European Food Safety Authority

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Opinion of the Scientific Panel on Additives and Products or Substances used in Animal Feed and the Scientific Panel on Genetically Modified Organisms on the safety and efficacy of the enzyme preparation Rovabio™ PHY AP/LC (3-phytase) as feed additive for chickens for fattening, laying hens, piglets and pigs for fattening in accordance with Regulation (EC) No 1831/2003

(Question No EFSA-Q-2005-281)

Adopted by

the FEEDAP Panel on 17 April 2007

and by

the GMO Panel on 22 March 2007

SUMMARY

EFSA received a request from the European Commission to assess the safety of the enzyme product Rovabio™ PHY LC/AP for the target animals, the consumer, user and the environment and the efficacy, when used under the proposed conditions. Rovabio™ PHY AP/LC is a feed additive with 3-phytase as its declared activity. It is produced by fermentation of the fungus *Penicillium funiculosum* 4.05b, which is genetically modified by introducing two genes, *i.e.* the gene encoding 3-phytase from *P. funiculosum* MW 163 and the selectable marker gene *tub1** from *P. funiculosum* IMI134756 that confers resistance to the fungicide benomyl. Multiple copies of the genes are randomly integrated into the fungal genome. No antibiotic resistance marker sequences are present in the final producer strain. The final enzyme preparation contains no cultivable producer organism and the level of newly introduced DNA is below the detection limit.

Rovabio™ PHY LC/AP is intended to be used in chickens for fattening, laying hens, weaned piglets, and pigs for fattening. The minimum proposed dose for incorporation in feeds is 300 RPU kg^{-1} (for laying hens) and 350 RPU kg^{-1} (for chickens and pigs for fattening and weaned piglets) for both forms. The maximum recommended dose for all species/categories is 500 RPU kg^{-1}.

There is a significant loss of phytase activity after incorporation of Rovabio™ PHY in premixtures or feeds at temperatures above 20°C. There is a concern that this low stability could lead to a significant under-dosing of the animals, despite the warnings included in the proposed product label.

Although most of the studies for efficacy and tolerance have been performed with the liquid formulation, these are considered to apply to both forms of the product. Evidence of efficacy of Rovabio™ PHY has been provided at a minimum dose of 300 RPU kg^{-1} for laying

hens, 350 RPU kg^{-1} feed for chickens for fattening and pigs for fattening and 250 RPU kg^{-1} feed for piglets.

Rovabio™ PHY is tolerated at seven times (chickens for fattening, laying hens), eight times (piglets) and five times (pigs for fattening) the maximum recommended dose. Safety for pigs for fattening is further reassured by the safety demonstrated at eight times the maximum recommended dose in piglets. Therefore, it is concluded that Rovabio™ PHY is safe for the target species when used at the recommended dose range.

Phytase as present in Rovabio™ PHY LC/AP is not mutagenic and did not show a toxic response of consumer relevance in a sub-chronic toxicity study. It was demonstrated to be slightly irritant for the eye and a potential sensitizer.

The active substance is a 3-phytase which can be considered essentially similar to naturally occurring phytases, and therefore no risk for the environment is foreseen.

Therefore, Rovabio™ PHY LC/AP is considered safe for the target species, the consumer, the user and the environment, when used under the conditions of use proposed by the applicant.

Key words: Phytase, *Penicillium funiculosum,* feed additive, enzyme, chickens for fattening, laying hens, piglets, pigs for fattening, efficacy, safety, genetically modified micro-organism, zootechnical additive, digestibility enhancer

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BACKGROUND

Regulation (EC) No 1831/2003[1] establishes the rules governing the Community authorisation of additives for use in animal nutrition. In particular, Article 4(1) of that Regulation 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 Adisseo France SAS[2] for authorisation of the product Rovabio™ PHY AP/LC to be used as a feed additive for chickens for fattening, laying hens, weaned piglets and pigs for fattening (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. 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 18 of April of 2006.

The additive Rovabio™ PHY AP/LC is a preparation of 3-phytase produced by the genetically modified micro-organism *Penicillium funiculosum* 4.05b (CBS 111433). 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, user and consumer and the environment of the product Rovabio™ PHY AP/LC, which is a preparation of 3-phytase produced by genetically modified micro-organism *Penicillium funiculosum* 4.05b (CBS 111433) when used under the conditions described in Table 1.

[1] OJ L 268, 18.10.2003, p.29.

[2] Adisseo France SAS, 42, Avenue Aristide Briand, 92160 Antony. France.

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

Additive	3-phytase produced by *Penicillium funiculosum* (CBS 111433)
Registration number/EC No/No (if appropriate)	-
Category of additive	Zootechnical additives
Functional group of additive	Digestibility enhancers

Description			
Composition, description	Chemical formula	Purity criteria (if appropriate)	Method of analysis (if appropriate)
ROVABIO™ PHY LC, liquid preparation Phytase: min. 1000 RPU[a] mL^{-1} Sorbitol: 30% NaCl : 6% Sodium acetate buffer 0.2 M: 10% Potassium sorbate: 0.1% **ROVABIO™ PHY AP**, powder preparation Phytase: min. 2500 RPU g^{-1} Maltodextrin: 35% Silicon dioxide: 47.4 % [a] RPU: One phytase unit is the amount of enzyme that releases 1 micromole inorganic phosphate per minute from sodium phytate as substrate under defined conditions (pH 5.5; 37°C).	-	Total viable count: < 5 x 10^4 CFU g^{-1} Coliforms: < 30 CFU g^{-1} *Escherichia coli*: absent in 25 g *Salmonella*: absent in 25 g Arsenic: < 3 ppm Lead: < 10 ppm Heavy metals as lead: < 40 ppm Mycotoxins: absent Antibiotic activity: absent Yeast and moulds: < 1 CFU g^{-1} Free from the viable production micro-organism	Phytase releases free inorganic phosphate from sodium phytate substrate. The released inorganic phosphate is determined colorimetrically. Phosphate reacts with ammonium molybdate and forms a phospho-molybdate complex. The phospho-molybdate complex is reduced by iron (II) sulfate and a blue complex is formed (molybdenum blue). The color intensity is determined relatively to a standard curve made from a calibrate solution of inorganic phosphate (potassium dihydrogen phosphate).

Trade name (if appropriate)	ROVABIO™ PHY AP and ROVABIO™ PHY LC
Name of the holder of authorisation (if appropriate)	Adisseo

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	-	350	500	-
Laying hens	-	300	500	-
Piglets (weaned)	-	350	500	-
Pigs for fattening	-	350	500	-

Other provisions and additional requirements for the labelling	
Specific conditions or restrictions for use (if appropriate)	**ROVABIO™ PHY AP** is to be incorporated only in mash feed. Shelf life In its original sealed container kept in a dry place: - at 4ºC: 6 months from date of production; - at room temperature (max. 30ºC): 3 months from removal from cold storage; Premix containing ROVABIO™ PHY AP should be used within 1 month after premix manufacturing date. Premix should be stored below 20ºC. A mash feed containing ROVABIO™ PHY AP incorporated via a premix should be stored below 20°C and should be used 1 month after manufacturing date. **ROVABIO™ PHY LC** is to be sprayed on pelleted or mash feed. Shelf life In its original sealed container kept in a dry place: - at 4ºC: 6 months from date of production - at room temperature (max. 30ºC): 3 months from removal from cold storage Feed containing ROVABIO™ PHY LC should be used 2 months after manufacturing date
Specific conditions or restrictions for handling (if appropriate)	**ROVABIO™ PHY LC** - Respiratory protection: combined respiratory protective device, for filtering gas and particles, with a specific canister. - Hand protection: impervious gloves - Eye protection: safety glasses with side-shields - Skin and body protection: impervious clothing - Hygiene measures: When using, do not eat, drink or smoke. Remove and wash contaminated clothing before re-use. Ensure adequate ventilation, especially in confined areas. **ROVABIO™ PHY AP** - Respiratory protection: combined respiratory protective device, for filtering gas and particles, with a specific canister. - Hand protection: impervious gloves - Eye protection: safety glasses with side-shields - Skin and body protection: impervious clothing - Hygiene measures: When using, do not eat, drink or smoke. Remove and wash contaminated clothing before re-use. Ensure adequate ventilation, especially in confined areas.
Post market monitoring (if appropriate)	ADISSEO is approved manufacturer and distributor of feed additives. (α FR 92.002.101). ADISSEO is also FAMI-QS certified. The tall-manufacturer for ROVABIO™ PHY AP/LC will be also approved and FAMI-QS certified. Traceability and procedures systems in place are in compliance with requirement of Regulations 183/2005/EC and

	178/2002/EC. All information (name and address of the supplier, name and address of the customer, date of transaction and delivery) are available immediately on request of authorities.
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
-	-	-	-

ASSESSMENT

1. Introduction

Phytase dephosphorylates phytic acid (myo-inositol hexaphosphate) by hydrolysing the bond between phosphate and myo-inositol in phytic acid and phytates. Between two-thirds and three-quarters of total plant phosphorus is phytate phosphorus but this is not directly available for monogastric animals as they do not produce significant amounts of phytase in their gastrointestinal tracts and the contribution from phytase of plant origin is low. As a consequence inorganic phosphate is added to their diets to meet their phosphorus requirements. Phytase added to feed releases more phosphate from the plant material in the diet reducing the need for the addition of phosphate and minimising phosphorus excretion and its burden on the environment.

Rovabio™ PHY LC/AP is a feed additive with 3-phytase as active ingredient intended to improve phosphorus utilization in animals fed cereal-based diets.

2. Characterisation of the product

Rovabio™ PHY LC/AP is a 3-phytase enzyme formulation marketed in two standard formulations (liquid and powder) and is obtained using a fermentation process with the genetically modified micro-organism *Penicilium funiculosum* as producing organism.

2.1. Qualitative and quantitative composition

Rovabio™ PHY LC is a liquid formulation containing at least 1000 RPU mL^{-1} with sorbitol (30%), NaCl (6%) and sodium acetate buffer (10%) as excipients and potassium sorbate (0.1%) as preservative.

Rovabio™ PHY AP is a powder formulation containing at least 2500 RPU g^{-1} with maltodextrin (35%) and silicon dioxide (47.4%) as excipients.

2.2. Physico-chemical properties and purity

The physico-chemical properties for Rovabio™ PHY LC are: viscosity 6 mPa s, specific gravity 1.18, pH 4.29; and for Rovabio™ PHY AP the bulk density is 295 kg m^{-3}. The particle size distribution for Rovabio™ PHY AP is < 20 µm: 4%; > 20 µm and < 50 µm: 6 %; > 50 µm and < 100 µm: 19%; > 100 µm and < 800 µm: 71%.

Rovabio™ PHY LC/AP 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, 2001). Thus, heavy metal contents as lead is < 40 mg kg^{-1}, arsenic < 3 mg kg^{-1} and lead < 10 mg kg^{-1}. For three batches tested the level of heavy metals was < 5 mg kg^{-1}. The applicant has supplied details of the methods.

Three batches of the final product were tested and no detectable amounts of any of the following mycotoxins were found: aflatoxins B1, B2, G1 and G2, ochratoxin A, sterigmatocystin, T2 toxin and zearalenone, patulin, citrinin, fumonisin B1 and B2.

The enzyme preparation does not possess inhibitory activity against *Staphylococcus aureus*, *Escherichia coli*, *Bacillus cereus*, *Bacillus circulans*, *Streptococcus pyogenes* and *Serratia marcescens* when diluted at 10% (V/V). The results of microbiological analysis of raw material showed that viable aerobic micro-organisms are < 1 cfu g^{-1}, coliforms < 10 cfu g^{-1}, yeasts and moulds < 1 cfu g^{-1}, and *Salmonella* and *Escherichia coli* were undetectable.

2.3. Stability

The stability of phytase in the Rovabio™ PHY product, feed premixtures and mixed feed has been studied in at least three batches, during several months. The results are summarised in Table 2.

Table 2. **Stability in months of phytase in Rovabio™ PHY product, feed premixtures and mixed feed, expressed as ≥ 90% recovery of the initial amount**

	Rovabio™ PHY AP		Rovabio™ PHY LC	
	20°C	30°C	20°C	30°C
Commercial product	3.4	2.9	4	3.2
Premixtures				
Poultry	0.9	0.4		
Pig	0.8	0.4		
Mixed pelleted feed				
Poultry	1	0.4	3	2.1
Pig	0.8	0.4	2.3	2

From Table 2 it can be concluded that Rovabio™ PHY has limited stability. In consequence the applicant proposes to label the product: "A premixture or mash feed containing Rovabio™ PHY AP should be stored below 20°C and used within one month after manufacturing date" and "Feed containing Rovabio™ PHY LC should be used within two months after manufacturing date".

The applicant states that the product can be mixed homogeneously in premixtures and feeds. However, no supporting data has been provided.

2.4. Characterisation of the production organism

2.4.1. Information relating to the genetically modified micro-organism

2.4.1.1. Characteristics of the recipient or parental micro-organism

The recipient strain is *Penicillium funiculosum* A1346. This fungal strain was isolated from the soil. Based on macroscopic and microscopic examinations the fungus was presumed to be close to *P. funiculosum*. The recipient strain is reported to produce at least three phosphatases (e.g. phytase), β-glucanase, xylanase and laminarinase which are secreted to the external medium. The results obtained with the final 3-phytase product from the genetically modified strain may thus, at least in part, result from synergy between 3-phytase and the other enzymatic activities ("hemicellulases") already present in the recipient strain.

No previous commercial use of this strain or any other strains of *P. funiculosum* is indicated in the dossier. Therefore, no history of safe use has been demonstrated for this fungus in the production of enzymes or in any other use. In general, the main concerns with any fungal strain are the production of antibacterials and/or toxins (specifically mycotoxins). No toxicological studies or studies on the production of antibiotics or mycotoxins have been undertaken with the recipient strain or reported for *P. funiculosum* in general; these were performed with the final product (see section 2.2).

Human or animal pathogenicity of *P. funiculosum* has not been reported in the literature. Fungi belonging to the genus *Penicillium* are used as sources of food enzymes and are not

referenced as pathogenic by Directive 2000/54/EC of the European Parliament and of the Council of 18 September 2000 on the protection of workers from risks related to exposure to biological agents at work.

2.4.1.2. Characteristics of the donor organism

The donor organism for the *PhyGo* gene was *P. funiculosum* MW 163 and for the *tub1** gene the donor was *P. funiculosum* IMI134756. Both are of the same species as the recipient organism. Internal transcribed sequence (ITS) comparison suggests that these two fungal strains are very similar to the recipient strain *P. funiculosum* A1346. In fact, *P. funiculosum* MW 163 is derived from *P. funiculosum* A1346 by mutagenesis. No further details are provided about this mutagenesis program but the strains are reported to have morphological and physiological differences.

2.4.1.3. Description of the genetic modification process

Penicillium funiculosum A 1346 protoplasts were transformed using Ca^{2+}-PEG method. The co-transformation included two linearised DNA fragments, purified from plasmids pOT ACS 38.1 and pFY3B. The final producer organism is called *P. funiculosum* 4.05b.

The first plasmid, pOT ACS 38.1, contained a copy of the *PhyGo* gene from *Penicillium funiculosum* MW 163, encoding 3-phytase. To produce this plasmid, a fragment containing the *PhyGo* coding sequence as well as the flanking promoter and terminator sequences was cloned into a commercial vector pCR-Blunt II-TOPO, which contains resistance genes for ampicillin (*bla*) and kanamycin (*neo*). The sequence of *PhyGo* gene is given. The *PhyGo* sequence was claimed to have a low percentage of identity with other phytases but to share sequence similarity with the *PhyA* genes encoding fungal phytases.

The second plasmid, pFY3B, contained the selectable marker gene *tub1** which encodes a slightly modified β-tubulin from *P. funiculosum* IMI134756; this confers resistance to the fungicide benomyl. To produce this plasmid, a fragment containing the *tub1** coding sequence as well as the flanking promoter and terminator sequences was cloned into a commercial vector, pBIISK, which contains *bla,* expression of which would confer ampicillin resistance in bacteria. The sequence of the *tub1** gene is given in the dossier.

The DNA fragments containing *PhyGo* and *tub1** were purified from the rest of the plasmids by agarose gel electrophoresis. The purity of the fragments was confirmed by agarose gel analysis. The absence of antibiotic resistance genes from the final producer organism *P. funiculosum* 4.05b was confirmed by PCR using primers specific for *bla* and *neo* and by Southern analysis using a vector mixture as the probe.

The genes were randomly integrated into the fungal genome. The copy number of the introduced genes is not known. A selectable marker, encoded by *tub1**, is present in the final producer organism.

Glycosylation and the possible microheterogeneity of the 3-phytase in the producer organism *P. funiculosum* 4.05b were not studied. The endogenous phytase in the recipient organism is apparently glycosylated. The phytase phyA of *Aspergillus niger* is heavily glycosylated and shows microheterogeneity in molecular weight distribution. It also has ten Cys residues which form five disulfide bridges. These characteristics are shared by other phytases. The disulfide bridges apparently contribute to the good stability of phytases. Glycosylation is suggested to assist in folding and transport of phytase through the membrane.

2.4.2. Information relating to the product purification process

The enzyme is extracellular and is recovered from submerged fermentation by filtration of the broth to separate the fungal biomass. This is done either in a rotary vacuum drum filter or a tangential microfiltration with organic membranes. The filtrate is concentrated by ultrafiltration, which removes e.g., small organic molecules and peptides (cut-off value between 6 and 50 kDa). Bactofiltration (deep filtration with plate and frame filter press; cut-off 0.45 µm) is done for the formulation or enzyme concentrate. Toxins would be expected to be removed from the final enzyme preparation during these filtration steps, if produced by the recipient micro-organism. The absence of the producer organism has been demonstrated using standard plate assay. The absence of recombinant DNA from the enzyme preparations has been demonstrated by quantitative PCR, the detection limit being 4 pg.µL^{-1} total DNA for the *tub1** fragment and 40 pg µL^{-1} for the *PhyGo* fragment.

2.5. Conditions of use

Rovabio™ PHY AP/LC is intended to be used in feed at a minimum recommended dose of 300 RPU kg^{-1} for laying hens and 350 RPU kg^{-1} for chickens for fattening, weaned piglets and pigs for fattening. A maximum recommended dose is set at 500 RPU kg^{-1} for all species and categories mentioned above.

2.6. 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.

3. Efficacy

3.1. Efficacy for chickens for fattening

Trial 1[3] Balance study

A total of 72 Ross one-day-old chickens were randomly distributed into six treatments (12 replicates of one bird each per treatment). The treatments resulted from the supplementation of a basal corn-soybean meal diet containing 0.22% available P and 0.51% Ca with 0, 250, 500, 750 or 1000 RPU Rovabio™ PHY LC kg^{-1} feedingstuffs (confirmed by analysis). For comparison, a positive control with 0.32% available P was included. The enzyme was sprayed onto the pellets. The birds were weighed on day 12 (beginning of the experiment) and on day 19 and 22. From day 20 to 22, excreta were collected daily for each bird, and after the collection period, it was pooled for analysis. At the end of the experiment, all birds were killed and both tibias removed from six birds per treatment and analysed for P and Ca content.

Supplementation with 500 RPU Rovabio™ PHY kg^{-1} feed, improved P and Ca retention when compared to the negative control, and increased the content of ash, P and Ca in the tibia (Table 3).

[3] Technical dossier/Section III/Annex 2

Table 3. **Effect of Rovabio™ PHY LC on retention of P and Ca and on composition of tibia of chickens for fattening from 12 to 22 days of age**

Rovabio™ PHY (RPU kg^{-1})	Analysed phytase content (RPU kg^{-1})	Retention (%)		Tibia content (mg g^{-1})		
		P	Ca	Ash	P	Ca
0	-	43.9^{a}	56.5^{b}	122.3^{a}	19.7^{a}	38.4^{a}
250	172	46.0^{ab}	59.6^{bc}	130.7^{ab}	21.0^{ab}	41.9^{ab}
500	353	48.2^{b}	63.3^{cd}	134.2^{bc}	22.0^{bc}	43.4^{bc}
750	471	48.3^{b}	64.1^{d}	133.0^{b}	21.4^{ac}	42.6^{b}
1000	583	48.6^{b}	63.6^{d}	136.8^{bc}	22.3^{bc}	44.2^{bc}
Positive Control	-	40.8^{c}	46.9^{a}	143.7^{c}	23.3^{c}	46.7^{c}

$^{a, b, c, d}$: Values in a column with different superscripts differ significantly (P ≤ 0.05)

Trial 2[4] Balance study

A total of 48 one-day-old Ross male chickens were randomly distributed into four treatments (12 replicates of one chick per treatment). The experiment took place from day 12 to 22 of age. The treatments resulted from the supplementation of a basal corn soybean meal diet, containing 0.21% available P and 0.54% Ca with 0, 300 or 400 RPU Rovabio™ PHY LC kg^{-1} feedingstuffs (confirmed by analysis). For comparison, a positive control containing 0.31% available P was also included. The enzyme was sprayed onto the pellets.

The birds were weighed on day 12 (beginning of the experiment) and on day 19 and 22. From day 20 to day 22, excreta were collected daily for each bird and, after the collection period, it was pooled for analysis.

Supplementation with Rovabio™ PHY LC did not affect the digestibility of dry matter or energy in chickens for fattening. P retention was increased with the supplementation with 300 RPU Rovabio™ PHY kg^{-1} feed (Table 4), while that of Ca was only increased with levels of Rovabio™ PHY of 400 RPU kg^{-1}.

Table 4. **Effect of Rovabio™ PHY LC on performance parameters and retention of P and Ca of chickens for fattening from 12 to 22 days of age**

Rovabio™ PHY (RPU kg^{-1})	Analysed phytase content (RPU kg^{-1})	Retention (%)	
		P	Ca
0	-	48.3^{a}	58.5^{b}
300	269	51.1^{b}	57.2^{ab}
400	311	51.9^{b}	60.5^{b}
Positive Control	-	46.1^{a}	53.3^{a}

$^{a, b}$: Values in a column with different superscripts differ significantly (P ≤ 0.05)

Trial 3[5] Balance study

Sixty one-day-old Ross male broilers were divided into six treatments consisting of ten animals individually housed per treatment. Birds were fed a starter corn-soybean meal diet from day 0 to 12 and a grower diet based on corn, soybean meal and extruded soybeans from day 12 to day 22. The treatments resulted from the supplementation of the negative basal diet (0.21% available P; 0.54 % Ca) with 0, 200, 250, 300 or 350 RPU Rovabio™ PHY LC kg^{-1} feedingstuffs (confirmed by analysis). For comparison purposes, a positive control

[4] Technical dossier/Section III/Annex 3

[5] Supplementary information November 2006/Annex B

(0.31 % available P) was also included. The animals were fed *ad libitum*. Total excreta were collected between 19 and 22 days of age.

Retention of P and Ca are reported in Table 5. Two animals (one in negative and one in positive control) died during the experiment due to strangulation in the cage.

Table 5. **Effect of Rovabio™ PHY LC on retention of P and Ca in chickens for fattening**

Rovabio™ PHY (RPU kg^{-1})	Analysed phytase content (RPU kg^{-1})	Retention (%)	
		P	Ca
0	-	47.6^{ab}	54.8^{b}
200	166	49.2^{bc}	56.6^{bc}
250	225	50.3^{bc}	58.0^{bc}
300	270	51.7^{c}	60.5^{c}
350	358	51.7^{c}	60.3^{c}
Positive Control	-	44.4^{a}	49.3^{a}

$^{a, b, c}$: Values in a column with different superscripts differ significantly (P ≤ 0.05)

Retention of P and Ca was significantly improved by the supplementation with 300 RPU Rovabio™ PHY LC kg^{-1} feedingstuffs or higher, in comparison to the negative control.

Trial 4[6] *Growth performance*

A total of 1920 one-day-old Ross 308 male chickens were randomly distributed to six treatments (eight replicates of 40 birds per treatment). The treatments resulted from the supplementation of a basal diet containing 0.25% available P with 0, 350, 500 or 650 RPU Rovabio™ PHY LC kg^{-1} feed (confirmed by analysis). Two more treatments included a positive control containing 0.35% available P, and the positive control supplemented with 500 RPU Rovabio™ PHY kg^{-1} feed. The feeds were given in two phases (starter 0-21 days; grower 21-42 days). The enzyme was sprayed on top of the pellets. Parameters measured included feed intake, weight gain, and feed conversion. Mortality and health condition were monitored.

Table 6. **Effect of Rovabio™ PHY LC on performance parameters of chickens for fattening (0-42 days)**

Rovabio™ PHY (RPU kg^{-1})	Analysed phytase content (RPU kg^{-1})	Feed intake (g)	Weight gain (g)	Feed/gain (g g^{-1})
0	-	3574^{a}	2076^{a}	1.72^{b}
350	245/323	3820^{b}	2318^{b}	1.65^{a}
500	223/404	3951^{bc}	2380^{b}	1.66^{a}
650	490/427	4074^{cd}	2500^{c}	1.63^{a}
Positive control	-	4218de	2511^{c}	1.68^{ab}
Positive control + 500	420/341	4337^{e}	2633^{d}	1.65^{a}

$^{a, b, c, d}$: Values in a column with different superscripts differ significantly (P ≤ 0.05)

Addition of 350 RPU Rovabio™ PHY kg^{-1} feed significantly improved feed intake, weight gain and feed conversion of the birds when compared to the control animals fed the P deficient diet (Table 6). Supplementing the positive control diet with 500 RPU Rovabio™ PHY kg^{-1} feed increased weight gain of birds compared to the unsupplemented treatment. Mortality was low and not related to the supplementation with phytase.

[6] Technical dossier/Section III/Annex 4

Conclusions on chickens for fattening

Evidence of efficacy of Rovabio™ PHY LC in chickens for fattening has been provided in two balance studies and in one growth study. The results support the efficacy of Rovabio™ PHY at a minimum dose of 350 RPU kg^{-1}.

3.2. Efficacy for laying hens

Trial 1[7] Digestibility study

A total of 50 ISA Brown laying hens (age: 34 weeks) were randomly distributed into five dietary treatments (ten replicates of one hen per treatment). The dietary treatments resulted from the supplementation of a basal corn-soybean meal diet (0.16% available P) with 0, 150, 300 or 500 RPU Rovabio™ PHY LC kg^{-1} feed (confirmed by analysis). A positive control containing 0.26% available P was also included. The enzyme was sprayed onto the mash diet. The animals were fed the diets for 15 days. After 17 days of the beginning of the experiment, the animals were killed and ileal contents collected for analysis.

Supplementation with 300 RPU Rovabio™ PHY kg^{-1} feed significantly ($P \leq 0.05$) improved ileal P digestibility (Table 7).

Table 7. **Effect of Rovabio™ PHY LC on ileal digestibility of P in laying hens**

Rovabio™ PHY (RPU kg^{-1})	Analysed phytase content (RPU kg^{-1})	Apparent ileal P digestibility (%)
0	-	35.9[b]
150	141	49.0[ab]
300	272	57.4[a]
500	410	58.3[a]
Positive Control	-	60.1[a]

[a, b]: Values in a column with different superscripts differ significantly ($P \leq 0.05$)

Trial 2[8] Digestibility study

A total of 40 ISA Brown laying hens (age: 54 weeks) were randomly distributed to four treatments (ten replicates of one layer per treatment). The dietary treatments resulted from the supplementation of a corn-soy-bean meal basal diet containing 0.16% available P and 3.2% Ca, with 0, 200, 300 or 500 RPU Rovabio™ PHY LC kg^{-1} feed (confirmed by analysis). The enzyme was sprayed onto the mash diet. The animals were fed the experimental diets for ten days, after which they were sacrificed and ileal contents collected. The ileal contents of two hens were pooled, resulting in five replicates per treatment.

Ileal digestibility of P was significantly increased only with levels of Rovabio™ PHY LC of 300 and 500 RPU kg^{-1} (Table 8).

[7] Technical dossier/Section III/Annex 5

[8] Technical dossier/Section III/Annex 6

Table 8. **Effect of Rovabio™ PHY LC on ileal digestibility of P and Ca of laying hens**

Rovabio™ PHY (RPU kg^{-1})	Analysed phytase content (RPU kg^{-1})	Apparent ileal digestibility (%)	
		P	Ca
0	-	44.6^{a}	48.7^{ab}
200	161	52.6^{ab}	44.0^{b}
300	272	53.3^{b}	54.4^{ab}
500	395	56.2^{b}	63.0^{a}

$^{a, b}$: Values in a column with different superscripts differ significantly (P≤ 0.05)

Trial 3[9] Production study

A total of 240 18-week-old brown Hysex laying hens were randomly allocated to five dietary treatments (12 replicates of four birds each per treatment). The experimental diets resulted from the supplementation of a corn-soybean meal basal diet with 0, 200, 300, 500 or 650 RPU kg^{-1} feed (confirmed by analysis). The liquid formulation of the enzyme was used and mixed in the mash feed. The experiment lasted for 24 weeks. The parameters measured comprise body weight, feed intake, feed conversion, egg production, and number of eggs cracked, dirty and with soft shell. Health status was monitored daily.

Supplementation with Rovabio™ PHY LC did not affect feed intake, final weight, weight gain or mean egg weight during the experimental period (Table 9). Supplementation with 200 or more RPU kg^{-1} feed significantly (P ≤ 0.05) improved egg production (total and percentage), egg mass (total per cage) and feed conversion. No differences were observed in the number of dirty, cracked or soft-shelled eggs. No animals died during the experiment.

Table 9. **Effect of Rovabio™ PHY LC on performance of laying hens (18-42 weeks)**

Rovabio™ PHY (RPU kg^{-1})	Analysed phytase content (RPU kg^{-1})	Final weight (g bird^{-1})	Egg production		Egg weight (g)	Egg mass (g bird^{-1} day^{-1})	Feed conversion (egg/feed)
			(n cage^{-1})	(%)			
0	-	1935	577.3^{a}	85.9^{b}	55.1	53.1^{b}	0.410^{b}
200	157	1977	597.1^{b}	88.9^{a}	56.2	53.8^{a}	0.426^{a}
300	225	1868	598.4^{b}	89.1^{a}	56.2	53.8^{a}	0.428^{a}
500	368	1918	612.3^{b}	91.1^{a}	56.2	54.6^{a}	0.433^{a}
650	469	1947	602.8^{b}	89.7^{a}	56.2	53.8^{a}	0.427^{a}

$^{a, b}$: Values in a column with different superscripts differ significantly (P ≤ 0.05)

Conclusions on laying hens

Evidence of efficacy of Rovabio™ PHY LC in laying hens has been provided in two digestibility studies and in one long term study. The results support the efficacy of Rovabio™ PHY at a minimum dose of 300 RPU kg^{-1}.

3.3. Efficacy for piglets

Four studies carried out at two different locations were presented in the dossier.

Trial 1[10] Digestibility trial

[9] Technical dossier/Section III/Annex 7

[10] Technical dossier/Section III/Annex 8

In the first study, 12 piglets (German Landrace x Piétrain) in the post weaning stage (8.5 kg) were randomly allocated to four groups of three animals each and housed individually in metabolic cages. The test set-up was a fully randomised block design; each treatment group was tested on six animals. The piglets were fed a corn/barley/soy phosphorus-deficient diet (0.43% total P corresponding to 0.2% available P). Rovabio™ PHY LC was administered via the feed at concentrations of 0, 250, 500 and 1000 RPU kg^{-1} (confirmed by analysis). There was an adaptation period and a quantitative collection period of faeces for one week. Statistical analysis was done via the GLM procedure.

The main effect was an increase in P and Ca apparent digestibility with significant effects in all treated groups compared to the negative controls (Table 10).

Table 10. **Effect of Rovabio™ PHY LC on P and Ca digestibility in piglets**

Rovabio™ PHY (RPU kg^{-1})	Analysed phytase content (RPU kg^{-1})	Faecal digestibility (%)	
		P	Ca
0	0	42.0[c]	55.5[c]
250	246	59.1[b]	64.1[b]
500	544	64.2[b]	68.6[ab]
1000	1009	70.4[a]	72.0[a]

[a, b, c]: Values in a row not sharing a common superscript are significantly different (P≤0.001)

Trial 2[11] *Digestibility and performance trial*

A total of 36 piglets (German Landrace x Piétrain) in the post weaning stage (28 days old, mean body weight 7.4 kg) were randomly allocated to three groups of twelve animals each, according to origin, sex and live weight and kept individually in pens. They were fed a P-deficient diet (corn/barley/soy) containing 0.45% total P corresponding to 0.2% available P. Liquid Rovabio™ PHY LC was administered via the feed at concentrations of 0, 250, 500 U kg^{-1} (confirmed by analysis). Feed was given dry *ad libitum*. During the test, which lasted six weeks (from 7.4 to 26.5 kg live weight), animals were weighed weekly, daily gains recorded and feed/gain ratio calculated. In the third test week faeces were collected. Digestibility was measured with 0.5% insoluble ash as inert marker added to the feed. At the end of the test, the phalanx media of the right front leg was dissected from six animals from each group and analysed. Statistical analysis was done via the GLM procedure.

Table 11. **Effect of Rovabio™ PHY LC on performance, mineral digestibility and bone mineral content in piglets (0-6 weeks)**

Rovabio™ PHY (RPU kg^{-1})	Analysed phytase content (RPU kg^{-1})	Daily feed intake (g day^{-1})	Average daily gain (g day^{-1})	Feed/gain (g g^{-1})	Faecal digestibility (%)		Bone (mg g^{-1})	
					P	Ca	P	Ca
0	0	682[b]	391[b]	1.75[a]	49.9[c]	63.5[b]	32.9[c]	68.7[b]
250	264	772[a]	451[a]	1.72[a]	54.7[b]	63.7[b]	39.2[b]	80.9[a]
500	485	765[a]	452[a]	1.70[a]	63.9[a]	69.4[a]	42.8[a]	84.6[a]

[a, b, c]: Values in a row not sharing a common superscript are significantly different (P≤0.001)

The main effects (Table 11) were a dose-related increase in average daily gain and apparent P digestibility (with all groups being statistically significantly different one from the other). Furthermore, bone P and Ca content was significantly increased at the recommended dose of Rovabio™ PHY LC.

[11] Technical dossier/Section III/Annex 9

Trial 3[12] Digestibility and performance trial

A total of 24 piglets (crossbreeds) in the post weaning stage (body weight 10 – 12 kg) were randomly allocated to four groups of six animals individually housed each. They were given a P-deficient feed containing 0.12% available P. An additional positive control group was given feed containing 0.22% available P. Rovabio™ PHY LC was administered via the feed at concentrations of 0, 250, 350 RPU kg^{-1} (confirmed by analysis) throughout the entire treatment period (3 weeks). The animals were fed at 2.5 times the maintenance need. During the test, animals were weighed weekly, feed intake and daily gains recorded and feed/gain ratio calculated. During the third week, faeces were collected quantitatively. Data were analysed by ANOVA.

No effects were observed at any of the doses for feed intake, body weight gain or feed/gain ratio (0-3 weeks). However, P digestibility was significantly greater in both treated groups than in negative controls (Table 12). No dose-response relationship was evident. Ca digestibility was not significantly different.

Table 12. **Effect of Rovabio™ PHY LC on P and Ca digestibility in piglets**

Rovabio™ PHY (RPU kg^{-1})	Analysed phytase content (RPU kg^{-1})	Faecal digestibility (%)	
		P	Ca
0	-	51.1[a]	54.9
250	319	57.1[bc]	60.3
350	381	59.4[c]	60.0
Positive control	-	53.3[ab]	59.9

[a, b, c]: Values in a row not sharing a common superscript are significantly different ($P \leq 0.001$)

Trial 4[13] Digestibility trial

Forty piglets were divided into five treatments consisting of eight animals individually housed per treatment (weight at start: 8.8 kg). The treatments resulted from the supplementation of a corn/soybean meal basal diet (0.15 % available P; Ca 0.65 %) with 0, 200, 350 or 500 RPU Rovabio kg^{-1} feedingstuffs (confirmed by analysis). A positive control (0.25 % available P; Ca 0.65%) was also added. The enzyme was sprayed on the pelleted feed. Feeding was restricted. Following an adaptation period of five days, faeces were collected quantitatively for another five days. At the end of the trial, piglets were killed and samples were taken from the anterior left metacarpus. Faecal digestibility of P and Ca and bone mineralization were measured. There was no mortality during the trial.

Table 13. **Effect of Rovabio™ PHY LC on P digestibility and bone mineralization in piglets**

Rovabio™ PHY (RPU kg^{-1})	Analysed phytase content (RPU kg^{-1})	Faecal P digestibility (%)	Bone P content (mg g^{-1})
0	-	32.5[a]	19.2[a]
200	164	39.1[ab]	20.0[ab]
350	316	45.1[bc]	22.3[c]
500	440	51.4[c]	22.4[c]
Positive Control	-	39.9[ab]	21.5[bc]

[a, b, c]: Values in a column with different superscripts differ significantly ($P \leq 0.05$)

[12] Technical dossier/Section III/Annex 10

[13] Supplementary information November 2006/Annex C

Supplementation with Rovabio™ PHY LC at 350 RPU kg^{-1} feedingstuffs significantly improved P digestibility and P bone content in comparison to the negative control (Table 13).

Conclusions on efficacy for piglets

Evidence of efficacy of Rovabio™ PHY LC in piglets has been provided in four digestibility studies. The results support the efficacy of Rovabio™ PHY at a minimum dose of 250 RPU kg^{-1}.

3.4. Efficacy for pigs for fattening

Four studies carried out at three different locations were presented in the dossier.

Trial 1[14] Digestibility trial

Eight pigs (German Landrace x Piétrain) in the fattening stage (castrated males, 57.3 kg) were randomly allocated to four groups of two animals each, kept individually in metabolic cages. The test set-up was a fully randomised block design. The test was conducted in three runs, so each treatment group was tested on six animals. The experimental treatments resulted from the supplementation of a P-deficient feed (0.13% available P) with Rovabio™ PHY LC at 0, 250, 500 and 1000 RPU kg^{-1} (confirmed by analysis). The animals were fed restrictively. Each test run consisted of a adaptation and collection (faeces) period of one week. Statistical analysis was done via the GLM procedure.

Table 14. **Effect of Rovabio™ PHY LC on P and Ca digestibility in pigs for fattening**

Rovabio™ PHY (RPU kg^{-1})	Analysed phytase content (RPU kg^{-1})	Faecal digestibility (%)	
		P	Ca
0	0	24.0^{d}	33.7^{b}
250	249	37.3^{c}	35.4^{b}
500	454	44.8^{b}	41.2^{ab}
1000	847	52.0^{a}	47.2^{a}

$^{a, b, c, d}$: Values in a row not sharing a common superscript are significantly different ($P \leq 0.001$)

The main effects were an increase in apparent P digestibility with significant effects noted in treated animals *vs.* negative controls (Table 14). A dose-related effect was observed, with significant inter-group differences at all levels. Ca digestibility was only significantly higher at the highest tested dose of Rovabio™ PHY LC.

Trial 2[15] Bone mineralization trial

Forty growing pigs (from 35 to 85 kg) were divided into five treatments consisting of eight animals individually housed per treatment. The treatments were a positive control (0.22 % available P) and a negative control (0.12 % available P) supplemented with Rovabio™ PHY LC at 0, 250, 350 or 600 RPU kg^{-1} feedingstuffs (confirmed by analysis). The enzyme was sprayed on the pelleted feed. Feeding was *ad libitum*. At the end of the trial pigs were slaughtered and samples were taken from the anterior left metacarpus. Bone mineralization was measured.

Supplementation with Rovabio™ PHY LC at 350 RPU kg^{-1} feedingstuffs significantly improved bone P content in comparison to the negative control (Table 15).

[14] Technical dossier/Section III/Annex 11

[15] Supplementary information November 2006/Annex D

Table 15. **Effect of Rovabio™ PHY LC on bone mineralization in pigs**

Rovabio™ PHY (RPU kg^{-1})	Analysed phytase content (RPU kg^{-1})	Bone P content (mg g^{-1})
0	-	32.9^{a}
250	250	36.3^{ab}
350	341	39.1^{b}
600	630	37.5^{b}
Positive Control	-	39.1^{b}

$^{a, b}$: Values in a column with different superscripts differ significantly (P≤ 0.05)

Trial 3[16] Performance and digestibility trial

A total of 40 growing pigs (cross breeds) were randomly allocated to five groups of eight animals each, individually housed in cages. They were given a P-deficient feed containing 0.10% available P. An additional positive control group was given feed containing 0.20% available P. The total P content in the feed was 0.35% (0.43% in positive controls). Rovabio™ PHY LC was administered via the feed at concentrations of 0, 200, 300, 400 RPU kg^{-1} (confirmed by analysis) throughout the entire treatment period. Feeding was restricted. Faeces samples were taken after two and four weeks for Ca and P digestibility studies. Digestibility was measured with 0.5% HCl-insoluble ash as inert marker added to the feed. The animals were slaughtered after the four-week administration period and the left metacarpus was analysed for mineral content. Statistical analysis was done via the ANOVA procedure.

Table 16. **Effect of Rovabio™ PHY LC on mineral digestibility (total period) and bone mineral content in pigs for fattening (0-4 weeks)**

Rovabio™ PHY (RPU kg^{-1})	Analysed phytase content (RPU kg^{-1})	Faecal digestibility (%)		Bone content (mg g^{-1})	
		P	Ca	P	Ca
0	-	32.5^{a}	49.0^{b}	30.6^{a}	63.9^{a}
200	225	38.2^{b}	42.2^{ab}	33.6^{b}	70.0^{ab}
300	396	44.3^{c}	47.6^{b}	35.1^{b}	75.0^{b}
400	423	47.5^{c}	48.2^{b}	34.9^{b}	73.2^{b}
Positive Control	-	36.3^{b}	38.3^{a}	35.1^{b}	73.0^{b}

$^{a, b, c}$: Values in a row not sharing a common superscript are significantly different (P ≤ 0.001)

There were no significant effects of Rovabio™ PHY LC on body weights (0-4 weeks). P digestibility, measured over the whole period, was significantly higher in the three treated groups than in the negative controls but there were no significant differences between the two higher doses (300 and 400 RPU kg^{-1}) (Table 16). Ca digestibility was unchanged. Bone P content was significantly improved from the lowest dose on, while bone Ca content was significantly improved at doses above 300 RPU kg^{-1} Rovabio™ PHY LC.

Trial 4[17] Performance and digestibility trial

A total of 128 male and female pigs for fattening (Landrace; 22.8 kg body weight on average) were divided into nine blocks according to initial weight and sex. Each block consisted of four pens with three males or four females per pen. The four treatments consisted of a positive control with normal P level (total P: 0.472% for grower diet, i.e.,

[16] Technical dossier/Section III/Annex 12

[17] Technical dossier/Section III/Annex 13

0.23% available P, 0.41% for finisher diet, i.e., 0.19% available P) and the supplementation of a negative control diet with low P content (total P: 0.37% for grower diet, i.e., 0.13% available P, 0.31% for finisher diet, i.e., 0.09% available P) with 0, 500 or 5000 RPU Rovabio™ PHY LC kg^{-1} feedingstuffs. However, the analysed activity in feed was only approximately 50% of the intended dose. Body weight and feed intake were recorded at weeks 5, 9 and 16. In week 13, titanium dioxide was added to the feed as inert marker and after 10 days faeces were collected per pen for digestibility measurements. At the end of the trial (16 weeks), the left metacarpus of 12 animals per treatment was dissected and analysed for minerals. For statistical analysis, a randomised block design was used.

Rovabio™ PHY LC, at both levels, significantly improved daily weight gain, feed/gain ratio and bone P content compared to the negative control (Table 17).

Table 17. **Effect of Rovabio™ PHY LC on performance, digestibility and bone minerals in pigs for fattening (0-16 weeks)**

Rovabio™ PHY (RPU kg^{-1})	Analysed phytase content (RPU kg^{-1})	Daily feed intake (g day^{-1})	Average daily gain (g day^{-1})	Feed/gain (g g^{-1})	Faecal digestibility (%)		Bone content (mg g^{-1})	
					P	Ca	P	Ca
0	-	1419[b]	498[c]	2.85[c]	16.9[c]	51.8[a]	10.2[c]	27.2[ab]
500	280	1748[a]	647[b]	2.70[b]	24.1[c]	54.3[a]	11.4[b]	27.3[a]
5000	2500	1875[a]	742[a]	2.53[a]	64.8[a]	50.1[a]	12.5[a]	26.6[b]
Positive control	-	1888[a]	733[a]	2.58[ab]	38.0[b]	41.8[b]	12.3[a]	27.5[a]

[a, b, c]: Values in a row not sharing a common superscript are significantly different ($P \leq 0.001$)

Conclusions of efficacy for pigs for fattening

Evidence of efficacy of Rovabio™ PHY LC in pigs for fattening has been provided in four studies. The results support the efficacy of Rovabio™ PHY at a minimum dose of 350 RPU kg^{-1}.

4. Safety

4.1. 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 trait which can be expressed or no longer expressed

The newly introduced genes in the producer strain *P. funiculosum* 4.05b encode 3-phytase and β-tubulin. Stability of the expression was verified by comparing phytase activity in the initial stock and six successive conidia generations grown in liquid medium. The production strain was characterized using BIOLOG MICROLOG SYSTEM™ microbial identification technology with 95 carbon source utilization tests. The potential effects of glycosylation are considered in the general assessment of the safety of the enzyme preparation (sections 4.3 and 4.4).

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

The producer strain *P. funiculosum* 4.05b contains *PhyGo* and *tub1** genes with their natural 5' and 3' flanking sequences derived from *P. funiculosum* MW 163 and

P. funiculosum IMI134756, respectively. There are multiple copies of the introduced genes randomly integrated in the fungal genome; the number and location of the copies is not known. The formation of hybrid proteins cannot be excluded. However, in the event that such proteins could be formed and eventually co-purified with the secreted phytase, their potential harmful effects would have been evaluated in the general assessment of the safety of the enzyme preparations for the target animals and humans. The absence of vector DNA was demonstrated. The molecular characterisation of the genetic modification as such did not trigger any particular safety concerns regarding the final product.

4.1.2. Conclusions regarding the genetic modification

No sequences that cause concern in terms of toxin or antibiotic production were added to the producer strain *Penicillium funiculosum* 4.05b. The Panel is of the opinion that the molecular characterisation of the genetic modification as such does not trigger any particular safety concerns regarding the final product.

4.2. Safety for the target species

4.2.1. Safety for chickens for fattening[18]

A floor-pen tolerance study was conducted with 960 male Ross 308 broilers from 0 to 42 days of age. The birds were randomly distributed to three experimental treatments (eight replicates of 40 birds per treatment). The feeds were given in two phases (starter: 0-21 days; grower: 21-42 days). The treatments resulted from the supplementation of a corn-soybean basal diet (0.25% available P) with Rovabio™ PHY LC at 0, 500 or 5000 (10X maximum recommended dose) RPU kg^{-1} feed. The liquid formulation of the enzyme was added to feed after pelleting. Parameters measured included animal performance and daily health monitoring. At the end of the experiment, ten birds from the control and the tolerance groups were randomly selected and killed for post-mortem examination.

Enzyme activity in feed was analysed and found to be 223 and 3507 RPU kg^{-1} feed, representing approximately 0.4X and seven times the maximum recommended dose.

Supplementation with seven times the maximum recommended dose of Rovabio™ PHY significantly increased feed intake, weight gain and feed conversion when compared to the control. Mortality was low (1.6%) and not affected by phytase addition, and *post-mortem* examination of the dead and sacrificed birds showed no alterations related to the treatments.

Since chickens for fattening tolerated seven times the maximum recommended dose, it is concluded that use of the product at the recommended dose range is safe for chickens for fattening.

4.2.2. Safety for laying hens[19]

A tolerance study was conducted with 144 18-week-old brown Hysex laying hens, which were randomly distributed to three treatments (12 replicates of four hens per treatment). The treatments resulted from the supplementation to a basal corn-soy diet with 0, 500 or 5000 RPU Rovabio™ PHY LC kg^{-1} feed (10X maximum recommended dose). The liquid formulation of the enzyme was added to the mash feed. The experiment lasted for 24 weeks. Performance parameters as well as daily monitoring of animal health were

[18] Technical dossier/Section IV/Annex 2
[19] Technical dossier/Section IV/Annex 3

recorded. At the end of the experiment, blood samples were taken from eight hens per treatment for haematology and clinical chemistry.

Enzyme activity in feed was analysed and found to be 368 and 3397 RPU kg^{-1} feed, representing approximately 0.7 times and seven times the maximum recommended dose, respectively.

Performance parameters and egg quality were not negatively affected by the supplementation with seven times the maximum recommended dose of Rovabio™ PHY. Blood haematology and clinical chemistry were not affected, with the exception of the mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration, which were increased in both groups supplemented with the phytase. These changes are considered to be in the normal range.

Since laying hens tolerated seven times the maximum recommended dose, it is concluded that use of the product at the recommended dose range is safe for laying hens.

4.2.3. Safety for piglets[20]

Ninety six newly weaned piglets (Landrace; 7.3 kg body weight) were randomly distributed by weight into 6 blocks. Each block contained four pens with four animals each. Each treatment comprised 22 to 24 piglets. The four experimental treatments consisted of a positive control (available P: 0.28-0.34 %), and a negative control (available P: 0.18-0.24%), supplemented with Rovabio™ PHY AP at 0, 500 (1X) or 5000 (10X) RPU kg^{-1} Rovabio™ PHY AP. The diets (pre-starter days 0-14; starter days 14-28) were presented in mash form and offered *ad libitum*. Body weight and feed intake were measured at the start, at day 14 and at the end of the trial (day 28). Morbidity, clinical signs and mortality were checked at least once daily throughout the study. On day 28 blood samples were taken from one pig in each pen for clinical chemistry and haematology analysis; additionally, six piglets from the control group and six piglets from the tolerance group were killed for macroscopic examinations. For statistical analysis, a randomised block design was used with the pen as experimental unit.

Enzyme activity measured in feed was 520 and 4028 RPU kg^{-1}, representing one time and eight times the maximum recommended dose, respectively.

No mortality was recorded during the trial. Rovabio™ PHY AP at both levels did not negatively affect animal performance. Although there are some significant differences between treatments for some haematological/blood biochemical values (RBC, AP, serum P, serum Ca), but were within the physiological range for piglets. Macroscopic observations did not reveal any abnormality.

Since piglets tolerated eight times the maximum recommended dose, it is concluded that use of the product at the recommended dose range is safe for piglets.

4.2.4. Safety for pigs for fattening[21]

The tolerance test for pigs for fattening was performed in conjunction to efficacy trial 4. The four experimental treatments consisted of a positive control, and the supplementation of the negative control with Rovabio™ PHY LC at 0, 500 (1X) or 5000 (10X maximum recommended dose) RPU kg^{-1}. Animals were checked at least once daily throughout the study. At the end of the trial (16 weeks), blood samples were taken from one pig in each pen for clinical chemistry and haematological analysis; all pigs were killed and

[20] Technical dossier/Section IV/Annex 4
[21] Technical dossier/Section IV/Annex 5

macroscopic examination was carried out. For statistical analysis, a randomised block design was used (GLM procedure) with the pen as experimental unit.

Enzyme activity in feed was about 50% of that intended (284 and 2500 RPU kg^{-1}).

Six pigs were removed during the study due to lameness (one from treatment 500), meningitis (one from treatment 5000) or sudden death (two from treatment positive control; one from negative control; one from treatment 500) and not used for the calculations. Rovabio™ PHY LC at both levels did not negatively affect animal performance. Although there are some significant differences between treatments for some blood biochemical values (GTP, AP, serum P), they are either an expected consequence of treatment or fall within physiological range for pigs and are not considered an adverse effect. Observations at necropsy did not reveal any treatment-related abnormalities.

Since pigs tolerated five times the maximum recommended dose, it is concluded that use of the product at the recommended dose range is safe for pigs for fattening. Safety for pigs for fattening is further reassured by the safety demonstrated at eight times the maximum recommended dose in piglets.

4.2.5. Conclusions on safety for the target species

There is some concern that the low stability of Rovabio™ PHY could lead to a significant under-dosing of the animals, especially in the tolerance studies. A conservative approach has been taken in assessing the safety for the target species by using the analysed phytase activities in the feeds.

Rovabio™ PHY is well tolerated at seven times (chickens for fattening, laying hens), eight times (piglets) and five times (pigs for fattening) the maximum recommended dose. Safety for pigs for fattening is further reassured by the safety demonstrated at eight times the maximum recommended dose in piglets.

Therefore, it is concluded that Rovabio™ PHY is safe for the target species when used according to the proposed conditions of use.

4.3. Safety for the consumer

All the toxicity studies were carried out with phytase concentrate (see section 2.4.2).

4.3.1. Genotoxicity studies

4.3.1.1. Reverse bacterial mutation assay

A reverse bacterial mutation test was performed under OECD guideline 471 using *Salmonella* Typhimurium strains TA98, TA100, TA102, TA1535 and TA1537, and strain WP2uvrA of *Escherichia coli* in the presence and absence of liver preparation from phenobarbitone and β-naphthoflavone treated rats. Concentrations of phytase were up to 5000 µg $plate^{-1}$ (corresponding to the recommended maximum test-concentration for soluble, non-cytotoxic substances).

It was concluded that the phytase concentrate is not mutagenic at any concentration used in these tests, either with or without metabolic activation.

4.3.1.2. *In vitro* test for genetic damage in mammalian species (chromosomal aberration assay)

Two chromosome aberration tests were conducted under OECD guidelines number 473 using Chinese hamster ovarian cells which were exposed to phytase tested both with and

without metabolic activation. The concentrations of phytase ranged from 500 to 5000 µg mL^{-1} in the first study and from 400 to 4000 µg mL^{-1} in the second study. Cells treated with phytase either with or without activation showed no significant numbers of cells showing structural chromosomal aberration to those observed in concurrent solvent controls.

It was concluded that the phytase concentrate did not induce structural chromosomal aberrations in this *in vitro* cytogenetic test system.

4.3.2. Acute oral toxicity study

In a GLP-compliant 'limit test' conducted under OECD guideline 423 a group of three female Wistar rats were given phytase concentrate broth at a dose level of 20000 mg kg^{-1} bw (44319 RPU kg^{-1} bw). Since all treated animals survived a further group of three additional females were treated with the same dose. In both steps, there was no mortality. In one animal a moderate activity decrease with piloerection was noted between 15 minutes and one hour after treatment. No other clinical signs were observed at any time during the study. The mean body weights and the body weight gains were in the normal range during the 14-day observation period. Based on this study the enzyme preparation can be classified as non-toxic.

4.3.3. Sub-chronic oral toxicity study

4.3.3.1. Two-week oral toxicity study

In a GLP-compliant study conducted under OECD guideline 408, three groups of five male and five female Wistar rats were given by oral gavage phytase concentrate broth at a dose of 70, 900 or 11850 RPU kg^{-1} bw day^{-1} for two consecutive weeks. Clinical observations were made once daily. Body weight was measured on days 0, 3, 7, 10 and 14 of the study. A gross pathology examination was conducted at the end of the treatment period and selected organs were weighed. Data on haematology and clinical chemistry for individual animal were not provided. There was a transient decrease in body weight gain in the high dose group during the first week of the study. No pathological alterations related to the test substance were found in any treated group of animals. No mortality occurred during the study. From this study a NOAEL of 900 RPU kg^{-1} bw day^{-1} can be identified.

4.3.3.2. 13-week oral toxicity study

In a GLP-compliant study conducted under OECD guideline 408, three groups of ten male and ten female Wistar rats were given phytase concentrate broth by oral gavage at doses of 100, 1000 or 10000 RPU kg^{-1} bw day^{-1} for 13 consecutive weeks. The animals were checked daily for mortality and clinical signs. Neurobehavioral effects were assessed by a battery of functional tests in all rats at the end of the treatment period. Body weight and food consumption were recorded before the beginning of the study, then once weekly. Haematology, clinical chemistry and urine analysis were carried out at the end of the study. There were no clinical signs of toxicity and no effects on mean daily food intake, body weight, body weight gain, ophthalmology, haematology, clinical chemistry or urine analysis. Organ weights were similar between groups and no mortality occurred. Macroscopic and microscopic examinations revealed no evidence of treatment-related effects. The NOAEL from this study is 10000 RPU kg^{-1} bw day^{-1}.

4.3.4. Conclusions regarding consumer safety

Based on the studies conducted with phytase concentrate broth this product is not mutagenic and shows no effects which would be of concern for consumer safety.

4.4. Safety for the user

4.4.1. Skin irritation

A GLP-compliant study according to OECD guideline 404 was performed in rabbits to assess the skin irritation effect of phytase concentrate broth. Groups of three New Zealand white rabbits were each applied a single dermal dose of 0.5 mL of the test substance. The skin was examined for evidence of irritation after 24, 48 and 72 h following the removal of the test substance. The animals were examined at 1, 24, 48 and 72 h after the patch removal. There was no evidence of irritation symptoms or other clinical signs.

4.4.2. Eye irritation

A GLP-compliant study according to OECD guideline 405 was performed in rabbits to assess the eye irritation effect of Phytase concentrate broth. Groups of three New Zealand white rabbits were each administered a single dose of 0.1 mL of the test substance. The eyes were examined at 1, 24, 48 and 72 h after the application. One hour after treatment slight redness and slightly increased discharge excretion was present although these symptoms were reversible. Based on the results, the product is concluded to be slightly irritant to the eye.

4.4.3. Skin sensitisation

A GLP-compliant study according to OECD guideline 406 was performed to assess the potential skin sensitization potency of Phytase concentrate broth. Group of ten female Dunkin Hartley guinea pigs was subjected to sensitization procedures in a two-stage operation, i.e., an intradermal treatment and a topical application. The test substance was used in concentration of 5% for intradermal injections and in undiluted state for dermal sensitization treatment. The 50% of the animals challenged with the product showed patchy, confluent, intense erythema and swelling on the skin surface. The product is concluded to be a dermal sensitizer.

4.4.4. Inhalation toxicity

No acute inhalation toxicity study was performed. However, as respiratory protection is always recommended for enzyme preparations due to potential respiratory sensitization, and the systemic toxicity profile indicates no adverse effects, the omission of such a study is acceptable.

4.4.5. Conclusions regarding user safety

All these studies were performed with a concentrated batch of the active substance phytase, but not with the final product as marketed.

There was evidence of eye irritation and skin sensitization when a concentrated phytase solution was used; therefore the product should be regarded as an irritant to the eyes and as a skin sensitizer for users of the product.

4.5. Safety for the environment

The producer organism is removed from the product and the recombinant DNA is below the limit of detection (40 pg μL^{-1}). The active substance is a 3-phytase, which can be considered essentially similar to naturally occurring phytases. Phytases are proteins and are normally degraded in the gastrointestinal tract and the environment. Therefore no further environmental risk assessment is required.

5. Post-market monitoring

No risks associated with the use of the product are foreseen, and therefore there is no need for specific requirements other than those specified by the Feed Hygiene Regulation and Good Manufacture Practice.

CONCLUSIONS

There is a significant loss of phytase activity after incorporation of Rovabio™ PHY in premixtures or feeds at temperatures above 20°C. There are concerns that this low stability could lead to a significant under-dosing of the animals, despite the warnings included in the proposed product label.

Although most of the studies for efficacy and tolerance have been performed with the liquid formulation, these are considered to apply to both forms of the product.

Evidence of efficacy of Rovabio™ PHY has been provided at a minimum dose of 300 RPU kg^{-1} for laying hens, 350 RPU kg^{-1} feed for chickens for fattening and pigs for fattening and 250 RPU kg^{-1} feed for piglets.

Rovabio™ PHY is tolerated at seven times (chickens for fattening, laying hens), eight times (piglets) and five times (pigs for fattening) the maximum recommended dose. Safety for pigs for fattening is further reassured by the safety demonstrated at eight times the maximum recommended dose in piglets. Therefore, it is concluded that Rovabio™ PHY is safe for the target species when used at the recommended dose range.

The introduced genes do not trigger any particular safety concerns in terms of toxins or antibiotics. The final enzyme preparation contains no cultivable producer organisms and the level of the newly introduced DNA is below the limit of detection.

Phytase as present in Rovabio™ PHY LC/AP does not pose a genotoxic hazard and did not show a toxic response of consumer relevance in a sub-chronic toxicity study. It is concluded that there is no evidence for concern regarding consumer safety.

All the studies performed to assess user safety were based on a concentrated batch of the active substance phytase, but not on the final products. There was evidence of irritation for the eye and delayed contact hypersensitivity for the skin. As no acute inhalation toxicity study was provided the product should be regarded as potentially toxic by inhalation.

The active substance is a 3-phytase which can be considered essentially similar to naturally occurring phytases and therefore no risk for the environment is foreseen.

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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 Rovabio™ PHY AP/LC

In the current application authorisation is sought for ROVABIO™ PHY under the category zootechnical additives, digestibility enhancers, group 4(a), according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought to use ROVABIO™ PHY for chickens for fattening, laying hens, weaned piglets and fattening pigs, according to EFSA-Q-2005-281. ROVABIO™ PHY is an enzyme preparation, available in powder form (ROVABIO™ PHY AP) and in liquid form (ROVABIO™ PHY LC). The enzyme is produced by *Penicillium funiculosum* 4.05 b (CBS 111443). The feed additive has a target activity of minimum 2500 RPU/g for ROVABIO™ PHY AP and of minimum 1000 RPU/ml for ROVABIO™ PHY LC, where 1 RPU is the amount of enzyme that releases 1 µM inorganic ortho-phosphate per minute from sodium phytate as substrate at pH 5.5 and 37° C. It is intended to be mixed into compound feedingstuffs to a level of 250 RPU/kg.

For the determination of the enzyme activity of 3-phytase, in the *feed additive,* ROVABIO™ PHY*,* a colorimetric method is proposed by the applicant. The principle of the method is that after diluting the feed additive and performing an enzyme kinetics test in the presence of sodium phytate, the amount of phosphate released is measured via the formation of a phosphomolybdate complex by reduction of iron (II). The amount of phosphate released is read off a calibration curve constructed using inorganic phosphate. The method is considered suitable for official control purposes.

For the determination of the enzyme activity in *premixtures* and in *feedingstuffs* the applicant proposes an adapted version of the colorimetric method applied for feed additives. In the adapted version an initial step of extraction of the active agent from the premixture or feedingstuff is included.

When tested on *premixtures* for chicken and piglet feed the method's performance characteristics include relative repeatability standard deviation (RSD_r) values of 1.1-4.2 %; and recovery rates between 34 and 67 %.

When tested on *feedingstuffs* (chicken and piglet feed) the method's performance characteristics include RSD_r values of 1.0-7.6 %; and recovery rates between 100 and 131 %.

The applicant's method follows well known principles for the determination of phytase activity in various matrices, and transferability of the method has been verified by testing the method with a second laboratory. It should be noted though that other analytical methods for the determination of phytase activity in premixtures and feedingstuffs exist, which have been validated in inter-laboratory studies. These include a method proposed by the Association of German Agricultural Analytical and Research Institutes (*Bestimmung der Phytaseaktivität in Futtermitteln und Vormischungen (Determination of the phytase activity in feedingstuffs and premixtures)* Method book III of VDLUFA „The chemical analysis of feedingstuffs"; Method Number 27.1.2; 4th Auxiliary supply 1997 ; VDLUFA ISBN 3-922712-66-7, in German] which resulted in a relative between-laboratory standard deviation for reproducibility (RSD_R) of about 12 % for feedingstuffs and 8.4 % for a mineral premixture. Another method [Engelen et al. (2001) J. AOAC Int., 84, 629-633] obtained RSD_R values ranging from 14.0 to 27.6 % for feedingstuffs. However, data regarding ROVABIO™ pertaining to these two methods was not submitted by the applicant.

The European Association of Feed Additive Manufacturers (FEFANA) developed a method, suitable for the analysis of all phytase products currently authorised within the EU, which follows the same principle as the applicant's method, in order to allow for the

measurement of phytase activity in feedingstuffs, regardless of the specific phytase product used. The FEFANA method has been validated in an inter-laboratory study which was performed on feedingstuffs containing different 3- or 6- phytase products. The obtained values for the RSD_R, ranging from 5 to 14 %, are considered acceptable for the intended use. This method is currently under evaluation to become a standard of the European Committee for Standardisation (CEN). For these reasons, the CRL asked the applicant to compare the proposed in-house method with the inter-laboratory validated FEFANA method for chicken and piglet feed. The applicant provided results, showing a comparison between the proposed in-house method and a, to some extent modified, FEFANA method (different sample weight, different extraction solutions, buffer used during detection stage instead of water). With the modified FEFANA method, performance characteristics comparable to the method proposed by the applicant were obtained. While it is likely that the FEFANA method would be suitable for official control purposes for determining the enzyme activity of ROVABIO™ PHY in feedingstuffs, the data provided by the applicant concerns a *modified* version of the FEFANA method. For this reason, the CRL has no evidence of the suitability of the FEFANA method for official control purposes for this particular feed additive. Taking into account these facts, for determination of the enzyme activity of ROVABIO™ PHY in feedingstuffs, the CRL recommends the applicant's own method for official control purposes.

Regarding identification and characterisation of the additive, methods pertaining to the enumeration and detection of micro-organisms, identification of the strain including DNA profile for *Penicillium funiculosum* 4.05 b (CBS 111443), and determination of mycotoxins are provided.

No further testing or validation is required.