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Research Article

Risk Assessment of a Novel Arabinofuranosidase/Xylanase Enzyme Combination Intended to be Used in Food Animals

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Abstract

T Although poultry production shows relatively high feed conversion efficiency compared to other livestock species, a substantial proportion of dietary nitrogen remains undigested and is excreted as manure, contributing to environmental nitrogen losses. Enzymes capable of degrading arabinoxylans, particularly xylanase and arabinofuranosidase, have therefore been extensively investigated for their ability to improve feed digestibility and animal performance. A novel enzyme combination comprising endo-1,4-β-xylanase and α-L-arabinofuranosidase, produced by fermentation of *Talaromyces versatilis* strains, was developed with the objective of optimizing zootechnical performance while reducing nitrogen excretion. Literature data and meta-analysis results demonstrate that xylanase supplementation improves body weight gain, feed conversion ratio, nutrient digestibility, and apparent metabolizable energy, while reducing digesta viscosity and increasing nitrogen digestibility. Consumer safety was evaluated through a standard battery of *in vitro* genotoxicity assays, including the bacterial reverse mutation test (OECD TG 471), an *in vitro* chromosome aberration assay in Chinese hamster V79 cells (OECD TG 473), and an *in vitro* micronucleus test in mouse lymphoma L5178Y cells (OECD TG 487). The enzyme combination was administered twice daily by oral gavage at dose levels up to approximately 1000 mg Total Organic Solids (TOS)/kg body weight/day. User and worker safety was assessed using validated *in vitro* skin and eye irritation models (OECD TG 439 and TG 492). In conclusion, the novel xylanase/arabinofuranosidase enzyme combination demonstrates a favorable safety profile for consumers and users, while offering significant potential benefits in terms of zootechnical performance and environmental nitrogen reduction.

Keywords: Xylanase; Arabinofuranosidase; Enzyme; Poultry; Consumer; Safety

INTRODUCTION

The Farm to Fork strategy of the European Commission aims to reduce Nitrogen (N) losses from agriculture by 50% by 2030 and this, as regards the food system, is consistent with the wider ambition of the Colombo Declaration to halve nitrogen waste from all sources by 2030 [1]. Globally, the livestock sector has been estimated to account for a third of the world's nitrogen emissions and current production levels of livestock alone exceed planetary boundaries for nitrogen [2]. The feed employed is in many cases inefficiently converted into animal protein, with highest conversion rates for poultry meat (22%) and eggs (31%) [3,4]. The nitrogen that is not converted to food is excreted as manure and then applied to croplands and pastures where it may be lost by volatilization, denitrification, leaching, or run-off.

With the rise of large-scale industrialized livestock systems, manure disposal has become increasingly concentrated, reducing its uptake by crops [5]. Also, the production of animal and crop products has become increasingly specialized and separated, making it economically impractical to reuse manure N efficiently as an N input [6]. Unconstrained granivore systems offer the greatest possibilities for increasing nitrogen use efficiency (NUE), in particular by improving the nutrient digestibility of poultry by employing feed additives that contain enzymes which focus on the Non-Starch Polysaccharides (NSP) contained in wheat-based poultry diets. NSP act as anti-nutritional factors that reduce poultry performance via a negative effect on intestinal physiology. NSP-degrading enzymes are therefore commonly used in poultry feed to help hydrolyze the undigested fraction and thus improve energy and nutrients availability. Arabinoxylans are the main NSP present in wheat and corn, comprising 7.3 and 4.7% of the DM and accounting for 52 and 65% of total NSP, respectively. The anti-nutritional effect of wheat is mediated by its NSP constituents that raise the viscosity of gut contents and may modulate the microflora [7]. An increase in intestinal digesta viscosity is associated with enhanced bacterial fermentation and by reduced digestion and absorption of nutrients by the host. A combination of arabinofuranosidase and xylanase has been shown to improve the dry matter digestibility of corn and wheat in vitro and in vivo in broiler chickens and laying hens. Since current literature estimates that only up to 35% of the undigested fraction in poultry feed is digested by exogenous enzymes, it is reasonable to expect up to a 7% improvement in global feed digestibility with combined arabinofuranosidase and xylanase supplementation [8].

A dataset of 140 points obtained from 53 articles was analyzed using a mixed model methodology [9]. The results showed that ration supplementation with xylanase enzyme positively affects broiler chickens' growth performance and nutrient digestibility. The total Body Weight Gain (BWG) and Average Daily Gain (ADG) of broiler chickens increased by a quadratic response ($p < 0.05$) while feed consumption when expressed as total feed intake (FI; $p < 0.05$) was decreased and tended to fall when linearly expressed as an average daily feed intake

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(ADFI; $p < 0.10$). Consequently, xylanase supplementation in broiler ration reduced Feed Conversion Ratio (FCR). Xylanase was also shown to increase the digestibility of nutrients, namely dry matter, crude protein, starch, gross energy, fat, phosphorus, and calcium. In addition, and as expected, a decrease was also observed in digesta viscosity. Other effects of environmental interest, also confirmed by Pirgozliev et al. [5], include the increased digestibility of nitrogen and this results in a significant decrease in free nitrogen release in manure. Xylanase resulted in a significant increase in nitrogen-corrected apparent metabolizable energy (AMEn) which reached 5% with 16 ppm and even 6 to 8% when the caloric content of the diet was increased up to 3,070 kcal/kg [10]. The addition of carbohydrases can thus improve the AMEn value of a feed ingredient through improvements in fat and starch digestibility. The combined effects of xylanase can indeed break down arabinoxylans, the main NSP in wheat and therefore xylanase is the favored carbohydrase as an enzyme supplement for wheat-based diets, but other enzymes acting more specifically on arabinoxylans are also of interest.

Based on these observations, a combination of arabinofuranosidase and xylanase was developed with a ratio likely to optimize zootechnical productivity (by decreasing FCI) and produce related effects including improved nitrogen digestibility and fluidity of the digestive contents. Before proceeding with efforts to optimize the liquid and solid formulations of these enzymes, it was essential to ensure not only zootechnical efficacy and environmental benefits, but also safety for users (farmers, preparers of supplemented feed) and consumers of animal products derived from poultry receiving supplemented feed. For fermentation products like enzymes, a basic set of toxicity studies should be provided to the regulatory agencies consisting of genotoxicity/mutagenicity tests and a subchronic (90-day) oral toxicity study. As it is assumed that all enzymes are respiratory sensitizers, no further investigations were performed to test individual immunotoxicological effects which are due to individual responses than cannot in all cases be predicted by experimental models.

MATERIALS AND METHODS

The enzyme developed possesses endo-1.4-β-xylanase (EC 3.2.1.8) and α-L-arabinofuranosidase (EC 3.2.1.55) activities and was obtained from fermentation broths of Talaromyces versatilis strains. One 3,5-dinitrosalicylic acid (DNS) unit of xylanase is defined as the release of one µmole of xylose per minute from a substrate (beechwood xylan). One Fluorometric Unit (FU) of arabinofuranosidase is defined as the amount of enzyme needed for the release of one nanomole of 4-Methylumbelliferone per minute from a substrate (4-Methylumbelliferyl α-L-arabinofuranoside). Endo-1.4-β-xylanase and α-L-arabinofuranosidase potency corresponds to ca. 73,000 DNS units and 2,000,000 FU per gram of pure enzyme, representing quantitatively 93% and 7% w/w of the total enzyme content, respectively. Other components may be added to obtain solid (powder) or liquid forms. Formulated additives were shown in preliminary trials to display optimized effects when incorporated in poultry feed at 200 DNS units and 600 FU/kg of feed.

All toxicological studies focusing on consumer safety (genotoxicity studies and sub-chronic repeated dose oral toxicity study) were performed with a liquid enzyme concentrate form (43 mg TOS/g). In compliance with regulatory requirements for similar additives, safety for consumers was assessed by three in vitro genotoxicity studies: bacterial reverse mutation test (OECD TG 471), Chromosome Aberration Assay using Chinese hamster V79 lung cells (OECD TG 473) and mammalian cell micronucleus test OECD TG 487. A sub-chronic repeated dose oral toxicity study in rats (0ECD TG 408, 90-day oral study) was performed to provide in vivo data. Safety for users/workers was assessed using in vitro tests, including assessment of eye irritation (OECD TG 492) and skin irritation (OECD TG 439) for two test formulations, a powder (35 mg TOS/g) and a liquid form (19 mg TOS/ml).

In order to distinguish between the proportion of the enzyme preparation derived from the source material and that contributed by diluents and other ingredients, individual specifications for the tested enzyme combination require a statement of percentage Total Organic Solids (TOS) which is defined as follows: % TOS = 100 (A + W + D), where A = % ash, W = % water and D = % diluents and/or other ingredients, in accordance with e.g. 29th meeting of the Joint FAO/WHO Expert Committee on Food Additives. This concept overcomes the problem that enzyme preparations of different activities and forms were used in the toxicological studies or will be used in future feed additives. In the present case, for user safety studies, the solid formulation used contained approximately 3.5% TOS w/w and the liquid formulation contained approximately 1.9% TOS w/w, similar to formulations likely to be added to poultry feed. For in vivo investigations in rats and in vitro genotoxicity studies, the enzyme concentrate used contained 4.3% TOS w/w.

Genotoxicity Tests

► The Ames test (OECD TG N°471) is designed to evaluate genotoxic (mutagenic) potential by measuring the ability of the test substance to induce reverse mutations at the histidine locus of several strains of Salmonella typhimurium and at the tryptophan locus of Escherichia coli WP2 uvrA strain in the presence and absence of an exogenous metabolic activation system (S9). This bacterial reverse mutation test detects point mutations induced by chemicals causing base changes or frameshift mutations in the genome of amino acid-requiring strains of auxotrophic bacterial strains which are unable to grow on minimal medium - containing inorganic salts and glucose as a carbon source - except for spontaneous revertants. In the presence of a mutagenic agent, some of these strains may be converted to prototrophs after a reverse mutation to the wild type. These revertants can grow and form colonies in minimal medium. An increased number of the revertant colonies indicates mutagenic activity of the test item. Four Salmonella typhimurium strains were used to differentiate between base-pair (TA1535, TA100) and frame shift (TA1537, TA98) mutations. The Escherichia coli WP2 uvrA tryptophan (trp) reversion system measures trp- → trp+ reversions and thus detects mutagens that cause base-pair substitutions (AT to GC). These bacteria do not possess the mammalian enzyme system that is known to convert promutagens into mutagenic metabolites. An exogenous metabolic activation system was therefore added in the form of a mammalian microsomal enzyme activation mixture (liver extract, S9 fraction). The activation system uses nicotinamide-adenine dinucleotide phosphate (NADP+)-cytochrome P450-dependent mixed function oxidase enzymes of liver obtained from rats pre-treated with phenobarbital and β-naphthoflavone, two inducers of several drug-metabolizing enzymes.

Concentrations of 5000, 1581, 500 and 158.1 µg TOS/plate were tested, corresponding to the highest concentration recommended in OECD TG N°471 and a geometric regression of $1/\sqrt{10}$.

► The in vitro chromosome aberration test (OECD TG 473) is used to identify agents that cause structural chromosome aberrations in cultured mammalian cells. Structural aberrations may be of two types, chromosome or chromatid (the latter representing the target for the majority of chemical mutagens). Duplicate cultures with 300 well-spread metaphase cells were analyzed for each tested substance: enzyme combination, negative (vehicle) and positive control sample (ethyl methanesulfonate and cyclophosphamide). A 3-hour treatment with metabolic activation (in the presence of S9-mix) and a 3-hour treatment without metabolic activation (in the absence of S9-mix) were performed. Sampling was performed 20 hours after the beginning of exposure in both cases. Based on preliminary tests, the examined concentrations were 5000 (the highest recommended concentration in OECD TG 473), 2000 and 1000 µg TOS/mL.

► The in vitro micronucleus test is a genotoxicity test that uses the

detection of micronuclei (MN) in the cytoplasm of interphase cells as endpoint. The micronuclei observed in the cytoplasm of interphase cells may originate from acentric fragments (chromosome fragments lacking a centromere) or whole chromosomes that are unable to migrate with the rest of the chromosomes during the anaphase of cell division. The assay thus has the potential to detect the activity of both clastogenic and aneugenic genotoxic chemicals in appropriately treated cells, allowing the detection of negative and positive results. A concentrated powder containing the enzyme combination was tested in the in vitro micronucleus test using mouse lymphoma L5178Y TK+/- 3.7.2 C cells up to the recommended maximum concentration for non chemically defined substances according to the OECD Technical Guideline (TG) No. 487 (5 mg/ml) using duplicate cultures, with 2000 cells being scored for each concentration with contemporaneous assessment of negative (vehicle) and positive control samples. Three conditions were tested: 3-hour exposure with metabolic activation (in the presence of S9-mix) and 3-hour and 24-hour exposure without metabolic activation (in the absence of S9-mix). The examined concentrations were 5000, 4000, 2000, 1000, 500 and 250 µg Total Organic Solids (TOS, i.e. quantification of enzymes after evaporation of water and full mineralization at ca. 600°C) per ml.

Repeated Dose Toxicity in Rats

► A repeated dose toxicity study was performed in rats in full compliance with OECD TG No. 408. The combined enzyme was administered orallly by gavage twice daily for 90 consecutive days. The study consisted of a high dose of 1010.5 mg TOS/bw kg/day, two lower doses corresponding to a $1/\sqrt{10}$ descending sequence: 107.5 and 301 mg TOS/kg BW/day and a 0 mg/kg control group. In total, 40 male and 40 female Wistar rats were included. All animals were observed twice daily for mortality, morbidity and behavior changes, except on the day of necropsy (day 91) when observations were made once. Animals were weighed individually at the start of treatment and weekly thereafter, including on the day of necropsy. Feed consumption per cage was measured once at the end of the pre-treatment phase and weekly thereafter. This was used to calculate the mean feed consumption per animal per week. All animals underwent necropsy upon completion of the 90-day treatment period (Day 91). Ophthalmoscopy was performed before study start (on all animals) and in the last week of the treatment (Control and High dose animals). Functional Observation Battery (FOB) and measurement of locomotor activity was performed during the last week of treatment for all the animals. Blood and urine samples for clinical pathology and thyroid hormone samples were collected prior to necropsy from all animals. All animals underwent full pathological examination. Abdominal, thoracic and cranial cavities were examined for abnormalities and the organs were removed, examined and weighed. Both absolute and relative-to-body weight (as measured prior to terminal sacrifice) organ weights were determined. Estrus cycle evaluation, sperm analysis and histopathology investigations of all sampled organs were performed.

User Safety Studies

► The irritation potential of an enzyme additive may be predicted by measuring its cytotoxic effect, as reflected in the MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Thiazolyl blue; CAS number 298-93-1] assay, on EpiDerm™ a reconstituted human skin model. This method is approved by international regulatory agencies as a replacement for the identification of irritants in the in vivo Rabbit skin assay (OECD TG No. 404) and is specifically approved within OECD TG No. 439. EpiDerm™ is a three-dimensional human skin model comprising a reconstructed epidermis with a functional stratum corneum. Its use for skin irritation testing involves topical application of test materials to the surface of the epidermis and the subsequent assessment of their effects on cell viability. Cell viability determination is based on cellular mitochondrial dehydrogenase activity which is measured by MTT reduction and its subsequent conversion into a blue formazan salt that is quantitatively measured after extraction from tissues. Disks of EpiDerm™ were treated with each enzyme combination formulation (powder and liquid forms used for the genotoxicity and sub-chronic toxicity studies, respectively) and incubated for 1 hour (25 minutes at room temperature and 35 minutes at 37°C with 5% CO2 in a >95% humidified atmosphere). Exposure was terminated by rinsing with Dulbecco's Phosphate Buffered Saline (DPBS). The epidermis units were then incubated at 37°C for 24 hours with 5% CO2 in a >95% humidified atmosphere and at 37°C for 18 hours with 5% CO2 in a >95% humidified atmosphere. The viability of each disk was assessed by incubating the tissues for 3 hours with MTT solution at 37°C with 5% CO2, protected from light, in a > 95% humidified atmosphere. The precipitated formazan crystals were then extracted by shaking with isopropanol for 2 hours at room temperature and quantified spectrophotometrically at 570 nm. DPBS-treated epidermis was used as negative control and 5% (w/v) Sodium Dodecyl Sulphate (SDS) solution-treated epidermis was used as positive control (three units/control). Viability for each treated tissue was expressed as a % relative to the negative control. If the mean relative viability after 1 hour exposure and 42 hours post incubation was less than or equal to (≤) to 50% of the negative control, the test item was considered to be irritant to skin. The reduction in cell viability in treated tissues was compared to negative controls and expressed as a percentage. The % reduction in viability was used to predict the irritation potential of the test item.

► The potential of the test item or formulations to cause ocular irritation or serious eye damage was assessed as based on the protocol published by MatTek Corporation: "EpiOcularTM Eye Irritation (OCL-200EIT) Test for the prediction of acute ocular irritation of chemicals: Identification of chemicals not requiring classification and labeling for eye irritation or serious eye damage" (adopted version 2021) and the study design followed OECD TG Guideline No. 492. The EpiOcularTM tissue construct is a non-keratinized epithelium composed of stratified normal human keratinocytes in a three-dimensional structure. It models the cornea epithelium with progressively stratified, but not cornified, cells. Its use for eye irritation testing involves a topical application of test items on the surface of the cornea epithelial construct for different exposure incubations. Cell viability determination is based on cellular mitochondrial succinate dehydrogenase activity measured (within the mitochondria of viable cells) by the reduction and the conversion of a yellow dye, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], into a blue formazan salt. Disks of EpiOcularTM (two units) were treated with the test item and incubated at 37°C, 5% CO2 in a >95% humidified atmosphere for 30 minutes. At the end of the treatment period, each tissue was rinsed with DPBS, incubated for 12 minutes at room temperature to remove any remaining test item from the tissue, blotted on absorbent material, and then incubated for another 120 minutes at 37°C, 5% CO2 in a >95% humidified atmosphere. After the incubation, the units were transferred into MTT solution and incubated for a further approximately 3 hours to determine cell viability. The precipitated formazan crystals were then extracted using isopropanol and quantified spectrophotometrically at 570 nm. Distilled water- and Methyl Acetate-treated epithelium were used as negative and positive controls, respectively (two units / control). For each treated tissue, the viability was expressed as a % relative to the negative control. For each enzyme combination formulation (powder and liquid forms used for the genotoxicity and sub-chronic toxicity studies, respectively), the mean optical density of two treated tissues was determined and expressed as relative percentage of viability of the negative control.

RESULTS

Genotoxicity

1. Ames test: No precipitate was detected on the plates in the main tests for any of the examined bacterial strains, with and without metabolic activation. Slightly reduced background lawn was observed

in Salmonella typhimurium strains without metabolic activation at the highest concentration, but this was considered as having no impact on the outcome. The number of revertant colonies did not show any biologically relevant increase compared to the vehicle/solvent controls. No reproducible dose-related trends were noted and there was no indication of any test item-related effect. The mean values for revertant colonies in the negative (vehicle/solvent) control plates were within the general historical control range, the reference known mutagens employed showed the expected increase in the number of revertant colonies and the viability of the bacterial cells was checked by a plating experiment in each test. At least five analyzable concentrations were available for all strains; the study may therefore be considered as valid.

The reported data in the Ames test (OECD TG N°471) showed that the enzyme combination did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used. It may thus be concluded that the enzyme combination has no mutagenic activity in this well known in vitro mutagenicity test in prokaryotic cells.

2. Chromosome Aberration Test (OECD TG 473): no precipitate, no relevant changes in pH or osmolality and no cytotoxicity were observed with and without metabolic activation at all tested concentrations. None of the concentrations caused a significant increase in the number of cells with structural chromosome aberrations with or without metabolic activation when compared to the appropriate negative (vehicle) control value in the experiments. The positive control substances caused a statistically significant increase in the number of structural aberrations with or without metabolic activation, thus demonstrating the sensitivity of the test system and validating the test procedures.

It may be concluded that no induction of chromosome aberrations in Chinese hamster V79 cells was observed following exposure with the enzylme combination up to, and including, the highest recommended concentration of 5000 µg TOS/ml with and without S9 metabolic activation.

3. Micronucleus Assay: None of the tested concentrations caused any biologically or statistically significant increase in the number of micronucleated cells when compared to the appropriate negative (vehicle) control value in the experiments with and without metabolic activation. The negative (vehicle) control data were within the acceptable range for the spontaneous frequency of micronucleated cells. The positive control substances caused a statistically significant increase in the number of micronucleated cells in the experiments with or without metabolic activation, thus demonstrating the sensitivity of the test system. The evaluated concentration range was therefore considered to be adequate and it was concluded that the enzyme combination did not induce any statistically or biologically relevant increase in the frequency of micronucleated mouse lymphoma L5178Y TK+/- 3.7.2 C cells with or without metabolic activation.

Repeated Dose Toxicity (90-day study) in Rats

No test product-related clinical signs or mortality were noted during the study. The test product had no effect on body weight, body weight gain or food consumption. No effects were observed by ophthalmoscopic or neuromuscular paraclinical investigations and no changes were recorded for clinical pathology (hematology, coagulation, blood and urine chemistry). No test product-related effects were observed in the animals' estrus cycle and all the females showed normal distribution of the estrus phases. There were no changes in the T3, T4 and TSH hormone evaluation. Post-mortem examinations failed to reveal any organ weight, macroscopic or microscopic changes. Under the conditions of this 90-day repeated dose toxicity study in Wistar rats, the enzyme combination administered twice daily by oral gavage did not cause any adverse effects even at the highest tested daily dose which was slightly above 1000 mg TOS/kg BW, i.e. about 2000 mg pure enzyme combination per kg of body weight. The NOAEL was thus established at the Highest tested dose level (1010.5 mg TOS/bw kg/day).

User/Worker Safety

1. Skin irritation: Following exposure with either the solid (powder) or the liquid forms (same formulations as those used in the genotoxicity and sub-chronic toxicity studies, respectively), mean cell viability was close to 100% when compared to the negative control. The study met the validity criteria and was therefore considered to be valid. As the result was above the 50% threshold, both enzyme combination formulations may be considered to be non-irritant to the skin.

2. Eye irritation: The study met the validity criteria ans was therefore considered to be valid. Following exposure to either the powder or liquid form, mean cell viability was close to 100% compared to the negative control (after adjustment for non-specific color potential). This is above the 60% threshold and it may therefore be considered that each enzyme combination formulation was non-irritant to Reconstructed human Cornea-like Epithelium (UN GHS Classification: No Category).

DISCUSSION

A combination of arabinofuranosidase and xylanase was developed with a ratio likely to optimize zootechnical productivity (by decreasing FCI) and produce related effects including improved nitrogen digestibility and fluidity of the digestive contents. The development of this combined enzyme attempts to respond to the objectives set by the European Commission and the Colombo declaration aimed at reducing free nitrogen emissions into the environment by 50% by 2030. This is expected to be obtained by improving nutrient digestibility using enzymes focused on the Non-Starch Polysaccharides (NSP) contained in wheat-based poultry diets. NSP act as anti-nutritional factors that reduce poultry performance via a negative effect on intestinal physiology. The test enzyme combination was designated to help hydrolyze the undigested fraction and thus improve energy and nutrients availability as both arabinofuranosidase and xylanase have been shown to improve the dry matter digestibility of corn and wheat in broiler chickens with up to 7% improvement in global feed digestibility being expected. The anticipated zootechnical and environmental benefits should not, however, be offset by toxicological effects that make their use risky, particularly for the consumer or potentially exposed human users/workers.

Enzymes are generally considered as safe via oral exposure due to their inherent presence in human and animal food, their digestion in the gastrointestinal tract and their natural production and presence in the gastric juices of animals [11,12]. Evidence suggests that microbial enzymes do not produce acute or subchronic toxicity and are not genotoxic. The results of the genotoxicity assays performed on this novel combination of xylanase and arabinofuranosidase are consistent with this, confirming that these enzymes produced in a safe production organism bear no relevance to potential genotoxicity. The combination did not cause a positive mutagenic response with any of the test strains in either the presence or absence of metabolic activation, and an in vitro micronucleus assay in mouse lymphoma L5178Y TK+/- cells gave a negative response under the test conditions. In the subchronic 90-day repeated dose oral toxicity study, no evidence of in vivo toxicity was found in male or female rats when the enzyme combination was administered at dose-levels above the maximum dose suggested in OECD TG 408, i.e. >1000 mg TOS/kg BW/day, which corresponds to a dose close to 2000 mg/kg BW/day when considering the estimated actual molecular weight of the xylanase and arabinofuranosidase combination. The NOAEL of at least 1000 mg TOS/kg bw/day from this subchronic toxicity study can also be considered in conjunction with information on the level of enzyme intended to be used in the diet and with feed consumption data to generate a safety margin for the use of the enzyme in poultry feed. Estimated daily feed intakes of broilers (scaled to body weight) are

higher than those of pigs: the default value for average daily feed intake in poultry, generally speaking, as determined by EFSA, is 79 g DM/kg BW (average feed dry matter content of 88 %) [13], and the maximum daily feed intake of poultry as determined by the NRC is 65 g DM/kg bw. The present calculation is based on the EFSA value for broiler feed intake as this is the highest of all these values and therefore the most conservative. When the test combined enzyme preparation is added to poultry feed, the likely recommended inclusion rate will be 200 DNS units of endo-1,4-β-xylanase and 600 FU of α-L-arabinofuranosidase per kg of complete feeding stuff. Based on this, it can be estimated that an individual animal could be exposed to 18 DNS Units and 54 FU Units/kg bw/day. This is equivalent to about 0.36 mg TOS/ kg bw/day (based on an expected enzyme activity of the combination of 100/300 DNS/FU units/mg TOS). The safety margin is conventionally calculated by dividing the NOAEL by the highest estimated exposure level, i.e. 1010.5 mg TOS/kg bw/day ÷ 0.36 mg TOS/kg bw/day = 2807. A safety margin value of 100 or higher (e.g., 200 for EFSA [14]) is generally considered as protective of human health, as demonstrated by the 100-fold safety factor applied to food ingredients by the US FDA (21 CFR 170.22). On this basis, the calculated safety margin of 2807 is indicative of an acceptable margin for the test enzyme combination when used in animal feed at the recommended dose of 200 DNS/600 FU units/kg feed. An increase to +25% of these values in the event of higher potency for some production batches with a similar inclusion level in feed would be of no toxicological concerns with a safety margin value of 2105, far above the safety threshold. In addition, powdered or liquid formulations were shown to be non-irritative for the skin and eyes, which provides additional proof of safety for users or workers exposed to ready-for use additives containing the enzyme combination.

CONCLUSION

The results of these studies demonstrated that a novel bacterial combination of xylanase and arabinofuranosidase was not associated with any adverse effects in rats at dose levels up to >1000 mg TOS/kg bw/day in a 90-day repeated dose subchronic oral toxicity study conducted in accordance with the current OECD 409 guideline. In vitro genotoxicity testing additionally revealed that this enzyme combination is non-genotoxic. A safety margin above 2000 was calculated from the established NOAEL, an estimate of broiler consumption, and assuming incorporation of the enzyme combination into animal feed at the recommended level of 4000 xylanase DNS Units and 12000 arabinofuranosidase FU Units/kg complete feeding stuff. These findings support the safety of the tested enzyme combination for the intended use as an animal feed additive. The tested enzyme combination may thus be used safely in food producing chickens. Results of further investigations focused on zootechnical productivity, including effects on e.g. release of free nitrogen into the environment, and on the safety margins of each test formulation of the enzyme combination will subsequently be used to assess the Benefit-Risk ratio for broiler chickens and laying hens. Since the present risk analysis is not species-specific, the same conclusions can be extrapolated to other food-producing species, for example, pigs.

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