containing the 5' and 3' transcription initiator and terminator signals, respectively, of the *gla*A gene from the host *A. niger* GAM 53. Restriction endonuclease digestion was used to release the cDNA expression cassettes, which were then used to transform *A. niger* ISO-502. Transformants were screened for over-production of endo 1, 4-β-glucanase and the expression unit from one such strain was cloned into a standard *A. niger* expression vector, pGBTOP. Using an identical protocol to that employed in the generation of strain XEA64, one of the transformants was found to contain three in tandem integrated copies of the *cea* expression unit flanked by one *amd*S expression unit in one of the Δ*gla*A loci. Removal of the *amd*S marker was achieved by growing transformants on agar containing fluoroacetamide. The resulting strain was subject to an additional co-transformation with the *cea* and *amd*S expression cassettes. Counterselection to remove the *amd*S marker gene was successful and one of the transformants, LU12990, was selected. It contained several copies of the *cea* expression unit in tandem integrated in one of the Δ*gla*A loci. Absence of *E. coli* vector and *amd*S sequences was confirmed by Southern analysis. Further classical selection was used to fill up three extra empty Δ*gla*A loci with several tandemly integrated *xem*A expression cassettes, resulting in the final production organism, LU13118, containing multiple copies of the *cea* gene.[9]

2.2.2. Information relating to the production process

Each of the enzymes is produced separately by submerged fermentation using standard procedures. Following fermentation, sodium benzoate at pH 4.2 was added for six hours after which the cell material was removed by several filtration steps. After concentration of each enzyme by ultrafiltration, the individual preparations are mixed and then processed further into either the solid or the liquid product.

2.3. Stability and homogeneity

The stability of both forms of Natugrain® TS (three batches each) was tested[10] for up to 12 months at 20 ºC or for six months at 35 ºC. Results showed only a limited loss of xylanase activity of the solid form when stored at 35 ºC for six months (xylanase: 80 %, glucanase: 100 %), while practically no changes in the enzyme activities were observed at 20 ºC. No loss of activity was observed for the liquid form.

The stability of Natugrain® TS in premixtures for commercial turkey feed (three batches) was studied at 20 and 35 ºC. After 26 weeks of storage at 20 ºC the enzyme activities were 108 % and 102 % and after 12 weeks at 35 ºC, 84 % and 104 % for xylanase and glucanase, respectively.

The stability of Natugrain® TS (two batches) was tested in commercial poultry mash feed (for 16 months at 20 ºC or six months at 35 ºC) and pelleted feed (for 16 months at 20 ºC or ten months at 35 ºC), and no loss of activity was observed. The stability of Natugrain® TS L was tested in pelleted feed for 16 weeks at 20 ºC (xylanase: 100 %, glucanase: 100 %) and 16 weeks at 35 ºC (xylanase: 99 %, glucanase: 88 %). There is no data about the stability of Natugrain® TS and TS L in pig feed. However, stability in pig feed can be extrapolated from poultry feed because of the similarity in composition.

The stability studies of Natugrain® TS during pelleting of poultry feed (pelleting temperature: 80-85 ºC) showed that approximately 88 % of xylanase and glucanase activities were retained.

[10] Technical dossier/Section II. 2.1.4

The homogeneity of Natugrain® TS in vitamin/mineral premixtures was tested and showed an average variation coefficient below 10 %. Natugrain® TS distributed uniformly in the poultry feed (mash and pellets), with an average variation coefficient < 9 %. There is no data available about the homogeneity of Natugrain® TS L in pellets.

2.4. Conditions of use

Natugrain® TS is intended for use in feeds containing more than 30 % wheat, barley, rye and/or triticale at a recommended dose of 280-840 TXU xylanase and 125-375 TGU glucanase kg^{-1} (50-150 mg Natugrain kg^{-1}) complete feed for chickens for fattening and ducks for fattening, and 560-840 TXU xylanase and 250-375 TGU glucanase kg^{-1} (100-150 mg Natugrain kg^{-1}) for weaned piglets, turkeys for fattening and laying hens.

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 feed. The Executive Summary of the CRL report can be found in the Appendix.

3. Efficacy

3.1. Efficacy for piglets (weaned)

Three trials were conducted in two locations to support the efficacy of Natugrain® TS in piglets. The trials had a very similar design and are summarised in Table 2. In all three trials, basal diets were supplemented with Natugrain® TS at 0 or 100 mg kg^{-1} complete feedingstuffs. The enzyme activities were confirmed by analysis. All the basal diets were based on wheat/barley/rye; in trial 2, two basal diets were used, wheat/barley-based diets with or without rye. The statistical analyses in trial 2 showed no interaction between the type of diet and enzyme supplementation; therefore, the results showed in Table 2 are the mean values obtained regardless of the type of basal diet. Average initial weights of the piglets were 6.1 kg (trial 1), 9.4 kg (trial 2) and 8.3 kg (trial 3). The trials lasted for 42 days. Final body weight, daily weight gain, daily feed intake and feed to gain ratio were measured or calculated. GLM procedure was used for statistical evaluation of the data.

Table 2. **Summary of the performance trials on the efficacy of Natugrain® TS in weaned piglets**

Trial	No. of animals *Replicates x pigs per treatment*	Natugrain® TS (mg kg^{-1} feed)	Daily weight gain (g day^{-1})	Daily feed intake (g day^{-1})	Final body weight (kg)	Feed/gain (kg kg^{-1})	Mortality (n)
1[11]	144	0	335^{a}	529	19.2^{a}	1.58^{a}	5
	18 x 4	100	384^{b}	551	21.1^{b}	1.44^{b}	5
2[12]	150	0	475^{a}	846^{a}	29.1^{a}	1.80	0
	15 x 5	100	498^{b}	869^{b}	30.1^{b}	1.76	0
3[13]	240	0	447^{a}	873	27.1^{a}	1.97^{a}	0
	20 x 6	100	474^{b}	889	28.2^{b}	1.88^{b}	0

$^{a, b}$: Means in the same column for a given trial with different superscript are significantly different ($P < 0.05$)

In all three experiments, supplementation with Natugrain® TS at the minimum recommended dose significantly improved daily weight gain and final body weight. Feed intake was increased in one trial (trial 2) and feed to gain ratio was significantly improved in two trials (1 and 3) at the minimum recommended dose. No animals died in trials 2 and 3, while in trial 1 five animals died in each treatment (6.9 %).

Conclusion on efficacy for piglets

Based on data from three trials in which the performance of piglets was positively affected by supplementation with the minimum dose proposed (100 mg kg^{-1}) of Natugrain® TS, it is concluded that there is evidence of efficacy of this product in piglets at the minimum dose proposed.

3.2. Efficacy for chickens for fattening

Three trials were made with chickens for fattening in two locations. Natugrain® TS L was added post-pelleting to the wheat/barley- (trials 1 and 2) or wheat/rye-based (trial 3) diets at the intended doses of 0, 50, 100, 150 mg kg^{-1} (trial 1), 0, 50, 150 and 1500 mg kg^{-1} (trial 2) and 0, 50 and 100 mg kg^{-1} (trial 3). In trial 2, a concentrated form of the enzyme was used for the 1500 mg kg^{-1} dose. The enzyme activities were confirmed by analysis. In all cases, one-day-old male chickens were used and the trials lasted for 42 (trial 1) or 35 days (trials 2 and 3). Final body weight, daily weight gain (trials 2 and 3), daily feed intake and feed to gain ratio were measured or calculated. GLM procedure was used for statistical evaluation of the data.

Since all experiments followed a similar design, the main results are summarised in Table 3.

[11] Technical Dossier/Section III/Reg 2
[12] Technical Dossier/Section III/Reg 4
[13] Technical Dossier/Section III/Reg 6

Table 3. **Summary of the performance trials on the efficacy of Natugrain® TS L in chickens for fattening**

Trial	No. of animals *Replicates x birds per treatment* Duration	Natugrain® TS (mg kg^{-1}feed)	Daily weight gain (g day^{-1})	Feed intake (g day^{-1})	Final body weight (g)	Feed/gain (kg kg^{-1})	Mortality and removal (n)
1[14]	480 *15 x 8* 42 days	0	-	96[a]	1959[a]	2.08[a]	0
		50		104[b]	2365[b]	1.84[b]	2
		100		105[b]	2429[b]	1.82[b]	4
		150		103[b]	2446[b]	1.77[b]	4
2[15]	720 *12 x 15* 35 days	0	61.5[a]	110[a]	2198[a]	1.79[a]	8
		50	64.6[b]	112[a]	2309[b]	1.74[b]	7
		150	64.2[b]	109[a]	2293[b]	1.69[b]	16
		1500	64.8[b]	103[b]	2315[b]	1.59[b]	7
3[16]	540 *12 x 15* 35 days	0	52.0[a]	114	1866[a]	2.20[a]	9
		50	62.2[b]	116	2222[b]	1.87[b]	9
		100	64.3[b]	116	2298[b]	1.80[b]	12

[a, b]: Means in the same column for a given trial with different superscript are significantly different ($P < 0.05$)

Supplementation with Natugrain® TS L at the minimum recommended dose (50 mg kg^{-1}) significantly improved the final weight and feed to gain ratio in all three experiments, daily weight gain in two experiments (trials 2 and 3) and increased feed intake in one experiment (trial 2). Mortality was generally low (< 5 %) and irrespective of the use of Natugrain, except in trial 2 at the dose level of 150 mg kg^{-1} (mortality + culled animals = 8.9 %) where a high culling rate was caused by leg problems.

Conclusions on efficacy for chickens for fattening

Based on data from three trials in which the performance of chickens for fattening was positively affected by supplementation with the minimum recommended dose of Natugrain® TS L (50 mg kg^{-1}), it is concluded that there is evidence of efficacy of this product in chickens for fattening at this dose.

3.3. Efficacy for turkeys for fattening

Four efficacy trials were conducted with one-day-old turkeys for fattening (females in trials 1, 2 and 4 and males in trial 3) in two different locations. Natugrain® TS was added to the wheat/barley-based diets at the doses of 0 or 100 mg kg^{-1}. The solid formulation of the product was used in the first feeding phase and Natugrain® TS L during the other feeding phases (2-5 in trials 1, 2 and 4 and 2-6 in trial 3). The enzyme activities were confirmed by analysis in all cases. Final body weight at week 17 (trials 1, 2 and 4) or at week 22 (trial 3), daily weight gain, daily feed intake and feed to gain ratio were measured or calculated. GLM procedure was used for statistical evaluation of the data. The design and main results of the four trials are summarised in Table 4.

[14] Technical Dossier/Section III/Reg 18
[15] Technical Dossier/Section III/Reg 20
[16] Technical Dossier/Section III/Reg 22

Table 4. **Summary of the performance trials on the efficacy of Natugrain® TS and Natugrain® TS L in turkeys for fattening**

Trial Sex	No. of animals *Replicates x birds per treatment* Duration (weeks)	Natugrain® TS Natugrain® TS L (mg kg^{-1} feed)	Daily weight gain (g day^{-1})	Feed intake (g day^{-1})	Final body weight (kg)	Feed/gain (kg kg^{-1})	Mortality and removal (n)
1[17] ♀	360 *15 x 12/10/8** 17	0 100	96.0 96.0	254.0 248.1	11.4 11.4	2.65[a] 2.59[b]	18 16
2[18] ♀	360 *15 x 12/10/8** 17	0 100	95.4 96.8	257.9 251.5	11.3 11.5	2.70[a] 2.61[b]	12 6
3[19] ♂	300 *15 x 10/9/8/6** 22	0 100	139.0 142.0	406.0 404.0	21.4 21.9	2.92[a] 2.83[b]	10 9
4[20] ♀	360 *15 x 12/10/8** 17	0 100	102.0 103.3	287.7[a] 277.9[b]	12.2 12.4	2.83[a] 2.68[b]	22 11

* The number of animals was reduced at weeks 5 and 13 in trials 1, 2 and 4 and at weeks 10,14 and 18 in trial 3.

[a, b]: Means in the same column for a given trial with different superscript are significantly different ($P < 0.05$)

Supplementation with Natugrain® TS and Natugrain® TS L significantly improved feed to gain ratio at the minimum recommended dose (100 mg kg^{-1} diet) in all four trials. The daily feed intake was significantly reduced by the addition of the enzymatic preparation to the diet in one trial (trial 4). Mortality (including removal) was generally lower in the Natugrain® groups and within an acceptable level; it was mainly caused by leg problems.

Conclusions on efficacy for turkeys for fattening

Based on data from four trials in which supplementation with the minimum recommended dose (100 mg kg^{-1}) of Natugrain® TS /L significantly improved feed to gain ratio, it is concluded that there is evidence of efficacy of this product in turkeys for fattening at this dose.

3.4. Efficacy for laying hens

Four efficacy trials were conducted in two locations. Natugrain® TS L was added post-pelleting to the wheat/rye (trials 1 and 3), wheat/barley (trial 2) or wheat/barley/rye-based diets (trial 4) at the intended dose of 0 or 100 mg kg^{-1}. The enzyme activities were confirmed by analysis in all cases. Daily feed intake, egg production, feed to egg ratio, laying rate and egg weight were measured or calculated. GLM procedure was used for statistical evaluation of the data. The design and main results of the four trials are summarised in Table 5.

[17] Technical Dossier/Section III/Reg 8
[18] Technical Dossier/Section III/Reg 11
[19] Technical Dossier/Section III/Reg 14
[20] Technical Dossier/Section III/Reg 16

Table 5. **Summary of the performance trials on the efficacy of Natugrain® TS L in laying hens**

Trial	No. of animals *Replicates x birds per treatment* Duration (weeks)	Natugrain® TS L (mg kg^{-1} feed)	Feed intake (g day^{-1})	Egg mass production (g day^{-1})	Feed/egg (g feed/ g egg)	Layin g rate (%)	Egg weight (g egg^{-1})	Mortality (n)
1[21]	1152 *9 x 64* 52	0 100	122.5[a] 116.9[b]	57.3 57.4	2.14[a] 2.04[b]	90.4 90.3	63.4 63.6	69 63
2[22]	1152 *9 x 64* 26	0 100	115.1 113.8	57.4 57.8	2.00[a] 1.97[b]	93.9 94.1	61.2 61.4	32 24
3[23]	720 *24 x 15* 48	0 100	106.8 106.1	52.6[a] 53.4[b]	2.03[a] 1.99[b]	87.8 88.6	59.9 60.2	19 9
4[24]	720 *24 x 15* 48	0 100	102.6 102.5	51.9[a] 53.2[b]	1.98[a] 1.93[b]	85.2[a] 87.5[b]	60.9 60.8	1 4

[a, b]: Means in the same column for a given trial with different superscript are significantly different ($P < 0.05$)

Supplementation of laying hens' diets with Natugrain® TS L at the minimum dose proposed (100 mg kg^{-1} diet) significantly improved feed to egg ratio in all four trials. Additionally, daily feed intake was significantly lower in one trial (trial 1), egg mass production increased in two trials (3 and 4) and laying rate increased in one trial (trial 4) as a result of Natugrain® TS L supplementation. Mortality was generally low, except in trial 1 where mortality was higher due to technological problems though still within an acceptable level.

Conclusion on efficacy for laying hens

Based on data from four trials in which supplementation with Natugrain® TS L at 100 mg kg^{-1} significantly improved feed conversion, it is concluded that there is evidence of efficacy of this product in laying hens at the proposed minimum dose.

3.5. Efficacy for ducks

Efficacy has been demonstrated in chickens and turkeys for fattening at a dose of 50 and 100 mg kg^{-1}, respectively. Since the mode of action of xylanases and glucanases are the same in those species as in ducks, no further demonstration of efficacy in this minor species is needed. Therefore, based on the data provided for chickens for fattening it is considered that efficacy of Natugrain® TS in ducks can be assumed at 50 mg kg^{-1}.

[21] Technical Dossier/Section III/Reg 24
[22] Technical Dossier/Section III/Reg 26
[23] Technical Dossier/Section III/Reg 28
[24] Technical Dossier/Section III/Reg 30

4. Safety

4.1. The safety aspects of the genetic modification

4.1.1. 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

A. niger XEA64, used for the production of the thermostable endo1, 4-β-xylanase, contains twenty-eight copies of the *xem*A expression cassette integrated into the host chromosomal DNA. *A. niger* LU13118, which produces the thermostable endo 1, 4-β -glucanase contains thirty-six copies of the *cea* expression cassette integrated into the host chromosomal DNA.

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

The applicant has provided a description of the cloning procedures from which the composition of the insertion cassettes can be deduced. All inserted DNA is derived from *T. emersonii* and from the host *A. niger* GAM53 strain. No *E. coli* sequences remain in the production strains, as demonstrated by Southern analysis.[25]

Conclusions regarding the genetic modification

The recipient *A. niger* strain has a long history of safe use. The inserted DNA sequences are derived from *T. emersonii* and from the recipient *A. niger* strain, and no sequences that cause concern are introduced. It is concluded that the genetic modification does not raise any particular safety concern.

4.2. Safety for the target species

4.2.1. Safety for piglets

A tolerance study[26] was conducted with a total of 300 21-day-old weaned piglets (sex ratio 1:1) divided into ten replicates of ten animals per treatment. Feeding started with a weaning diet (days 1-14) followed by wheat/barley-based pre-starter feed (15-42 days). The diets were either unsupplemented or supplemented with the highest recommended dose (150 mg kg^{-1} diet) of Natugrain® TS and 100 times (equivalent to 15000 mg kg^{-1} diet) the maximum recommended dose using a concentrated form of the enzyme preparation. The enzyme activities were confirmed by analysis. Feed intake, daily weight gain and feed to gain ratio of the piglets were measured and calculated. Mortality and health status were monitored throughout the experiment.

There were no adverse effects observed on animal health status (mortality and culling: 2, 3 and 0 %) or performance (weight gain averaged approximately 250 g day^{-1}).

[25] Technical dossier/Suppl. Info. Aug08/Annex1
[26] Technical Dossier/Section IV/Reg 08

4.2.2. Safety for chickens for fattening

The tolerance study[27] with chickens for fattening was made over a 35-day period. Wheat and barley-based diets (starter and finisher) were supplemented with the highest recommended dose (150 mg kg^{-1} diet) of Natugrain® TS and 100 times (equivalent to 15000 mg kg^{-1} diet) the maximum recommended dose using a concentrated form of the enzyme preparation. The enzyme activities were confirmed by analysis. Feed intake, daily weight gain and feed to gain ratio of the chickens were measured and calculated. Mortality and health status were monitored throughout the experiment.

There were no adverse effects observed on animal health status (mortality: 4.4, 8.9 and 3.9 %) or performance (body weight at 35 days: approximately 2.3 kg).

4.2.3. Safety for turkeys for fattening

A six-week tolerance study[28] was conducted with 390 female turkeys for fattening (14 to 56 days of age) in three treatment groups with 13 replicates of ten turkeys per replicate. The wheat-soybean meal-based diet was supplemented with the highest recommended dose (150 mg kg^{-1} diet) of Natugrain® TS and 100 times (equivalent to 15000 mg kg^{-1} diet) the maximum recommended dose using a concentrated form of the enzyme preparation. Enzyme activities were confirmed by analysis. Feed intake, daily weight gain and feed to gain ratio of the turkeys were measured and calculated. Mortality and health status were monitored throughout the experiment.

There were no adverse effects observed on animal health status (mortality: 5.4, 6.9 and 3.9 %) or performance (body weight at 56 days: approximately 4 kg).

4.2.4. Safety for laying hens

An eight-week tolerance study[29] was conducted with 144 laying hens (60-week old) divided into eight replicates of six hens per treatment. Wheat/barley-based diets were fed unsupplemented or supplemented with the highest recommended dose (150 mg kg^{-1} diet) of Natugrain® TS or 100 times (equivalent to 15000 mg kg^{-1} diet) the maximum recommended dose using a concentrated form of the enzyme preparation. Enzyme activities were confirmed by analysis. Body weight, laying rate, egg weight, daily egg mass, feed to egg ratio and egg quality parameters (shell breaking power, deformability, Haugh-unit, yolk colour) were measured or calculated. Mortality and health status were monitored throughout the experiment.

There were no adverse effects observed on animal health status (no mortalities), performance (daily egg mass: 60 g) and egg quality parameters.

4.2.5. Conclusions on the safety for the target species

Natugrain® TS is safe for piglets, chickens and turkeys for fattening and laying hens at the maximum recommended dose. No specific tolerance study was performed in ducks. However, given the fact that the safety of Natugrain® TS has been established in chickens for fattening, turkeys for fattening and laying hens with a wide margin of safety (100), the safety for ducks for fattening can be assumed based on the tolerance trials performed in the major species.

[27] Technical Dossier/Section IV/Reg 02
[28] Technical Dossier/Section IV/Reg 04
[29] Technical Dossier/Section IV/Reg 06

4.3. Safety for the consumer

Two mutagenicity/clastogenicity studies and one 90-day repeated dose oral toxicity study in rats were performed to assess safety for the consumer.

4.3.1. Bacterial reverse mutation assay

The enzyme preparation was tested[30] in *Salmonella* Typhimurium strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* WP2uvrA, both with and without microsomal enzyme activation, according to OECD guideline 471. There was no evidence for genotoxicity of the test article in this study, using up to 5000 µg plate^{-1}.

4.3.2. Mouse micronucleus assay

The enzyme preparation (powder form dissolved in purified water) was tested[31] *in vivo* for chromosome damage (clastogenicity and aneugenic effects) in a mouse micronucleus assay according to guideline OECD 474. Exposure was by gavage for two consecutive days to five males per dose group at levels of up to 2000 mg kg^{-1} body weight; bone marrow was harvested 24 hours later. While there was a clear response in the positive controls, there was no evidence for chromosome damage (micronuclei) in mouse bone marrow cells.

4.3.3. 90-day oral toxicity study

A 90-day oral toxicity study was conducted with Natugrain® TS (powder).[32] Groups of ten Wistar rats per sex per group were given daily 0, 1000, 4000 or 16000 mg kg^{-1} diet. The study was conducted in accordance with the current OECD guideline 408. Clinical condition, food intake and body weight were monitored regularly throughout the study. At the end of the study, a battery of functional observational tests was conducted and blood and urine samples collected for examination. All animals were brought to necropsy and subject to macroscopic examination with a full range of tissues being weighed. Histological examination was confined to the control and high dose groups but covered a wide range of tissues. In addition, gross lesions were also studied histologically.

There was some dose-related decrease in food consumption and body weight gain in males, but without any other signs of toxicity in the broad range of endpoints mentioned. Therefore, it is concluded that the results showed no evidence of substance-related specific adverse effect up to the highest dose tested, equivalent to 1153 mg kg^{-1} or 1336 mg kg^{-1} body weight for males and females, respectively.

4.3.4. Conclusions on safety for the consumer

Based upon a lack of mutagenicity in two assays and the absence of any relevant effects in a 90-day study, it is concluded that the use of Natugrain® TS as a feed additive would pose no risk for the consumer.

4.4. Safety for the user

An acute dermal irritation study in the rabbit, according to OECD guideline 404, was conducted with Natugrain® TS powder[33] and showed that this preparation was non-irritant.

[30] Technical Dossier/Section IV/Reg 12
[31] Technical Dossier/Section IV/Reg 13
[32] Technical Dossier/Section IV/Reg 16
[33] Technical Dossier/Section IV/Reg 15

In an acute eye irritation study[34] with Natugrain® TS powder in rabbit, conducted in accordance to OECD guideline 405, absence of irritation/corrosion potential was demonstrated.

No acute inhalatory toxicity test was provided; however, in the absence of adverse effects by oral, dermal or mucosal route, and particle size being > 75 µm, risk from inhalatory exposure is not expected. Moreover, since precautionary measures are taken to prevent respiratory sensitisation from enzyme feed additives this would be sufficient to minimise other exposures. In the absence of a skin sensitisation test, the preparation must be considered as a potential skin sensitiser.

4.4.1. Conclusions for the safety for the user

It is concluded that no particular concern for the safety of the users is expected from Natugrain® TS, provided that standard protective measures for enzyme preparations are implemented.

4.5. Safety for the environment

The final enzyme preparation contains no cultivable production organisms as tested in the final product before formulation.[35, 36] The absence of recombinant DNA was demonstrated in the final solid and liquid enzyme products by PCR. Fragments of 540 bp and 572 bp were amplified to detect the xylanase and glucanase genes, respectively. The detection limits were 100 ng DNA g^{-1} in the solid enzyme product and 17 ng DNA mL^{-1} in the liquid product.[37, 38]

The active components of Natugrain® TS 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.

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[39] and Good Manufacturing Practices.

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

The molecular characterisation of the genetic modification does not trigger any particular safety concerns. The final enzyme preparation contains no cultivable producer organisms and the level of recombinant DNA is below the limit of detection.

The solid and liquid forms of the product are considered to be equivalent in terms of efficacy and safety at the same dose.

The efficacy of Natugrain® TS has been demonstrated at dose levels of 50 mg kg^{-1} in chickens for fattening and 100 mg kg^{-1} in laying hens, turkeys for fattening and piglets. Efficacy is also

[34] Technical Dossier/Section IV/Reg 14
[35] Technical dossier/Suppl. Info. Aug08/Annex2
[36] Technical dossier/Suppl. Info. Oct08/Annex1
[37] Technical dossier/Suppl. Info. Aug08/Annex3
[38] Technical dossier/Suppl. Info. Oct08/Annex2
[39] OJ L 35, 8.2.2005, p.1

assumed in ducks for fattening at 50 mg kg^{-1} based on the efficacy for chickens and turkeys for fattening.

Natugrain® TS are considered to be safe in chickens for fattening, laying hens, turkeys for fattening, ducks for fattening and piglets at the respective maximum proposed doses.

Based upon a lack of mutagenicity in two assays and the absence of any relevant effects in a 90-day study, it is concluded that the use of Natugrain® TS as an additive in animal feed would pose no risk for the consumer.

The solid form of the product is non-irritant for the eye and skin. It is presumed to be a potential skin and respiratory sensitiser. Although no study is provided on dermal sensitisation or on the effects of inhalation exposure, any precaution taken to protect the user from respiratory sensitisation would be sufficient to protect against any other adverse effects.

The active ingredient of Natugrain® TS 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.

RECOMMENDATION

In the Register entry, the activity of the liquid preparation should be expressed in TXU and TGU mL^{-1}.

DOCUMENTATION PROVIDED TO EFSA

1. Natugrain® TS/ Natugrain® TS L solid and liquid preparations containing thermostable endo-xylanse & thermostable β-glucanase. October 2007. Submitted by BASF Aktiengesellschaft.
2. Supplementary information. Natugrain® TS/ Natugrain® TS L solid and liquid preparations containing thermostable endo-xylanse & thermostable β-glucanase. July 2008. Submitted by BASF SE.
3. Supplementary information. Natugrain® TS/ Natugrain® TS L solid and liquid preparations containing thermostable endo-xylanse & thermostable β-glucanase. October 2008. Submitted by BASF SE.
4. Evaluation report of the Community Reference Laboratory for Feed Additives on the methods(s) of analysis for Natugrain® TS/ Natugrain® TS L.
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 Natugrain® TS

In the current application authorisation is sought for *Natugrain®TS* and *Natugrain®TS L*, under the category 'zootechnical additives' and the functional group 4(a), according to the classification system of Annex I of Regulation (EC) No 1831/2003. Authorisation is sought to use *Natugrain®TS* and *Natugrain®TS L* as a digestibility enhancer for piglets (weaned), laying hens, chicken and turkeys for fattening and ducks.

The two active agents of *Natugrain®TS* and *Natugrain®TS L* are (1) thermostable endo-1,4-β-xylanase, produced by a strain of *Aspergillus niger*-CBS 109.713, and (2) thermostable endo-1,4-β-glucanase, produced by a strain of *Aspergillus niger*-DSM 18404. The additive is intended to be marketed as powder (*Natugrain®TS*) and as liquid formulation (*Natugrain®TS L*). Both formulations contain an endo-1,4-β-xylanase activity of 5600 TXU/g product and an endo-1,4-β-glucanase activity of 2500 TGU/g product. They are intended to be mixed into *premixtures* and/or *feedingstuffs* to obtain a minimum endo-1,4-β-xylanase activity level of 280 up to a maximum recommended of 840 TXU per kg *feedingstuffs* and a minimum endo-1,4-β-glucanase activity level of 125 to a maximum recommended of 375 TGU per kg *feedingstuffs*. Enzymatic activity of endo-1,4-β-xylanase is expressed in thermostable xylanase units (TXU). One TXU is defined as the amount of enzyme that liberates 5 µmol of reducing sugars (xylose equivalents) from wheat arabinoxylan per minute at pH = 3.5 and 40°C. Enzymatic activity of endo-1,4-β-glucanase is expressed in thermostable glucanase units (TGU). One TGU is defined as the amount of enzyme that liberates 1 µmol of reducing sugars (glucose equivalents) from barley betaglucan per minute at pH = 3.5 and 40°C.

For the determination of the activity of endo-1,4-β-xylanase in the *feed additive*, *premixtures* and *feedingstuffs*, the applicant proposes an *in-house* validated viscosimetric method. Endo-1,4-β-xylanase catalyses the hydrolysis of glycosidic bonds in the substrate wheat arabinoxylan to yield xylose and reduces consequently the viscosity of sample solution. The decrease in viscosity of sample solution, expressed in terms of a drop time, is a measure for the endo-1,4-β-xylanase activity and is determined using a falling ball viscosimeter at pH = 3.5 and 55°C. The quantification is performed using an endo-1,4-β-xylanase standard curve based on reference enzyme provided by the applicant. The method performance characteristics, determined for the *feed additive, premixtures* and *feedingstuffs* matrices are: - a relative standard deviation for repeatability (RSD_r) ranging from 2.4 to 5.7%; - a relative intermediate precision (RSD_R) ranging from 3.4 to 11.8%; - a recovery rate ranging from 82 to 115%; - a limit of detection (LOD) of 11 TXU/kg *feedingstuffs*; - a limit of quantification (LOQ) of 36 TXU/kg *feedingstuffs*.

For the determination of the activity of endo-1,4-β-glucanase in the *feed additive*, *premixtures* and *feedingstuffs*, the applicant proposes an *in-house* validated viscosimetric method. Endo-1,4-β-glucanase catalyses the hydrolysis of glycosidic bonds in the substrate barley betaglucan to yield glucose and reduces consequently the viscosity of sample solution. The decrease in viscosity of sample solution, expressed in terms of a drop time, is a measure for the endo-1,4-β-glucanase activity and is determined using a falling ball viscosimeter at pH = 3.5 and 40°C. The quantification is performed using an endo-1,4-β-glucanase standard curve based on reference enzyme provided by the applicant. The method performance characteristics, determined for the *feed additive, premixtures* and *feedingstuffs* matrices, are: -a RSD_r ranging from 4.1 to 10.4%; - a RSD_R ranging from 7.5 to 12.3%; - the recovery rate ranging from 88 to 117%; - a LOD of 16 TGU/kg *feedingstuffs*; - a LOQ of 49 TGU/kg *feedingstuffs*.

Based on acceptable performance characteristics, both methods are considered to be suitable for official control purposes in the frame of authorisation.

Further testing or validation is not considered necessary.