Regulatory Toxicology and Pharmacology 99 (2018) 213–224

Contents lists available at ScienceDirect

Regulatory Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/yrtph

A novel glucuronoxylan hydrolase produced by fermentation is safe as feed additive: toxicology and tolerance in broiler chickens

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ARTICLE INFO

Keywords:
Glucuronoxylan hydrolase
Bacillus licheniformis
Toxicology
Safety
Feed additive
Broilers

ABSTRACT

The current study presents a safety evaluation of a novel glucuronoxylan hydrolase (EC 3.2.1.136) from *Bacillus subtilis* produced in *Bacillus licheniformis*. The glucuronoxylan hydrolase preparation did not exhibit irritative potential to the eye and skin when applied in *in vitro* models. The glucuronoxylan hydrolase preparation was non-mutagenic and non-clastogenic in *in vitro* tests. Oral administration of the glucuronoxylan hydrolase preparation to rats did not cause any adverse effect in a 90-days subchronic toxicity study. A tolerance study was performed with broiler chickens and confirmed that this glucuronoxylan hydrolase is safe for broiler chickens when fed at the maximum recommended dose, as well as at the 10 times higher dose.

In conclusion, there are no safety concerns with using this novel glucuronoxylan hydrolase as a feed additive as it is toxicologically inert and the glucuronoxylan hydrolase is well tolerated by broiler chickens. The beneficial safety evaluation of glucuronoxylan hydrolase is consistent with the fact that this type of enzyme is ubiquitous in nature.

1. Introduction

The structural carbohydrates of the plant cell wall are an important energy source for animals, but the energy is not easily accessible for mammals and birds which do not produce all the necessary enzymes to break down the cell wall (Ravn et al., 2016). Adding enzymes to animal feed to help increase nutrient digestibility and optimize nutritional value of the diet is a common practice in modern feed formulation (Adeola and Cowieson, 2011). A wide range of enzymes is available to optimize nutrient utilization from feed ingredients; e.g. the use of phytases, glucanases, xylanases, hemi-cellulases and proteases (Adeola and Cowieson, 2011; Dersjant-Li et al., 2015; Cowieson and Roos, 2016) enhances the bioavailability of nutrients for the animal and/or for its intestinal microbiota (Józefiak et al., 2010; Bedford and Cowieson, 2012; Kiarie et al., 2013). Thus, employing enzymes can optimize the use of feed for efficient production of animal protein making enzymes essential contributors to the sustainability rating of meat production (Bundgaard et al., 2014; Leinonen and Kyriazakis, 2016).

To the best of our knowledge, molecules with Enzyme Commission number 3.2.1.136 (glucuronoarabinoxylan endo-1,4-β-xylanase) have not previously been used in animal feed. Glucuronoxylan hydrolase enzymes hydrolyze highly branched arabinoxylan polysaccharides into small oligomers, thus catalyzing the breakdown of cell walls hemicellulose of cereals like corn, rice and sorghum (Biely et al., 2016). In a similar fashion, xylanases of the GH10 and GH11 families hydrolyze linear and lowly substituted beta-1,4-xylan polysaccharides (Ravn et al., 2016, 2017; Biely et al., 2016). Xylanases have large commercial usage in the paper and textile industries, and also in food and feed production (Polizeli et al., 2005). Several bacterial xylanases are commercially available for uses as feed additives (Chakdar et al., 2016). Xylanases are readily biodegradable and the potential environmental toxicity of xylanases has been described to be minimal as shown in toxicity studies with algae, daphnia and fish as reported in the ECHA REACH registration dossier for xylanase (REACH Registration number for Xylanase 01-2120747946-38-0000).

The production host, *Bacillus licheniformis*, is already used as a production host for several enzymes currently used in feed (Pariza and Cook, 2010). The literature shows that *Bacillus licheniformis* is regarded as non-pathogenic and non-toxigenic (Pariza and Johnson, 2001; de Boer AS, 1994). *Bacillus licheniformis*, and several of the enzymes it produces, is accepted as a constituent of animal feed (AAFCO OP,

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https://doi.org/10.1016/j.yrtph.2018.09.024
Received 18 June 2018; Received in revised form 12 September 2018; Accepted 23 September 2018
Available online 25 September 2018
0273-2300/

2018). The European Food Safety Agency (EFSA) considers *Bacillus licheniformis* to meet the requirements for the qualified presumption of safety (QPS) (EFSA, 2007). According to the new guidance from EFSA "on the assessment of the safety of feed additives for the consumer" which entered into force on the 1st of May 2018, no studies concerning the safety of use for the additive for the consumers are required for enzymes produced by genetically modified microorganisms for which the recipient strain is considered by EFSA to qualify for QPS, and for which the molecular characterization of the event does not give rise to concern (EFSA, 2017).

The *Bacillus licheniformis* production strain used in this study derives from Ca63 (DSM 9552) and is genetically modified to produce a modified glucuronoxylan hydrolase from *Bacillus subtilis* (ATCC 6051).

In the present study, we report the *in vivo* tolerance test of this novel glucuronoxylan hydrolase (EC 3.2.1.136) in broiler chickens as prescribed by the European Food Safety Authority (EFSA, 2011).

Further, a series of toxicological studies were carried out to evaluate the safety of this novel glucuronoxylan hydrolase as suggested by Pariza and his colleagues (Pariza and Johnson, 2001; Pariza and Cook, 2010) and corresponding to the safety assessment of enzymes performed by Lichtenberg and colleagues (Lichtenberg et al., 2011; Lichtenberg et al., 2017). The toxicological studies presented here comply with the requirements of the European Union Regulation N° 1831/2003 on additives for use in animal nutrition and its corresponding guidelines (EFSA, 2012). Specifically, the enzyme preparation was subjected to a neutral red uptake cytotoxicity test in 3T3 cells, an *in vitro* eye irritation/corrosion study in chicken eyes, an *in vitro* skin irritation test using EpiDerm human reconstituted skin membranes, a bacterial reverse mutation assay, an *in vivo* micronucleus test and a subchronic oral toxicity study in rats. The objective of these studies was to demonstrate the safety of the glucuronoxylan hydrolase as a feed additive compiling the safety information on the production strain, and the results of a relevant toxicological studies as well as a target animal tolerance study in broiler chickens, as prescribed by the European Food Safety Authority (EFSA, 2011).

2. Materials and methods

2.1. Construction of the production strain

The wild type glucuronoxylan hydrolase gene is from *Bacillus subtilis* (ATCC 6051). The DNA sequence of the wild type gene was modified to change 5 amino acids to create the glucuronoxylan hydrolase product. The construct was cloned into *E. coli* using standard vectors with strictly defined and well-characterised DNA sequences that are known not to encode or express any harmful or toxic substances, to create a plasmid containing the glucuronoxylan hydrolase expression cassette. The glucuronoxylan hydrolase expression cassette was introduced through recombination into the *Bacillus licheniformis* recipient strain derived from Ca63 (DSM 9552) using a standard transformation procedure. The transformants were subsequently evaluated by gene sequencing to assess incorporation of the expression cassette and to ensure that no unintended sequences were incorporated into the genome of the selected production strain. The glucuronoxylan hydrolase protein expressed from the introduced genes in the final production strain was verified by mass spectroscopy to be 100% identical to the protein sequence encoded by the donor gene.

2.2. Preparation of the glucuronoxylan hydrolase test substance

The glucuronoxylan hydrolase preparation evaluated in the present study was carried out in an industrial pilot biotechnological set-up certified according to ISO 9001 and in accordance with the procedures used for the manufacturing of commercial enzyme products. In brief, the genetically modified *Bacillus licheniformis* production strain described in Section 2.1, was cultivated in a bioreactor using a medium made of sterilized food-grade ingredients with pH adjustment. At the end of the process, the product was separated from the production organism using a series of filtration and concentration steps. Finally, a series of chemical and microbial analysis were carried out to characterize the glucuronoxylan hydrolase preparation. The liquid fermentation broth was used for the toxicological studies. A dried solid form of the product was produced by granulation following well-established industrial production practices and was used in a tolerance study with broiler chickens.

Table 1
Composition analyzes of the glucuronoxylan hydrolase preparation for the toxicological studies. ND = not detected.

Composition analyzes of glucuronoxylan hydrolase for toxicological studies.	
Composition analyzes	
Enzyme activity GXH(A)/g	42944
Carbohydrate (anthron) (g/kg)	10.3
Carbohydrate (tryptophan) (g/kg)	15.4
Water (Karl Fisher) (% w/w)	89.1
Total Organic Solids (% w/w)	8.5
Dry Matter (% w/w)	10.9
Ash (% w/w)	2.4
N-total (% w/w)	0.92
Pb (ppm)	< 0.5
As (ppm)	< 0.3
Cd (ppm)	< 0.05
Hg (ppm)	< 0.05
Cu (ppm)	0.57
Total viable count CFU/g	< 100
Salmonella CFU/25 g	ND
Coliform bacteria CFU/g	< 4
Enteropathogenic *E. coli* CFU/25 g	ND
Sulphur-reducing clostridia CFU//g	< 10
Staphylococcus aureus CFU/g	ND
Bacillus licheniformis (Production strain detection)	ND

2.3. Characterization of the glucuronoxylan hydrolase test substance

Glucuronoxylan hydrolase activity is expressed in glucuronoxylan hydrolase units (GXH(A)). One GXH(A) is defined as the amount of enzyme required to liberate 7.55 nmole of reducing sugar measured as xylose equivalents per minute at 50 °C and pH 5.0. The glucuronoxylan hydrolase preparation was also analyzed for chemical and microbial status using standard methods (Table 1). Total organic solids (TOS) from the fermentation consists mainly of protein and carbohydrate components and was calculated as follows: TOS (%) = 100 - water (%) - ash (%). The TOS content of glucuronoxylan hydrolase was 8.5% (w/w).

The enzyme activity was 42944 GXH(A)/g for the toxicological studies. For the tolerance study in broiler chickens, the glucuronoxylan hydrolase was evaluated at a maximum recommended dose (11666 GXH(A)/kg diet) and a ten times overdose (116658 GXH(A)/kg diet) when added to standard starter, grower and finisher diets for a period of 6 weeks as described in 2.4.7.

2.4. Toxicological evaluation of glucuronoxylan hydrolase

The toxicological studies were performed in accordance with the current OECD guidelines and with Good Laboratory Practice (OECD, 1998a). The *in vivo* studies were performed in agreement with the regulations and ethical guidelines on the use of animals for experimental purposes of the local authorities of the countries where the studies were performed.

2.4.1. Subchronic oral toxicity in rat

A 13-week subchronic toxicity study in rats was conducted. Glucuronoxylan hydrolase was administered by oral gavage to assess the toxic potential of the enzyme. The study was carried out by Envigo

(Cambridgeshire, UK) following OECD guideline No. 408 (OECD, 1998b). Four groups of Wistar (RccHan™; WIST) rats obtained from Envigo RMS Ltd. each comprising ten males and ten females, received glucuronoxylan hydrolase at doses of 0.0, 1.0, 3.3 or 10.0 mL/kg body weight/day by daily oral gavage administration for 13 weeks. These doses were equivalent to 88.9, 293 and 889 mg TOS/kg body weight/day and 44880, 148167 or 449265 GXH(A)/kg body weight/day. A control group received reverse osmosis water (the vehicle) in the same volume-dose (10 mL/kg BW). The rats were 43–49 days old at commencement of treatment and were fed an expanded rodent diet throughout the study (Teklad 2014C, pelleted diet). Potable water was freely available via polycarbonate bottles fitted with sipper tubes. The animals were housed five of the same gender in each cage. Each animal was identified uniquely by microchip. The environment was kept at temperature within the range 20–24 °C and relative humidity within the range 40–70%, with a 12-h light and 12-h dark cycle except during designated procedures. The animals were acclimatized to these conditions for 14 days before treatment commenced. Autoclaved wood shavings were used as bedding and changed at appropriate intervals. Aspen chew blocks and plastic shelters were provided as environmental enrichment in each cage.

It was confirmed by analysis of samples obtained at weeks 1, 6 and 13 that the test formulations were homogeneous and stable at room temperature (21 °C) for at least 24 h. All formulations were, however, administered within 4 h of preparation.

Animals received the test substance or vehicle control formulations orally at a total volume-dose of 10 mL/kg body weight, using a suitably graduated syringe and a rubber catheter inserted via the mouth into the stomach. Clinical signs were recorded daily. Body weights and food consumption were recorded once weekly. Water consumption was monitored daily by visual inspection.

Observations and tests on each animal were performed at the same time of day on each occasion, and the observer was unaware of the study group of the animal under assessment. Before treatment commenced and weekly during the study each animal was removed from the home cage and examined for exophthalmos, fur appearance, lacrimation, piloerection, reaction to handling, ease of removal from the home cage, salivation and vocalization on handling. Each animal was then placed in a standard arena for 1 min, during which it was assessed for arousal, convulsion, gait, grooming, palpebral closure, posture, tremor, twitches, urination and counts for activity and rearing. Manipulations, which included assessment of approach response, auditory startle response, tail-pinch response, pupils, righting reflex and touch response and measurement of body temperature, body weight, grip-strength and landing foot-splay, were performed once before treatment commenced and again in week 13. Motor activity was measured before commencement and during week 13 by automated infrared sensor equipment that recorded individual animal activity over a 1-h period.

Ophthalmoscopy was performed on all animals before start of treatment and at termination on all control rats and those receiving 10.0 mL/kg body weight/day (889 mg TOS/kg body weight/day).

Blood samples were taken, after overnight food deprivation, in week 13 using BD Microtainers internally coated with spray-dried K2 EDTA (BD Cat#365974) and coagulation tubes were prepared at Envigo (plain polypropylene tubes pre-filled with sodium citrate at a ratio of 9 parts blood to 1 part citrate). EDTA treated samples were analyzed using a Bayer Advia 120 Analyzer for a range of hematological parameters (hematocrit, hemoglobin concentration, erythrocyte, total and differential leucocyte and platelet counts, mean cell volume, mean cell hemoglobin and mean cell hemoglobin concentration). Derived values (Hct, MCH and MCHC) were calculated in ClinAxys In ClinAxys the Rectic gated (g) parameters were used: HCTg = (Retic RBC x Retic MCV)/1000 and MCHg = (Retic CHCM x Retic MCV)/100. Citrate treated samples were analyzed for prothrombin and activated partial thromboplastin times using a Stago STA Compact Max analyzer. Additional blood samples were obtained at the same time, using BD Microtainers internally coated with spray-dried lithium heparin, also containing an inert polymer gel (for plasma separation) (BD Cat#365985). Using a Roche P Modular Analyzer with an ion-specific electrode, the concentrations of glucose, urea, creatinine, total cholesterol, total proteins, albumin (with calculation of the albumin to globulin ratio), sodium, and potassium were analyzed in the plasma. Further, the activities of alkaline phosphatase, alanine and aspartate amino-transferase and gamma-glutamyl transpeptidase were analyzed in the plasma.

After completion of the 13-weeks treatment period the rats were euthanized by carbon dioxide inhalation followed by exsanguination. They were dissected and examined macroscopically and a range of tissues collected. Weights were recorded for the adrenals, brain, epididymides, heart, kidneys, liver, ovaries, spleen, testes, thymus and uterus. Histopathological examination of the following tissues was performed on the high dose and control animals, adrenals, aorta (thoracic), brain, caecum, colon, duodenum, epididymides, eyes, femur, heart, ileum, jejunum, kidneys, liver, lungs, lymph nodes (mandibular and mesenteric), mammary area, oesophagus, ovaries, pancreas, pituitary, prostate, rectum, salivary glands, sciatic nerve, seminal vesicles, spinal cord, spleen, sternum, stomach, testes, thymus, thyroid (with parathyroids), trachea, urinary bladder, uterus (with cervix), and vagina. Grossly abnormal tissues were examined for all groups whereas histopathological examination of the mid- and low-dose groups were only done if abnormalities were identified.

A parametric analysis was performed if Bartlett's test for variance homogeneity (Bartlett, 1937) was not significant at the 1% level. The F1 approximate test was applied. This test is designed to detect significant departure from monotonicity of means when the main test for the comparison of the means is a parametric monotonic trend test, such as Williams' test (Williams, 1971, 1972). The test statistic compares the mean square, NMS, for the deviations of the observed means from the maximum likelihood means, calculated under a constraint of monotonicity with the usual error mean square, EMS. The null hypothesis is that the true means are monotonically ordered. The test statistic is F1 = NMS/EMS which can be compared with standard tables of the F distribution with 1 and error degrees of freedom. If the F1 approximate test for monotonicity of dose-response was not significant at the 1% level, Williams' test for a monotonic trend was applied. If the F1 approximate test was significant, suggesting that the dose response was not monotone, Dunnett's test (Dunnett, 1955, 1964) was performed instead. Significant differences between the groups compared were expressed at the 5% ($p < 0.05$) or 1% ($p < 0.01$) level. For organ weight data, analysis of covariance was performed using terminal body weight as covariate (Angervall and Carlström, 1963). The treatment comparisons were made on adjusted group means in order to allow for differences in body weight which might influence the organ weights.

2.4.2. In vitro isolated chicken eye test

Glucuronoxylan hydrolase was evaluated undiluted for eye irritation potential in the Isolated Chicken Eye (ICE) test in accordance with OECD guideline 438 (OECD, 2013). The study was carried out at TNO Triskelion (Zeist, The Netherlands). In addition, the test included a negative control (saline) and a positive control (BAC 5%). Chicken eyes were obtained from slaughter animals used for human consumption. The isolated chicken eyes were exposed to a single application of 30 µL of the Glucuronoxylan hydrolase test item for 10 s followed by a 20 mL saline rinse. Three main parameters were measured to disclose possible adverse eye effects: corneal thickness (expressed as corneal swelling), corneal opacity and fluorescein retention of damaged epithelial cells. In addition, histopathology of the corneas was performed.

2.4.3. In vitro skin irritation test using EpiDerm™ reconstructed skin membranes

In vitro skin irritation potential of glucuronoxylan hydrolase was

assessed using EpiDerm™ reconstructed skin membranes. The study was conducted in accordance with the OECD guideline 439 (OECD, 2015). The study was carried out at TNO Triskelion (Zeist, The Netherlands).

2.4.4. Bacterial reverse mutation assay

A bacterial reverse mutation assay (Ames test) was carried out at Covance (North Yorkshire, England) to assess for mutagenic activity.

The glucuronoxylan hydrolase enzyme preparation may contain free amino acids, including histidine and tryptophan. This can increase the apparent number of revertants. To avoid the risk of artefacts due to growth stimulation, a 'treat and plate' protocol was applied (Mahon et al., 1989; Thompson et al., 2005) as earlier described by Pedersen and Broadmeadow (2000). The study was conducted in accordance with the general recommendations in OECD Guideline 471 (1997).

The study utilized four histidine-requiring *Salmonella typhimurium* (*S. typhimurium*) strains and one tryptophan-requiring *Escherichia coli (E. coli) strain* capable of detecting induced frame-shift mutations (*S. typhimurium* TA1537 and TA98) or base pair substitutions (*S. typhimurium* TA1535, TA100 and *E.coli* WP2uvrApKM101). Strains TA98, TA1535 and TA1537 were originally obtained from the UK NCTC. Strains TA100 and WP2 uvrA pKM101 were derived from cultures originally obtained from MolTox Inc., USA. The genotypes of the bacterial test strains were confirmed as described by Maron and Ames (1983) and Green (1984). The experiment was conducted twice. Experiment 1 used a broad glucuronoxylan hydrolase concentration range from 16 to 5000 µg TOS/mL. In experiment 2, a narrowed glucuronoxylan hydrolase concentration interval covering the range 160–5000 µg TOS/mL to examine more closely those concentrations of glucuronoxylan hydrolase, approaching the maximum test concentration and therefore most likely to provide evidence of mutagenic activity. The tests were carried out in the absence and presence of a metabolic activation system (Mutazyme™ S9 mix from Molecular Toxicology Incorporated, USA). In total, four complete and independent experiments were performed.

For both experiments, the bacterial strains were exposed to all six concentrations of glucuronoxylan hydrolase, solvent control (sterile deionized water), and appropriate positive control substances (2-nitrofluorene, 4-nitroquinoline 1-oxide, N-methyl-N′-nitro- N-nitrosoguanidine, ICR-191 mutagen or 2-aminoanthracene, both obtained from Sigma-Aldrich, UK. See Table 10 for further details) in a phosphate buffered nutrient broth liquid culture (incubation mixture) in sterile tubes. The final concentrations of glucuronoxylan hydrolase were 16, 50, 160, 500, 1600 and 5000 µg TOS/mL for experiment 1 and 160, 300, 625, 1250, 2500 and 5000 µg TOS/mL for experiment 2. The current regulatory guidelines recommend 5000 µg TOS/mL as the maximum concentration (EFSA, 2014).

Incubation mixtures consisted of 4 mL nutrient broth No 2 (Oxoid, UK), 4 mL of either 0.2 M phosphate buffer (pH 7.4) (Fisher Scientific) or S9 mix, 1 mL bacterial culture, and 1 mL glucuronoxylan hydrolase or control solution. These mixtures were incubated for a period of 3 h at 37 °C. After incubation, test substance and all nutrients originating from the test substance and broth were removed by centrifuging twice and washing with phosphate buffer. After the last centrifugation, bacterial cells were re-suspended in 2 mL of phosphate buffer. Bacteria were plated out in triplicates on selective agar (Vogel Bonner E agar plates, E & O Laboratories Ltd) and the number of revertant colonies per plate was determined as described for the direct plate incorporation assay by Maron and Ames (1983). Furthermore, the number of viable bacteria in each culture was determined by plate count. The mean number of revertant colonies per plate and standard deviation were calculated for each set of replicate plate treatments, and the relative fold increase over the concurrent vehicle control was calculated for each test article and positive control mean plate count by dividing this mean value by the corresponding and concurrent mean vehicle control plate count.

Table 2
Composition of the experimental diets (in %).

Ingredients	Starter diet 0–10 days	Grower diet 10–21 days	Finisher diet 21–35 days
Maize	50.29	54.37	59.55
Soybean meal (48% CP)	40.77	36.57	31.54
Soy oil	5.05		
Animal fat		5.65	5.68
Dicalcium phosphate	1.98	1.76	1.64
Calcium carbonate	0.66	0.60	0.57
Salt	0.37	0.38	0.38
DL-methionine	0.30	0.20	0.20
L-lysine HCl	0.13		0.02
L-threonine	0.03		
Choline chloride	0.03	0.07	0.09
Minerals & vitamins[a]	0.30	0.30	0.30
Noxyfeed	0.02	0.02	0.02
Maxiban	0.0625	0.0625	
Monteban			0.01
Calculated nutrients			
ME, Kcal/kg	3050	3100	3150
Crude protein	22.9	21.0	19.0
Crude fibre	2.3	2.3	2.2
Ether extract	7.3	8.0	8.1
Avail. lysine	1.28	1.06	0.96
Avail. methionine	0.61	0.49	0.47
Avail. methionine + cysteine	0.93	0.79	0.74
Avail. threonine	0.81	0.72	0.65
Avail. tryptophan	0.23	0.21	0.18
Calcium	0.96	0.87	0.81
Total phosphorus	0.68	0.63	0.60
Non-phytate phosphorus	0.48	0.44	0.40
Sodium	0.16	0.16	0.16

[a] One kg of feed contains: Vitamin A: 12 000 IU; Vitamin D_3: 2400 IU; Vitamin E: 30 mg; Vitamin K_3: 3 mg; Vitamin B_1: 2.2 mg; Vitamin B_2: 8 mg; Vitamin B_6: 5 mg: Vitamin B_{12}: 11 µg; Folic acid: 1.5 mg; Biotin: 150 µg; Calcium pantothenate: 25 mg; Nicotinic acid: 65 mg; Mn: 60 mg; Zn: 40 mg; I: 0.33 mg; Fe: 80 mg; Cu: 8 mg; Se: 0.15 mg; BHT: 25 mg.

Table 3
Food intake during 13-week toxicity study in rats. M = males and F = females.

Group/Sex		Mean Food Consumption [g/animal/week]
1F	Mean	114
	SD	4.3
	N	2
2F	Mean	109
	SD	2.8
	N	2
	% of 1F	96
3F	Mean	108
	SD	3.6
	N	2
	% of 1F	95
4F	Mean	107
	SD	5.2
	N	2
	% of 1F	94
1M	Mean	158
	SD	4.9
	N	2
2M	Mean	151
	SD	0.6
	N	2
	% of 1 M	96
3M	Mean	149
	SD	4.8
	N	2
	% of 1 M	95
4M	Mean	147
	SD	7.0
	N	2
	% of 1 M	93

Table 4
13-week toxicity study in rats. Absolute organ weights (g) group mean values for animals killed after 13 weeks of treatment and standard deviation (SD) of males (M) and females (F).

		Terminal body weight	Adrenals	Brain	Epididymides	Heart	Kidneys	Liver	Spleen	Testes	Thymus
1M	Mean	412	0.056	2.121	1.332	1.105	2.027	13.710	0.683	3.741	0.360
	SD	32	0.008	0.085	0.138	0.201	0.192	1.514	0.073	0.443	0.056
2M	Mean	398	0.059	2.097	1.394	1.021	1.995	13.628	0.714	3.780	0.302
	SD	32	0.008	0.091	0.122	0.104	0.166	1.372	0.087	0.365	0.052
3M	Mean	387	0.060	2.067	1.417	1.006	2.012	12.775	0.704	3.858	0.297
	SD	24	0.007	0.069	0.109	0.074	0.170	1.031	0.093	0.139	0.063
4M	Mean	383	0.057	2.075	1.271	1.029	2.098	12.932	0.699	3.532	0.285
	SD	29	s0.009	0.092	0.232	0.098	0.223	2.037	0.104	0.558	0.057

		Terminal body weight	Adrenals	Brain	Heart	Kidneys	Liver	Ovaries	Spleen	Thymus	Uterus and Cervix
1F	Mean	233	0.064	1.935	0.761	1.360	8.305	0.085	0.576	0.279	0.558
	SD	17	0.011	0.053	0.062	0.108	0.845	0.023	0.082	0.046	0.196
2F	Mean	230	0.063	1.903	0.723	1.317	7.936	0.092	0.571	0.289	0.683
	SD	9	0.007	0.086	0.031	0.105	0.570	0.018	0.070	0.063	0.259
3F	Mean	229	0.065	1.872	0.757	1.348	8.196	0.086	0.530	0.249	0.512
	SD	22	0.010	0.047	0.064	0.087	0.852	0.013	0.123	0.042	0.178
4F	Mean	225	0.060	1.909	0.750	1.314	7.567	0.079	0.571	0.295	0.682
	SD	17	0.009	0.081	0.132	0.063	0.972	0.013	0.046	0.070	0.200

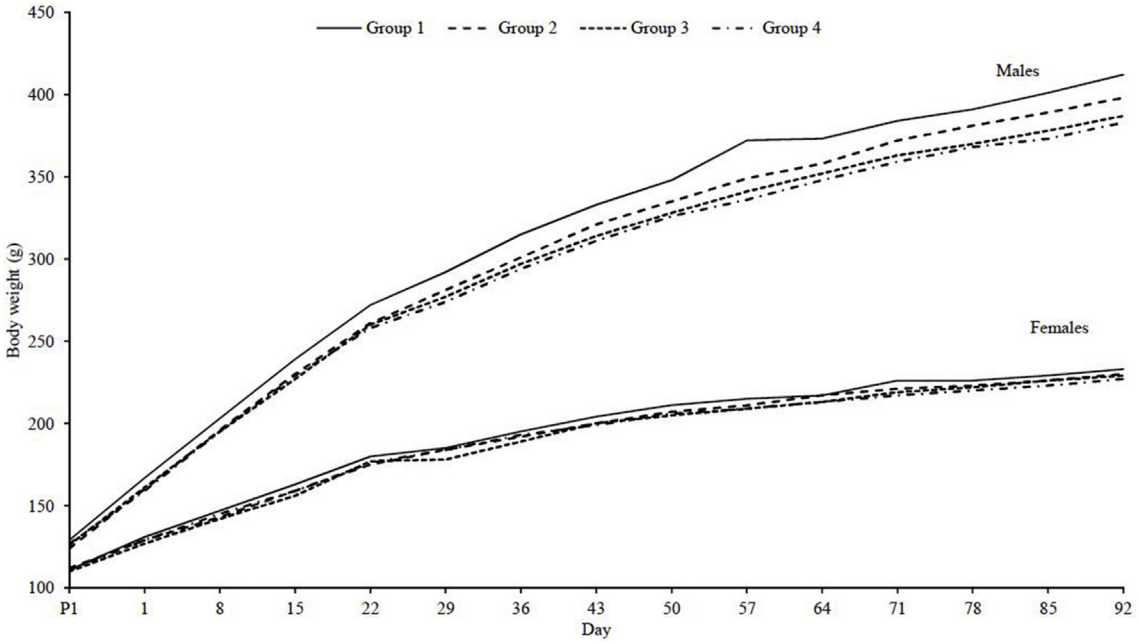

Fig. 1. Weight gain during 13-week toxicity study in rats.

2.4.5. In vitro micronucleus test in cultured human lymphocytes

Glucuronoxylan hydrolase was tested in an *in vitro* assay using human lymphocyte cultures prepared from pooled blood from two female donors. Blood donor details; age: 18–35 years, Selection criteria: Non-smokers, not heavy drinkers of alcohol, not on any form of medication (contraceptive pill excluded), not suspected of any virus infection, or exposed to high levels of radiation or hazardous chemicals. Cell cycle time: 13 ± 2 h.

The study was conducted according to OECD guideline 487 (OECD, 2016) at Covance Laboratories Ltd. (Harrogate, UK). Treatments – covering a broad range of concentrations separated by narrow intervals – were performed both in the absence and presence of metabolic activation with S9 - an Aroclor-induced rat liver metabolic activation system (Molecular Toxicology Incorporated, Boone, USA). The test article was formulated in purified water, and the highest concentration tested in the Micronucleus Experiment, 5000 μg TOS/mL (an acceptable maximum concentration for *in vitro* micronucleus studies according to current regulatory guidelines), was determined following a preliminary cytotoxicity Range-Finder Experiment.

Glucuronoxylan hydrolase was added 48 h after mitogen stimulation by phytohaemagglutinin (PHA) (Life Technologies UK). Cells were exposed to the test article for 3 h in the absence or presence of S9. In addition, a continuous 24 h treatment (equivalent to approximately 1.5–2 times the average generation time) in the absence of S9 was included. The following positive control chemicals were used: cyclophosphamide (CPA) (Acros Organics, Belgium) dissolved in dimethyl sulphoxide (DMSO) (Sigma–Aldrich, UK), vinblastine (VIN) (Sigma–Aldrich, UK) and mitomycin C (MMC) (Sigma–Aldrich, UK).

Whole blood cultures were established in tubes by placing 0.4 mL of pooled heparinised blood into 8.1 mL HEPES-buffered RPMI medium

Table 5
13-week toxicity study in rats. Hematology. Group mean values and standard deviation of males (M) and females (F). Significantly different from the controls:*: $P < 0.05$;**: $P < 0.01$.

Group/sex		Hct [L/L]	Hb g/dL	RBC $x10^{12}$/l	Retic $x10^{12}$/l	MCH pg	MCHC g/dL	MCV fL	RDW %	WBC $x10^9$/L	N $x10^9$/L	L $x10^9$/L	E $x10^9$/L	B $x10^9$/L	M $x10^9$/L	LUG $x10^9$/L	Plt $x10^9$/L	PT $x10^9$/L	APTT $x10^9$/L
1 M		0.428	16.3	8.27	0.146	19.8	38.2	51.8	12.1	6.23	1.04	4.94	0.10	0.01	0.12	0.01	856	25.9	18.9
	SD	0.0135	0.42	0.290	0.0160	0.72	0.83	1.75	0.56	1.211	0.405	0.827	0.092	0.008	0.056	0.007	129.9	2.33	1.59
2M		0.408**	15.8	8.01	0.149	19.7	38.6	51.1	12.4	6.80	1.03	5.54	0.08	0.02	0.12	0.01	920	23.9	19.7
	SD	0.0117	0.42	0.279	0.0146	0.83	0.68	1.98	0.45	0.948	0.152	0.950	0.022	0.007	0.025	0.007	123.6	2.86	1.92
3M		0.415**	16.1	8.08	0.154	19.9	38.8	51.3	12.1	7.15	1.27	5.59	0.11	0.02	0.16	0.01	929	23.5	19.0
	SD	0.0092	0.38	0.263	0.0094	0.40	0.45	0.99	0.38	1.667	0.570	1.098	0.058	0.012	0.096	0.006	96.7	2.20	1.38
4M		0.410**	16.0	7.84**	0.146	20.4*	39.1**	52.3	11.7	6.02	0.79	5.03	0.05*	0.02	0.12	0.01	831	23.7	19.1
	SD	0.0158	0.39	0.321	0.0245	0.62	0.83	1.09	0.34	1.130	0.277	1.029	0.010	0.008	0.052	0.007	141.5	2.84	1.43
1F		0.391	15.3	7.25	0.150	21.1	39.1	54.0	10.5	5.76	0.91	4.63	0.07	0.02	0.12	0.01	900	20.9	19.3
	SD	0.0119	0.44	0.358	0.0265	0.57	0.39	1.38	0.24	1.569	0.468	1.169	0.040	0.011	0.060	0.008	113.0	2.23	0.99
2F		0.388	15.0	7.24	0.154	20.7	38.5	53.6	10.6	5.72	0.86	4.65	0.08	0.02	0.10	0.02	903	22.0	18.1
	SD	0.0120	0.59	0.245	0.0223	0.75	0.68	1.30	0.26	1.339	0.282	1.142	0.038	0.011	0.035	0.005	76.1	1.49	1.22
3F		0.380	14.7	7.05	0.167	21.0	38.8	54.0	10.7	4.76	0.83	3.73	0.08	0.02	0.10	0.01	896	22.2	18.9
	SD	0.0193	0.57	0.440	0.0354	0.85	0.97	1.67	0.33	1.074	0.283	0.890	0.026	0.009	0.045	0.005	80.3	1.34	1.39
4F		0.390	15.0	7.23	0.153	20.7	38.4*	53.9	10.8	4.87	0.80	3.88	0.06	0.02	0.10	0.01	871	21.0	19.8
	SD	0.0174	0.64	0.303	0.0241	0.77	0.60	1.92	0.62	0.900	0.238	0.791	0.024	0.011	0.027	0.005	55.5	2.12	0.65

Hematocrit (Hct), Hemoglobin concentration (Hb), Erythrocyte count (RBC), Absolute reticulocyte count (Retic), Mean cell hemoglobin (MCH), Mean cell hemoglobin concentration (MCHC), Mean cell volume (MCV), Red cell distribution width (RDW), Total leucocyte count (WBC), Differential leucocyte count: Neutrophils (N), Lymphocytes (L), Eosinophils (E), Basophils (B), Monocytes (M), Large unstained cells (LUC), Platelet count (Plt), Prothrombin time (PT), Activated partial thromboplastin time (APTT).

(Life Technologies, UK) containing 10% (v/v) heat inactivated fetal calf serum (HIFCS, Life Technologies, UK) and 0.52% penicillin/streptomycin (Sigma–Aldrich, UK). PHA was included in the culture medium at a concentration of approximately 2% of culture to stimulate the lymphocytes to divide. Blood cultures were incubated at 37 ± 1 °C for 48 h and rocked continuously. Immediately prior to treatment, 0.1 mL culture medium was removed from all continuous cultures to give a final pre-treatment volume of 8.4 mL. In addition, 0.9 mL culture medium was added to all positive control cultures to give a final pre-treatment volume of 9.3 mL for continuous (24 + 0 h) cultures and 9.4 mL for pulse (3 + 21 h) cultures. S9 mix or KCl (0.5 mL per culture) was added appropriately. Cultures were treated with the test article or controls (1 mL per culture or 0.1 mL for positive control cultures). Cultures were incubated at 37 ± 1 °C for the designated exposure time. Several drops of suspension were gently spread onto multiple clean, dry microscope slides and the dried cells were stained for 5 min using filtered 4% (v/v) Giemsa (pH 6.8). Slides from the highest selected concentration and two lower concentrations were taken for microscopic analysis, such that a range of cytotoxicity was covered. For each treatment regime, two vehicle control cultures were analyzed for micronuclei. Positive control concentrations, which gave satisfactory responses in terms of quality and quantity of binucleated cells and numbers of micronuclei, were analyzed. After scoring, slides were decoded and the proportion of micronucleated binucleate (MNBN) cells at each treatment concentration was compared with the concurrent solvent (negative) control using the Fisher's Exact Test (one-sided analysis) with probability values of $p \leq 0.05$ accepted as significant. A Binomial dispersion test was conducted to assess homogeneity between replicate cultures (Richardson et al., 1989) Micronucleus frequency was also assessed against the historical solvent control (normal) range (95% reference range, based on percentiles of the observed data (Hayashia et al., 2011)).

2.4.6. Cytotoxicity test

To evaluate the cytotoxicity of glucuronoxylan hydrolase a Neutral Red Uptake (NRU) assay in 3T3 cells was performed according to OECD Guidance Document 129 (OECD, 2010) at Envigo CRS Limited (Cambridgeshire, UK). The growth of 3T3 cells treated with a range of concentrations of the test item was compared with vehicle control cultures after 48 h exposure both visually and using neutral red uptake.

2.4.7. Tolerance study in broiler chickens

In order to evaluate the tolerance of broiler chickens to the glucuronoxylan hydrolase, a tolerance study was carried out using broilers chickens in the experimental farm of IRTA in Mas Bover, Spain. The objective of the trial was to evaluate the dose related efficacy of a glucuronoxylan hydrolase on performance of broiler chickens and the tolerance to a high dietary inclusion level. The glucuronoxylan hydrolase was evaluated at a maximum recommended dose (11666 GXH(A)/kg diet) and a ten times overdose (116658 GXH(A)//kg diet) when added to standard starter, grower and finisher diets for a period of 6 weeks.

A total of 1920 day-of-hatch broiler chicks (Ross 308), obtained from a commercial hatchery were kept in a floor-pen facility and distributed by sex into 3 treatments allocated in 48 replicate groups of 40 birds each. The birds were fed starter, grower and finisher diets based on maize and soybean meal as the main feed ingredients, formulated to meet the National Research Council (NRC, 1994) nutrient recommendations. The composition of the basal diets, as well as the calculated and the analyzed nutrient contents are summarized in Table 2. Feeds were given for *ad libitum* consumption as crumbles in the starter phase and as 3 mm pellets in the grower and finisher phases. The quantity of feed required for each dietary treatment was supplemented on top with the glucuronoxylan hydrolase. The tested glucuronoxylan hydrolase was analyzed to have a glucuronoxylan hydrolase activity of 69857 GXH(A)/g product.

Table 6
13-week toxicity study in rats. Clinical Chemistry. Group mean values and standard deviation (in brackets) of males (M) and females (F). Significantly different from the controls:*: P < 0.05;**: P < 0.01. Alkaline phosphatase (ALP), Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Urea, Creatinine (Creat), Glucose (Gluc), total cholesterol (Chol), Sodium (Na), Potassium (K), Total protein (Total Prot), Albumin (Alb), Albumin/globulin ratio (A/G Ratio).

Group/Sex		ALP U/L	ALT U/L	AST U/L	Urea mmol/L	Creat µmol/L	Gluc mmol/L	Chol mmol/L	Na mmol/L	K mmol/L	Total Prot g/L	Alb g/L	A/G Ratio
1 M		83	50	74	7.81	28	9.22	2.38	142	4.3	68	37	1.15
	SD	15.0	10.5	19.9	1.752	2.1	0.833	0.384	0.9	0.17	2.1	1.1	0.062
2M		81	49	66	8.12	28	9.18	2.39	142	4.2	66	36	1.18
	SD	18.9	8.8	15.2	1.397	3.4	0.760	0.364	0.9	0.22	2.3	1.3	0.070
3M		77	55	81	7.79	28	9.04	2.29	142	4.2	67	37	1.23
	SD	13.0	7.1	19.7	0.826	2.7	0.927	0.349	1.0	0.23	2.1	0.9	0.078
4M		74	53	82	7.56	28	8.65	2.38	142	4.1	68	37	1.17
	SD	22.0	11.6	26.5	1.333	2.6	1.101	0.413	1.1	0.19	2.6	1.2	0.054
1F		35	51	82	7.45	29	7.98	2.19	143	3.9	73	42	1.41
		9.1	15.1	32.0	0.495	4.9	1.399	0.554	1.3	0.15	4.4	2.6	0.110
2F		32	43	71	7.74	33	8.84	1.97	142	4.0	70	42	1.45
	SD	6.3	12.0	30.3	0.835	4.3	1.090	0.386	0.9	0.28	3.2	1.8	0.086
3F		33	48	67	8.50	32	8.90	2.12	142	3.8	70	42	1.48
	SD	9.2	9.7	11.2	1.035	3.3	0.956	0.452	1.3	0.27	2.5	2.0	0.123
4F		33	36**	59*	7.67	32	8.75	2.11	143	3.8	72	42	1.42
	SD	8.5	6.4	7.7	0.693	3.0	0.589	0.475	1.8	0.30	2.8	1.6	0.081

Table 7
In Vitro Cytotoxicity Test: Neutral Red Uptake in BALB/c 3T3 Cell Culture. The viability of the cells is reported over a range of concentrations of glucuronoxylan hydrolase and sodium lauryl sulphate (positive control).

glucuronoxylan hydrolase (mg/mL)	Viability (%)	SLS (µg/mL)	Viability
0	100	0	100
0.01	84	30	124
0.03	88	60	135
0.1	87	75	123
0.3	89	90	90
1	96	95	59
3	78	100	40
10	73	110	13
30	19	120	10

The experiment involved 3 dietary treatments as follows: A – negative control fed non-supplemented basal diets; B – treatment receiving glucuronoxylan hydrolase at 11666 GXH(A)/kg diet; C – treatment receiving glucuronoxylan hydrolase at 116658 GXH(A)/kg diet (overdose). Each dietary treatment was assigned to 16 replicates (8 replicates with male birds and the other 8 with female birds).

Performance parameters monitored during the experimental period included live weight, live weight gain, feed intake, feed conversion ratio and mortality.

At the end of the experiment (42 days), blood samples from two birds per replicate of all treatments (96 samples) were taken for hematology and biochemical measurements.

Hematological examinations conducted involved the total number of red blood cells (RBC), blood hemoglobin concentration (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and total and differential number of white blood cells (WBC). In blood serum the following biochemical parameters were determined: glucose, bilirubin, cholesterol, uric acid, creatinine, albumin, globulin, total protein, amylase, creatine kinase, alkaline phosphatase, ALT, AST, and GGT (gamma glutamyl transferase).

In addition, gross pathology examination was conducted following blinding procedure on two preselected birds per replicate, to identify possible changes in peritoneal cavity, lungs, liver, kidney, heart, spleen and different parts of gastro-intestinal tract.

Results were analyzed as a randomized complete block design by two-way ANOVA. Differences among treatment means were investigate by Duncan's multiple range test and were considered significant at P < 0.05.

3. Results & discussion

3.1. Subchronic oral toxicity in rat

The systemic toxic potential of glucuronoxylan hydrolase at doses of 88.9, 293 and 889 mg TOS/kg/day was assessed by giving glucuronoxylan hydrolase orally by gavage to Wistar rats over a period of 13

Table 8
Ex vivo/in vitro eye irritation test using Isolated Chicken Eye Test.

Test material	Swelling %	Opacity	Fluorescein retention	Irritation categories[a]	Irritation Index[b]	Classifications (EU-CLP[c]/UN-GHS[d])
glucuronoxylan hydrolase	2	0.8	0.5	I; II; I	28	NC/NC
Bac 5%	38	3.0	3.0[e]	IV; IV; IV	158	1/1

[a] I = no effect; II = slight effect; III = moderate effect; IV = severe effect.

[b] Irritation Index = maximum mean corneal swelling + maximum mean opacity (x 20) + mean fluorescein score (x 20).

[c] EU-CLP: NC = not classified; Category 2 = Irritating to eyes; Category 1 = irreversible effects on the eye/serious damage to the eye. Regulation (EC) No 1272/2808 of the European Parliament and of the Council of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2806.

[d] UN-GHS: NC = not classified; Category 2B = mild irritant, causes eye irritation; Category 2A = irritant, causes eye irritation; Category 1 = irreversible effects on the eye/serious damage to the eye. United Nations-Economic Commission for Europe (UN/ECE) (2003). Globally Harmonised System of Classification and Labelling of Chemicals (GHS). UN, New York and Geneva, 2007.

[e] Severe loosening of epithelium.

Table 9
Skin irritation. OD570 of readings and calculated viability (average and standard deviation (SD)).

Group	substance	OD570 ± SD (%)	Viability ± SD (%)
Test Group	glucuronoxylan hydrolase	1.619 ± 0.073	100 ± 5
Negative control	PBS	1.613 ± 0.090	100 ± 6
Positive control	5% SDS	0.040 ± 0.001	2 ± 0

weeks. Analysis of total nitrogen content, N-total % was performed on three occasions during the study and showed that all animals had been adequately exposed to glucuronoxylan hydrolase at increasing doses. Clinical observations were performed continuously and no adverse signs of toxicity, ophthalmoscopy, and changes in the appearance or general behavior were observed that could be attributed to the treatment. There was a non-statistically significant trend towards slightly low body weight gain, when compared with controls, in males receiving 33 or 100% glucuronoxylan hydrolase and a similar trend for food intake (Table 3 and Fig. 1); however, this represented trends that were present before treatment commenced and was therefore not attributable to treatment.

The animals were subjected to a macroscopic necropsy. Specified organs and tissues were weighed, fixed and prepared for histopathological examination. The microscopic examination performed after 13 weeks of treatment revealed no test item related findings. The incidence and distribution of all findings were unrelated to treatment.

All intergroup organ weight differences were minor, not dose related, or were the result of single animals affecting the group mean value (Table 4). Although not statistically significant, there was an increase in kidney weight and a reduction of thymus weight in high dose males but neither finding was associated with any histopathological lesions in these tissues. Consequently, these trends were attributed to normal biological variation and were of no toxicological significance.

The hematological investigation in week 13 did not identify any toxicological significant differences from control. All inter-group differences from control, including those attaining statistical significance, were minor, lacked dose-relationship or were confined to one sex and were therefore attributed to normal biological variation (Table 5).

The biochemical analyses of plasma performed after 13 weeks of treatment did not reveal any changes in response to treatment with glucuronoxylan hydrolase. All inter-group differences from control, including those attaining statistical significance, were minor, lacked dose-relationship or were confined to one sex and were therefore attributed to normal biological variation (Table 6). In the treated females, there was a tendency towards slightly low alanine and aspartate aminotransferase activity, with statistical significance being attained at the high dose. Such trends of decreased plasma activities for these enzymes are only considered of toxicological importance if there is marked hepatotoxicity affecting enzyme synthesis in the hepatocytes and there was clearly no evidence for this from the histopathological investigation.

3.2. Cytotoxicity test

The growth of 3T3 cells treated with a range of concentrations of the test item was compared with vehicle control cultures after 48 h exposure both visually and using neutral red uptake.

Glucuronoxylan hydrolase demonstrated cytotoxicity compared to the vehicle control at the highest concentration of 30 mg/mL, slight cytotoxicity at concentrations 10–3 mg/mL and no cytotoxicity observed at the lower concentrations of 1–0.01 mg/mL. The estimated IC_{50} for glucuronoxylan hydrolase in the present assay was between 10 and 30 mg/mL (Table 7).

3.3. Skin and eye irritation

3.3.1. In vitro isolated chicken eye test

Microscopic examination of the corneas generally revealed very slight erosion and very slight vacuolation of the epithelium after exposure to glucuronoxylan hydrolase. The negative control eye did not show any corneal effect and demonstrated that the general conditions during the tests were adequate. Microscopic examination of the cornea did not reveal any abnormalities, apart from very slight vacuolation of the epithelium. Without any other effects observed in the cornea, the very slight vacuolation was considered a chance finding. The positive control BAC 5% caused severe corneal effects and demonstrated the ICE test validity to detect severe eye irritants (Table 8). Applying the classification criteria of the ICE, the classifications as “Not Classified” (UN-

Table 10
Bacterial reverse mutation assay; data from experiment 2 only. Mean number of revertant colonies per plate. Abbreviations; TOS:Total Organic Solids. 4-NQO:4-Nitroquinoline 1-oxide. NaN3:Sodium azide. 2-NF: 2-Nitrofluorene. ICR-191:Acridine Mutagen. 2-AA:2-Aminoanthracene.

Compound	μg/plate	S9	TA98	TA100	TA1535	TA1537	WP2 *uvrA* pKM101
Purified water	–	–	14.3	66.0	30.0	24.0	112.0
glucuronoxylan hydrolase	160	–	20.3	82.0	21.3	6.3	112.7
glucuronoxylan hydrolase	300	–	14.3	78.0	19.0	6.7	137.0
glucuronoxylan hydrolase	625	–	15.3	76.7	16.5	5.3	112.3
glucuronoxylan hydrolase	1250	–	17.7	84.7	16.7	6.7	141.0
glucuronoxylan hydrolase	2500	–	18.0	79.0	15.3	3.3	126.3
glucuronoxylan hydrolase	5000	–	18.0	100.3	27.7	14.7	125.7
2NF	25	–	1496.3				
NQO	1	–		601.3			
MNNG	2.5	–			1486.0		
ICR-191	1	–				1310.7	
MNNG	7.5	–					530.3
Purified water	–	+	11.0	87.7	22.0	3.3	110.3
glucuronoxylan hydrolase	160	+	12.3	66.0	16.0	6.7	136.7
glucuronoxylan hydrolase	300	+	13.7	72.0	23.0	10.3	145.3
glucuronoxylan hydrolase	625	+	14.3	80.7	25.3	6.3	131.0
glucuronoxylan hydrolase	1250	+	21.7	84.3	24.0	4.7	189.7
glucuronoxylan hydrolase	2500	+	17.7	81.7	23.7	6.7	161.0
glucuronoxylan hydrolase	5000	+	16.0	84.3	25.0	5.7	145.3
AAN	2.5	+	860.3	546.0		44.7	
AAN	5	+			97.7		
AAN	20	+					278.7

Table 11
In vitro micronucleus test in cultured human lymphocytes.

Treatment	Concentration (μg/mL)	Cytotoxicity (%) [c]	Mean MNBN Cell Frequency (%)	Historical Control Range (%)[b]	Statistical Significance
3 + 21 h -S-9	Vehicle[a]	–	0.30	0.20–1.00	–
	1000	10	0.65		NS
	3000	10	0.65		NS
	4000	19	0.65		NS
	5000	34	0.40		NS
	MMC, 0.20	34	4.35		$p \leq 0.001$
3 + 21 h + S-9	Vehicle[a]	–	0.55	0.20–1.07	–
	1000	2	0.65		NS
	3000	16	0.40		NS
	4000	13	0.45		NS
	5000	23	0.45		NS
	CPA, 3.00	34	1.65		$p \leq 0.001$
24 + 24 h -S-9	Vehicle[a]	–	0.18	0.10 to 0.90	–
	100.0	21	0.45		$p \leq 0.05$
	1000	37	0.30		NS
	3000	43	0.75		$p \leq 0.001$
	4000	34	0.40		NS
	VIN, 0.04	32	6.80		$p \leq 0.001$

*Positive control.
NS Not significant.
[a] Vehicle control was purified water.
[b] 95th percentile of the observed range.
[c] Based on replication index.

GHS and EU-CLP classifications) can be applied.

3.3.2. In vitro skin irritation test using EpiDerm™ reconstructed skin membranes

The cell viability after glucuronoxylan hydrolase treatment was found to be 100% with a standard deviation of 5% (Table 9). The negative control (PBS) was found to be 100% viable with a standard deviation of 6% whereas the positive control (SDS) induced a mean viability of 2% with a standard deviation of 0%. A test article is considered not to possess irritating properties if the cell viability is above 50%, hence the data of the present study indicate that the glucuronoxylan hydrolase is a non-irritant (UN GHS No Category).

3.4. Bacterial reverse mutation assay

The results of the bacterial reverse mutation assay (Ames test) are presented in Table 10. The positive control substances induced significant increases in revertant colony numbers, thereby demonstrating the sensitivity of the bacterial strains and the metabolizing potential of the S9 mix. No concentration-related and reproducible increases in revertant colonies were obtained with any of the bacterial strains exposed to glucuronoxylan hydrolase, either in the presence or absence of S9 mix. Glucuronoxylan hydrolase concentrations up to 5000 μg TOS/mL were tested. This is the recommended maximum concentration in current regulatory guidelines. Based on the present data, it is concluded that glucuronoxylan hydrolase is not mutagenic in the Ames test.

3.5. In vitro micronucleus test in cultured human lymphocytes

Micronuclei were analyzed at four concentrations and a summary of the data is presented in Table 11. Appropriate negative (vehicle) control cultures were included in the test system under each treatment condition. The proportion of micronucleated binucleate (MNBN) cells in these cultures fell within the current 95th percentile of the observed historical vehicle control (normal) ranges. Mitomycin C (MMC) and Vinblastine (VIN) were employed as clastogenic and aneugenic positive control chemicals respectively in the absence of rat liver S-9. Cyclophosphamide (CPA) was employed as a clastogenic positive control chemical in the presence of rat liver S-9. Cells were sampled in the Micronucleus Experiment at 24 h (CPA, MMC) or 48 h (VIN) after the start of treatment. All positive control compounds induced statistically significant increases in the proportion of cells with micronuclei.

Pulse 3 + 21 h treatment of cells with glucuronoxylan hydrolase, in the absence and presence of S-9 resulted in frequencies of MNBN cells which were similar to and not significantly ($p \leq 0.05$) higher than those observed in concurrent vehicle controls for all concentrations analyzed (both treatments). The MNBN cell frequency of all glucuronoxylan hydrolase treated cultures (all concentrations) fell within the 95th percentile of the normal ranges.

Following extended (24 + 24 h) treatment in the absence of S-9, weak increases in MNBN cells were observed at the low concentration (100 μg TOS/mL, inducing 21% cytotoxicity) and an intermediate concentration (3000 μg TOS/mL, inducing 43% cytotoxicity). However, these increases were small and the values of both replicate cultures at each of these concentrations fell within normal values. No increases were observed at the highest concentration (4000 μg TOS/mL) or at the intermediate concentration (1000 μg TOS/mL). The MNBN cell frequencies of all glucuronoxylan hydrolase treated cultures (all concentrations) fell within normal ranges. It was therefore considered that the observed statistical increases were of no biological importance.

It is concluded that glucuronoxylan hydrolase did not induce biologically relevant increases in micronuclei in cultured human peripheral blood lymphocytes following treatment in the absence and presence of S-9. Concentrations were either tested up to the regulatory recommended maximum of 5000 μg TOS/mL (3 + 21-h treatments), or were limited by the presence of post treatment precipitate to 4000 μg TOS/mL (24 h treatment).

3.6. Tolerance study in broiler chickens

Results of the tolerance study are summarized in Table 12. Performance of birds in this study was considered normal, as the body weight of the broiler chickens was close to the standard for the breed in all periods. During the starter and the grower periods, there was an improvement of the feed to gain ratio (FCR) of the broiler chickens receiving the diet supplemented with the 10 times the recommended dose when compared to the control diet. The improvement of the FCR was a combination of numerical effects on weight gain and feed consumption, suggesting greater availability of the nutrients as a result of enzyme supplementation. Performance in the overall study showed a significant

Table 12
Effects of the glucuronoxylan hydrolase supplementation on broiler performance and selected hematological and blood biochemical parameters.

Parameter	A	B	C
	Control	glucuronoxylan hydrolase (11666 GXH(A)/kg)	glucuronoxylan hydrolase (116658 GXH(A)/kg)
Performance parameters (male + female)			
Starter			
Final weight, day 0–14	478	473	482
Feed/gain ratio, day 0–14	1.164a	1.155a	1.142b
Grower			
Final weight, day 14–28	1489	1476	1504
Feed/gain ratio, day 14–28	1.502a	1.488ab	1.474b
Finisher			
Final weight, day 28–42	2838	2801	2822
Feed/gain ratio, day 28–42	1.748	1.728	1.76
Overall			
Final weight, day 0–42	2838	2801	2822
Feed/gain ratio, day 0–42	1.565	1.550	1.555
Heamatological parameters			
Red blood cells ($x10^6/\mu L$)	2.46E+06	2.41E+06	2.46E+06
Hemoglobin (g/100 mL)	12	12	12
Hematocrit (%)	30.8	30.3	30.3
Mean corpuscular volume (fl)	126	126	123
Mean corpuscular hemoglobin (pg/L)	48.4	49	48.5
Mean corpuscular hemoglobin concentration	38.7	38.9	39.4
Eosinophils (% WBC)	9.83	8.24	8.35
Basophils (% WBC)	3.10	4.00	3.10
Lymphocytes (% WBC)	35.5	38.9	39.5
Monocytes (% WBC)	4.17	5.31	3.32
Heterophils (% WBC)	47.4	43.5	45.7
Blood biochemical parameters			
Glucose (mg/dL)	231	232	225
Bilirubin (mg/dL)	0.115	0.113	0.118
Cholesterol (mg/dL)	140	139	142
Uric acid (mg/dL)	5.8	5.3	5.2
Creatinine (mg/dL)	0.23a	0.22b	0.22b
Albumin (g/L)	11	11	11
Globulin (g/L)	20	20	20
Total protein (g/L)	32	31	31
Amylase (U/L)	500	446	481
Alkaline phosphatase (U/L)	5336	5719	5460
Creatine phosphokinase (U/L)	48887a	43841ab	34526b
ALT (U/L)	2.9	2.9	2.7
AST(U/L)	507	502	432
GGT (U/L)	19	17	20

a,b Means within the same row without a common letter are significantly different ($P < 0.05$).
Mean of 8 replicates of males or 8 replicates of females.
Means followed by different letters are significantly different, Duncan's multiple range test, $P < 0.05$.

sex*treatment interaction for the FCR indicating that the females receiving the recommended dose of the enzyme preparation showed a significant improvement (2.1%) in this variable compared to the control group.

No clinical signs of any health problems were noted during this study. Mortalities were low across treatments and were not related to treatments as the level of mortality for the highest inclusion level of glucuronoxylan hydrolase was similar to that of the control group.

The results of blood hematology did not reveal any relevant changes due to the dietary administration of the glucuronoxylan hydrolase. There were no significant differences among the treatments for any of the variables examined. At the highest inclusion level of the glucuronoxylan hydrolase, an increase of mean corpuscular hemoglobin concentration (MCHC) and a decrease of mean corpuscular volume (MCV) was noticed, but they were not statistically different from the low dose and the control group. An increase of MCHC leads usually to an elevated content of hemoglobin which was not the case in this experiment. However, the values for the investigated parameters were near to the reference values found in the literature (Bounous and Stedman, 2000; Trîncă et al., 2012). The proportion of heterophils, eosinophils, basophils, lymphocytes and monocytes recorded in the study were close to the reference values (Hoffmann, 1961). Those hematology findings were not consistent with a treatment-related effect, and in the absence of a decreased hemoglobin concentration in the plasma of the treated groups, no anemia and safety concern could be reported with increasing inclusion level of the glucuronoxylan hydrolase.

The blood chemical analyses showed no significant differences among the treatments for any of the variables examined, except for creatine which was higher in the control group than in the treatments supplemented with the enzyme, although differences were very small. A significant reduction of creatine kinase activity was recorded in the broiler chickens receiving the ten times recommended dose.

A general necropsy was performed where special attention was drawn to the kidney, the liver, the spleen, the lungs and the heart. The avian necropsy did not show any adverse effects associated with the dietary treatments.

According to the results recorded in this study, the glucuronoxylan hydrolase was well tolerated even at ten times the maximum recommended dose.

4. Conclusion

The present work describes the methodologies and results of several toxicological studies performed to evaluate the safety of the novel microbial glucuronoxylan hydrolase as feed additive as well as the

tolerance study of the novel enzyme in broiler chickens. The protocols for the studies were selected to meet, at least, the regulatory requirements applicable for a feed enzyme in Europe.

The 6-week tolerance study confirmed that the glucuronoxylan hydrolase is safe for broiler chickens when fed at the maximum recommended dose, as well as at the 10 times higher dose. The results did not reveal any significant changes due to dietary administration of the glucuronoxylan hydrolase in terms of hematological and biochemical examinations and post mortem necropsy.

A toxicological evaluation of the glucuronoxylan hydrolase as prescribed by the European Food Safety Authority (EFSA, 2012) was carried out. The studies were performed at the highest dose levels required by the current OECD guidelines. Glucuronoxylan hydrolase administered by oral gavage to rats for 13 weeks did not cause any adverse effect. In addition, glucuronoxylan hydrolase did not exhibit irritative potential when applied to the eye using the *in vitro* isolated chicken eye test or to the skin using the *in vitro* three-dimensional EpiDerm™ model. Finally, glucuronoxylan hydrolase was found not to represent mutagenic or clastogenic potential when tested in relevant genotoxicological assays. Acute aquatic toxicity evaluated with daphnia and algae, combined with the biodegradability of xylanase enzymes in general shows a low safety concern for the aquatic environment (REACH dossier 01-2120747946-38-0000). Based on the tolerance trial in broilers, the toxicological data and the fact that the *Bacillus licheniformis* production organism derives from a safe strain lineage, it is concluded that there are no reasons for safety concerns when using this glucuronoxylan hydrolase as a feed additive.

In conclusion, glucuronoxylan hydrolase is an advantageous additive to chicken feed and cause no harm to the environment. The positive safety evaluation of glucuronoxylan hydrolase is in line with the fact that this type of enzyme is ubiquitous in nature. However, from a workers' safety perspective, glucuronoxylan hydrolase should, like any other enzyme, be handled according to the standard safety guidelines for enzymes to avoid respiratory sensitization.

Conflicts of interest

None of the authors has any conflicts of interest concerning this paper.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.yrtph.2018.09.024.

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