REGULATED PRODUCTS SAFETY ASSESSMENT

Safety Assessment on the Safety and Efficacy of a Feed Additive Containing Muramidase Produced by *T. Reesei* DSM 32338 for Its Use in Laying Hens (RP1499)

Food Standards Agency[1], Food Standards Scotland[2]

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FSA Research and Evidence

An application was submitted to the Food Standards Agency and Food Standards Scotland in March 2022 from DSM Nutritional Products ('the applicant') for the new use (extension of species) of an additive containing muramidase produced by *Trichoderma reesei* DSM 32338, under the category of 'zootechnical additives' and functional group 'other zootechnical additives'. The additive is proposed to be used at a minimum inclusion rate of 30,000 LSU(F)/kg of complete feed (12% moisture) for laying hens.

Under Assimilated Regulations (EU) No 2019/805 and No 2020/163, the additive is authorised for use in feed for chicken and minor poultry species for fattening, turkeys for fattening, and chickens, turkeys and other poultry species reared for breeding. In addition, the additive is authorised in the EU under the Commission Implementing Regulation No 2021/1431 for use in feed for weaned piglets. This application requested a new use (extension of species) in feed for laying hens. The FSA/FSS concluded that the additive was correctly identified and characterised as there were no changes from the previous assessment. No significant concerns were raised for the Identity, characterisation and condition of use section of the dossier.

The additive can be considered safe for the target species (laying hens) up to a dose of 60,000 LSU(F)/kg of complete feed. The additive is safe for consumers and the environment. The active substances are presumed to be respiratory sensitisers due to their proteinaceous nature. The liquid formulation of the additive is not considered an irritant to the eyes or skin. The solid formulation of the additive is not an irritant to the skin but should be considered a potential eye irritant.

Based on the three trials provided, the FSA/FSS concluded that muramidase has the potential to be efficacious as a zootechnical additive in laying hens when used at the minimum inclusion rate of 30,000 LSU(F)/kg complete feed.

This is a joint FSA and FSS publication.

1. Introduction

The FSA and FSS have undertaken an assessment for a feed additive containing muramidase produced by *Trichoderma reesei* DSM 32338 (DSM Nutritional Products, Heanor Gate Ind. Est., Heanor, Derbyshire, DE75 7 SG, United Kingdom) under assimilated Regulation (EC) No 1831/2003 (EC, 2003) in the category of 'zootechnical additives' and functional group 'other zootechnical additives' for its new use (extension of species) in laying hens.

Under Assimilated Regulations (EU) No 2019/805 and No 2020/163, the additive is authorised for use in feed for chicken and minor poultry species for fattening (EC, 2019), turkeys for fattening, and chickens, turkeys and other poultry species reared for breeding (EC, 2020). In addition, the additive is authorised in the EU under the Commission Implementing Regulation No 2021/1431 for use in feed for weaned piglets (EC, 2021). This application requested a new use (extension of species) for use in feed for laying hens.

In line with Article 8 of Assimilated Regulation No 1831/2003, the assessment has considered and concluded that the feed additive complies with the conditions laid down in Article 5, including: safety considerations for human, animal and environmental health; efficacy of the additive for its intended effect; potential impairment of the distinctive features of animal products. This, and the FSA/FSS guidance for the evaluation of feed additive applications, has formed the basis and structure for the assessment.

This safety assessment represents the opinion of the FSA and FSS.

Table 1. Table showing products included in this assessment

Title	Product type	Intended use/s	Intended species or categories of animals
Muramidase (EC 3.2.1.17) produced by *T. reesei* DSM 32338	Feed additive	Zootechnical additive	Laying hens

2. Assessment

2.1. Section II: Identity, characterisation and conditions of use

The additive is a muramidase produced by *Trichoderma reesei* DSM 32338, from this point on referred to as 'muramidase'. The additive is proposed to be authorised both as:

- Dry formulation: a dust-free granular powder meant for inclusion in premixture and compound feed with a guaranteed minimum activity of 60,000 LSU(F) (muramidase activity units) per gram of product.
- Liquid formulation: a liquid product meant for direct inclusion in compound feed, via post-pelleting application, with a guaranteed minimum activity of 60,000 LSU(F) per gram of product.

No changes to the formulation from the previous authorisation were proposed, but the applicant provided data from several batches supporting the identification values outlined in Table 2 for the solid formulation and Table 3 for the liquid formulation. The microbial contamination and the amounts of the detected impurities do not raise safety concerns.

Table 2. Identity table for solid formulation of muramidase

Composition	
Fermentation extract, enzyme or TOS[1]	18 %
Sodium sulphate	70 %
Cellulose	6 %
Kaolin	4 %
Sucrose	1 %
Water	1 %
Enzyme activity[2]	70800 - 74200 LSU(F)/g
Chemical-physical properties	
Dusting on Heubach filter	0.4 – 0.8 mg
Particle size distribution	> 850 µm – 2.4 – 18.7 % < 850 µm – 81.4 – 97.6 % < 425 µm – 11.9 – 35 % < 150 µm – 0 %
Loose density	1100 g/L
Tapped density	1300 g/L
Impurities	
Arsenic	< 0.3 mg/kg
Lead	≤ 0.5 mg/kg

Composition	
Cadmium	< 0.05 mg/kg
Mercury	≤ 0.05 mg/kg
E. coli	Absent in 25 g
Salmonella spp.	Absent in 25 g
Enterobacteriaceae	< 10 CFU/g
Coliforms	< 4 /g
Yeasts and filamentous fungi	≤ 10 CFU/g
Bacillus cereus	< 10 CFU/g
Paracelsins	< 0.01 mg/kg

[1] Total Organic Solids: sum of the organic compounds, excluding diluents, e.g. water, contained in the final enzyme preparation. This TOS fraction is considered as the active substance in toxicological studies.
[2] Enzyme activity values are sourced from the Certificates of Analysis provided by the applicant. The enzymatic activity may vary from batch-to-batch, but the applicant declared that the minimum enzymatic activity is 60,000 LSU(F) per gram of product.

Table 3. Identity table for liquid formulation of muramidase

Composition	
Fermentation extract, enzyme or TOS[1]	19 %
Potassium sorbate	0.05 %
Sodium benzoate	0.15 %
Sorbitol	22 %
Water	58.8 %
Enzyme activity[2]	80000 - 95000 LSU(F)/g
Appearance	
Clear brown solution	
Chemical-physical properties	
Density	1.1 g/ml
Viscosity at 25^{o}C	7 – 10 mPa.s
Impurities	
Arsenic	< 0.3 mg/kg
Lead	< 0.5 mg/kg
Cadmium	< 0.05 mg/kg
Mercury	< 0.05 mg/kg
E. coli	Absent in 25 g
Salmonella spp.	Absent in 25 g
Enterobacteriaceae	< 10 CFU/g
Coliforms	< 4 /g
Yeasts and filamentous fungi	≤ 20 CFU/g
Paracelsins	< 0.01 mg/kg

[1] Total Organic Solids: sum of the organic compounds, excluding diluents, e.g. water, contained in the final enzyme preparation. This TOS fraction is considered as the active substance in toxicological studies.
[2] Enzyme activity values are sourced from the Certificates of Analysis provided by the applicant. The enzymatic activity may vary from batch-to-batch, but the applicant declared that the minimum enzymatic activity is 60,000 LSU(F) per gram of product.

Using whole genome sequencing, the taxonomic identity of the production strain DSM 32338 as *T. reesei* was confirmed. The genetic modification of the production strain does not raise any safety concerns. The production process has not been modified since the previous authorisation; therefore, the conclusions that the production strain is safe remain relevant to this assessment.

Analysis of three production batches of the enzyme concentrate showed absence of the production microorganism and recombinant DNA in the muramidase concentrate. No gene coding for antibiotic production has been added in the construction of the production strain *T. reesei* (DSM 32338) according to the applicant. The applicant confirmed that no antibiotic is intentionally added along the manufacturing process of the enzyme. Furthermore, analysis of 4 batches for both product formulations showed levels of paracelsins below the limit of detection of analytical method, as the strain is known to produce peptaibols such as paracelsin A, C and D. Supporting documentation and certification relating to quality assurance and further information on Hazard Analysis and Critical Control Point (HACCP) principles, including critical control points and safety data sheets, were also provided upon request.

To achieve minimum declared activity (60,000 LSU(F)/g) after 24 months (solid form) and 18 months (liquid form), the product is overfilled up to 74,200 LSU(F)/g for the solid form and up to 95,000 LSU(F)/g for the liquid form. The stability of the additive during the pelleting process was assessed showing 83% retention in pellets at 80°C and 80% at 90°C. After 3 months storage at 25^{o}C, the stability of the pelleted feed was further assessed showing 91 and 92 % retention in pelleted feed when palleted at 80°C and 90°C, respectively. However, it was noted that the stability during pelleting process was only tested for 30-40 seconds, and it is therefore recommended to take this into consideration for labelling, as some of the uses may require longer conditioning times.

Based on the uniformity study, both enzyme preparations are found to mix homogenously in feed with a coefficient of variation around 10% for the dry powder form and around 5% for the liquid form. The proposed conditions of use of the additive are described in Table 4.

Table 4. Conditions of use of the additive proposed by the applicant

Species or category of animal	Min. content in complete feed	Max. content in complete feed	Withdrawal period
Laying hens	30,000 LSU(F)/kg	-	-
Other provisions and additional requirements for the labelling			
Specific conditions or restrictions for use	Recommended dose range 30,000 – 60,000 LSU(F) /kg. No contraindications.		
Specific conditions or	The liquid preparation (L) is for post-pelleting application, to be sprayed		

Species or category of animal	Min. content in complete feed	Max. content in complete feed	Withdrawal period
restrictions for handling	directly onto the compound feed using appropriate spraying equipment. The dry powder preparation (C) shall be mixed into the feed directly with the other feed materials or after incorporation in a premixture.		
Specific conditions for use	-		

2.1.1. Conclusions on Section II: Identity, characterisation and conditions of use

The additive was fully identified and characterised. It was concluded that the additive consisting of muramidase produced by *T. reesei* DSM 32338 did not raise any safety concerns regarding the genetic modification of the production strain. The microbial contamination and the amounts of the detected impurities do not raise safety concerns. The production strain and its recombinant DNA were not detected in the additive. It was noted that the pelleting stability for the additive was only tested for 30-40 seconds, and therefore this should be made clear on the label as some of the uses may require longer conditioning times. No further concerns were raised for Identity, characterisation and conditions of use section of the dossier.

2.2. Section III: Safety

2.2.1. Safety for the target species

Safety for the target species was assessed based on a 90-day sub-chronic toxicity study in rats. The FSA/FSS reviewed this study, submitted as part of the applicant's original application for authorisation, and resubmitted as part of this current application, to demonstrate safety in laying hens.

The study was performed following the Organisation for Economic Co-operation and Development (OECD) Test Guideline (TG) 408 and was compliant with good laboratory practice (GLP). Three groups of rats, each comprising 10 males and 10 females, received doses by oral gavage of 0.113, 0.374 or 1.132 g total organic solids (TOS)/kg body weight/day (equivalent to 38,462, 126,923 or 384,616 LSU (F)/kg body weight/day) for 13 weeks. A control group received the vehicle at the same volume-dose (10 mL/kg body weight) as the treated groups. The applicant concluded that oral administration of the additive for 13 weeks in rats up to doses of 384,616 LSU (F)/kg body weight/day was well tolerated and did not cause any adverse changes. A no-observed-adverse-effect level (NOAEL) of 384,616 LSU(F)/kg body weight/day was identified. The FSA/FSS agreed the NOAEL of 384,616 LSU(F)/kg body weight/day.

Using default feed intake values set in Guidance on the assessment of the safety of feed additives for the target species (EFSA, 2017) and an uncertainty factor of 100 applied to the NOAEL derived from the 90-day study, the applicant calculated the daily maximum safe concentration of the additive for laying hens. The FSA/FSS agreed with the applicant that the calculation confirms there are no safety concerns for laying hens when using the additive at a maximum recommended dose of 60,000 LSU(F)/kg food. The calculated values can be seen in Table 5.

The FSA/FSS concluded that the additive can be considered safe for the target species up to the maximum recommended level of 60,000 LSU (F)/kg, and there is no requirement for further studies.

Table 5. Safe feed concentration of muramidase in laying hens

NOAEL (90-day rat study)	384,616	LSU (F)/kg body weight/day
Uncertainty factor	100	
Safe daily intake for non-rat species	3,846	LSU (F)/kg body weight/day
Default feed intake level for laying hens	53	g dry matter/kg body weight
Max safe concentration in feed for laying hens	63,861	LSU (F)/kg feed

2.2.2. Safety for the consumer

Safety for the consumer has been evaluated in previous FEEDAP opinions, which did not identify any safety concerns for consumers (EFSA, 2018, EFSA, 2019). The data was reviewed by EFSA in 2018 and 2019, prior to the UK's exit from the EU; thus, these opinions are applicable to GB. These studies formed part of the assessment leading to the current authorisation of the additive in GB.

To support the assessment of safety for consumers, the applicant presented three tests. These studies were also submitted as part of the original application for authorisation:

- 90-day sub-chronic oral toxicity study (OECD TG 408)
- Bacterial reverse mutation test (OECD TG 471)
- *In vitro* Human Lymphocyte Chromosome Aberration Test (OECD TG 473)

The applicant provided an *in vitro* Mammalian Micronucleus Test in Human Peripheral Blood Lymphocytes upon request. The test was performed following OECD TG 487 and was claimed to be GLP compliant. The additive was tested at concentrations which were based upon the results of a preliminary cytotoxicity assay. Muramidase was applied at concentrations of 3000, 4000 and 5000 µg TOS/mL for 3 hours in the presence and absence of S9 metabolic activation and a continuous treatment of 20 hours

at muramidase concentrations of 100, 1000 and 3000 µg TOS/mL in the absence of S9 metabolic activation. The additive did not induce a significant increase in the frequency of binucleated micronucleated cells compared to a vehicle control. Therefore, the FSA/FSS concludes that the test item did not induce structural and numerical chromosome aberrations *in vitro* in human peripheral blood lymphocytes under the experimental conditions employed in this study.

Considering the newly submitted *in vitro* micronucleus test data, alongside the conclusions from the previous genotoxicity studies and the subchronic oral toxicity study that have supported the safety of the feed additive for the consumer, the FSA/FSS concluded that the additive can be considered safe for the consumer.

2.2.3. Safety for the user/worker

The applicant provided the following studies from the original authorisation to evaluate the safety of the additive for the user/ worker:

- *In Vitro* Skin Irritation: Reconstructed Human Epidermis Test Method (OECD TG 439)
- Isolated Chicken Eye Test Method for Identifying i) Chemicals Inducing Serious Eye Damage and ii) Chemicals Not Requiring Classification for Eye Irritation or Serious Eye Damage (OECD TG 438)

When these studies were assessed by the FEEDAP panel in 2018 it was determined that a conclusion on the irritancy potential of the additive could not be reached as the test item showed a lower enzyme activity than that of the additive.

In the current assessment, the applicant submitted a Bovine Corneal Opacity and Permeability Test Method for Identifying i) Chemicals Inducing Serious Eye Damage and ii) Chemicals Not Requiring Classification for Eye Irritation or Serious Eye Damage, and *In Vitro* Skin Irritation: Reconstructed Human Epidermis Test Method.

The skin irritation potential of the solid and liquid formulations of the additive was assessed separately using the *in vitro* EpiDerm™ Reconstructed Human Epidermis Test Method. The test was performed according to OECD TG 439 and was GLP compliant. In this study, the additive was used at 74,600 LSU(F)/g in the solid formulation and 93,100 LSU(F)/g in the liquid formulation (i.e. greater than the maximum specification of the additive). Under the experimental conditions reported, both solid and liquid formulations were classified as non-irritant in accordance with the UN GHS 'No Category'.

The eye irritation potential of the solid and liquid formulations was assessed separately using the Bovine Corneal Opacity and Permeability Assay. The test was performed according to OECD TG 437 and was GLP compliant. In this study the additive was used at 74,600 LSU(F)/g in the solid formulation and 93,100 LSU(F)/g in the liquid formulation (i.e. greater than the maximum specification of the additive). Under the reported experimental conditions, the *in vitro* irritancy score for the solid formulation was between 3 and 55 and did not allow for a stand-alone prediction of 'No Category' or 'Serious Eye Irritant, Category 1'. Therefore, the FSA/FSS could not conclude on the eye irritation potential of the solid formulation. Based upon the results obtained for the liquid formulation, under the reported experimental conditions, the liquid formulation is classified as non-irritant in accordance with UN GHS 'No Category'.

The FSA/FSS concluded that the liquid formulation of the additive is considered to be non-irritant to the skin and eyes. The solid formulation of the additive is considered to be non-irritant to the skin. The FSA/FSS cannot conclude on the eye irritation capability of the solid formulation; therefore, applying the principle of precaution, the solid formulation should be considered a potential eye irritant.

Due to the proteinaceous nature of the active substance, both forms of the additive are assumed to be respiratory sensitisers.

2.2.4. Safety for the environment

Safety for the environment has been assessed in previous FEEDAP opinions (EFSA, 2018, EFSA , 2019), when the UK was an EU member state, and these assessments remain applicable to the UK. The applicant has not provided any new information for the FSA/FSS to reconsider these conclusions. The FSA/FSS concluded that the proposed extension to species does not impact the safety of the additive which have been previously evaluated. The additive, under the proposed conditions of use, is considered safe in the environment.

2.2.5. Conclusions on safety

The FSA/FSS concludes that the use of the additive, under the proposed conditions of use, is safe for consumers and the environment. The liquid formulation of the additive is considered non-irritant to the eyes or skin. The solid formulation of the additive is non-irritant to the skin but should be considered a potential eye irritant. The proposed extension to species requested to this authorisation did not raise any further safety concerns. The FSA/FSS concluded that the additive is safe for laying hens up to the maximum inclusion level of 60,000 LSU(F)/kg feed.

2.3. Section IV: Efficacy

2.3.1. Efficacy in hens

The applicant presented three long-term efficacy studies in laying hens. All three studies followed a complete randomised block design, with the cage as the experimental unit. Upon request, the applicant provided evidence that the animals were treated in compliance with welfare regulations, ensuring animal welfare was monitored throughout the course of the trials. The mortality and general health of the hens were monitored daily, with the most likely cause of death/culling also recorded. No concerns were raised in terms of mortality and culling rates. An overview of the format of the efficacy trials is provided in Table 6:

Table 6. Overview of muramidase efficacy trials in laying hens

Study	Replicates/ treatment	Intended dose (LSU(F)/kg feed)	Actual dose (LSU(F)/kg feed)	Duration
1	21 (5 hens per cage)	0 30,000	<2,000 29,000	140 days
2	24 (8 hens per cage)	0 15,000 30,000	<2,000 16,750 29,733	140 days
3	21 (15 hens per cage)	0 15,000 30,000	<2,000 12,800 30,300	84 days

Treatment with muramidase at the minimum recommended dose of 30,000 LSU(F)/kg feed resulted in an increase in laying index (%) in trials 1 and 3 and an increase in egg mass (g/per hen/day) in all three trials.

An increase in final body weight and an improvement in feed to egg mass conversion ratio (FCR) were observed in hens supplemented with 30,000 LSU(F) kg/feed in trial 1 and 2. However, the same benefits were not observed in trial 3 over the global study period, although it was noted that there was a significant improvement in FCR in the second half of the study. Egg quality characteristics were assessed in trial 2, and treatment with muramidase was found to have no adverse effect on the number of broken or dirty shells.

The FSA/FSS noted that most of the beneficial effects on zootechnical performance parameters that were observed in trials 2 and 3 were only seen at the higher dose, not at the lower dose of 15,000 LSU(F)/kg feed. The FSA/FSS therefore agreed with the applicant's proposed minimum inclusion rate of 30,000 LSU(F)/kg complete feed.

2.3.2. Conclusions on efficacy

Based on the three trials provided, the FSA/FSS concluded that muramidase has the potential to be efficacious in laying hens at a minimum inclusion rate of 30,000 LSU(F)/kg complete feed. This demonstrates that the additive favourably affects animal production, performance or welfare.

3. Analytical methods evaluation

Conclusions on the analytical methods are presented here as an extract from the Evaluation Report of the European Union Reference Laboratory (EURL) for Feed Additives on the Method(s) of the Analysis for muramidase produced by *T. reesei* DSM 32338 (EURL, 2018).

> For the quantification of muramidase activity in the feed additive, premixtures and feedingstuffs:
>
> - Fluorescence-based enzyme assay method that determines the enzyme-catalysed depolymerisation of a fluorescein-labelled peptidoglycan preparation at pH 6.0 and 30 °C.
>
> One LSU(F) unit is defined as the amount of enzyme that increases the fluorescence of 12.5 µg/ml fluorescein-labelled peptidoglycan per minute at pH 6.0 and 30 °C by a value that corresponds to the fluorescence of approximately 0.06 nmol fluorescein isothiocyanate isomer I.

The FSA/FSS accepts the EURL analytical method evaluation reports. The FSA/FSS determined the analytical methods proposed as appropriate for official controls for this feed additive. The analytical methods were approved in 2018, at the time in which the UK was still an EU member.

4. Conclusions

The additive is authorised for use in feed for chicken and minor poultry species for fattening, turkeys for fattening, and chickens, turkeys and other poultry species reared for breeding. This application requested a new use (extension of species) for use in feed for laying hens. The manufacturing process and the composition of the additive have not been modified since the previous assessments. The data was reviewed by EFSA in 2018, 2019 and 2021. Two of the opinions were carried out prior to the UK's exit from the EU; thus, these opinions are applicable to GB. The additional data provided in the current assessment was evaluated by the FSA and FSS. The additive was correctly identified and characterised. It was noted that the

pelleting stability for the additive was only tested for 30-40 seconds, and therefore this should be made clear on the label as some of the uses may require longer conditioning times.

The FSA/FSS concluded that the additive is safe for laying hens up to the maximum inclusion level of 60,000 LSU(F)/kg feed. The FSA/FSS concluded that the additive under the proposed conditions of use is considered to be safe for consumers and the environment. The liquid formulation of the additive is considered non-irritant to the eyes or skin. The solid formulation of the additive is non-irritant to the skin, but should be considered a potential eye irritant. The proposed extension to species (laying hens) requested for this authorisation did not raise any further safety concerns.

Based on the three trials provided, the FSA/FSS concluded that muramidase has the potential to be efficacious in laying hens at a minimum inclusion rate of 30,000 LSU(F)/kg complete feed.

The FSA/FSS accepts the EURL analytical method evaluation reports. The FSA/FSS determined the analytical methods proposed as appropriate for official controls for this feed additive.

Abbreviations

Acronym	Definition
ACAF	Advisory Committee on Animal Feedingstuffs
CFU	Colony forming units
EC	European Commission
EFSA	European Food Safety Authority
EURL	European Union Reference Laboratory
FEEDAP	Scientific Panel on Feed Additives and Products or Substances Used in Animal Feed
FSA	Food Standards Agency
FSS	Food Standards Scotland
GLP	Good Laboratory Practice
LSU(F)	Muramidase activity units
NOAEL	No observed adverse effect level
OECD	Organisation for Economic Co-operation and Development
WGS	Whole Genome Sequence

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