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## Safety and efficacy of muramidase from *Trichoderma reesei* DSM 32338 as a feed additive for chickens for fattening and minor poultry species

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### Abstract

The additive under assessment is a muramidase from *Trichoderma reesei* DSM 32338 which is to be used as a zootechnical additive in feed for chickens for fattening and minor poultry species. The production strain is a genetically modified microorganism. The introduced genetic sequences do not give rise to safety concerns and no viable cells and no DNA of the production strain were detected in the additive. The results of a tolerance trial in chickens for fattening showed that the birds tolerated well 10-fold the highest recommended level of 45,000 LSU(F)/kg feed. Therefore, the Panel concluded that the additive is safe for chickens for fattening and extrapolated the conclusion to minor poultry species for fattening. The enzyme filtrate used to formulate the additive was tested in genotoxicity studies and in a subchronic oral toxicity study. The results of these tests did not indicate any reason for concern for consumer safety arising from the use of the product as a feed additive. Therefore, the Panel concluded that the additive is safe for the consumers. The Panel could not conclude on the potential of the additive for skin/eye irritancy or for its skin sensitisation potential. Owing to the proteinaceous nature of the active substance, the additive is considered to have potential for respiratory sensitisation. The FEEDAP Panel concluded that the use of this product as a feed additive poses no risks to the environment. The additive is to be used as a zootechnical additive, functional group other additives. To support the efficacy, the applicant submitted three efficacy studies with comparable design. The results showed significant and positive improvements on the feed to gain ratio of the birds receiving the additive at the lowest recommended level (25,000 LSU(F)/kg feed). This conclusion was extrapolated to minor poultry species for fattening.

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## Summary

Following a request from European Commission, the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) was asked to deliver a scientific opinion on the safety and efficacy of muramidase from *Trichoderma reesei* DSM 32338 as a feed additive for chickens for fattening and minor poultry species.

The additive is a muramidase enzyme containing product produced by a genetically modified strain of *T. reesei* which is deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen with the accession number DSM 32338. The parental organism is considered safe as a production organism. The introduced sequences do not give rise to safety concerns and no viable cells and no DNA of the production strain were detected in the additive. The additive does not give rise to any safety concerns with regard to the genetic modification.

The results of a tolerance trial in chickens for fattening showed that the birds tolerated well 10-fold the highest recommended level of 45,000 LSU(F)/kg feed. Therefore, the Panel concluded that the additive is safe for chickens for fattening and extrapolated the conclusion to minor poultry species for fattening.

The enzyme filtrate used to formulate the additive was tested in genotoxicity studies and in a subchronic oral toxicity study. The results of these tests did not indicate any reason for concern for consumer safety arising from the use of the product as a feed additive. Therefore, the Panel concluded that the additive is safe for the consumers.

The Panel could not conclude on the potential of the additive for skin/eye irritancy or for its skin sensitisation potential. Owing to the proteinaceous nature of the active substance, the additive is considered to have potential for respiratory sensitisation.

The FEEDAP Panel concluded that the use of this product as a feed additive poses no risks to the environment.

The additive is to be used as a zootechnical additive, functional group other additives. To support the efficacy, the applicant submitted three efficacy studies with comparable design. The results showed significant and positive improvements on the feed to gain ratio of the birds receiving the additive at the lowest recommended level (25,000 LSU(F)/kg feed). This conclusion was extrapolated to minor poultry species for fattening.

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## 1. Introduction

### 1.1. Background and Terms of Reference

Regulation (EC) No 1831/2003<sup>1</sup> establishes the rules governing the Community authorisation of additives for use in animal nutrition. In particular, Article 4(1) of that Regulation lays down that any person seeking authorisation for a feed additive or for a new use of a feed additive shall submit an application in accordance with Article 7.

The European Commission received a request from DSM nutritional Products Ltd., Switzerland<sup>2</sup> for authorisation of the product muramidase produced by *Trichoderma reesei* DSM 32338, when used as a feed additive for chickens for fattening and minor poultry species (category: zootechnical additives; functional group: other zootechnical additives).

According to Article 7(1) of Regulation (EC) No 1831/2003, the Commission forwarded the application to the European Food Safety Authority (EFSA) as an application under Article 4(1) (authorisation of a feed additive or new use of a feed additive). EFSA received directly from the applicant the technical dossier in support of this application. The particulars and documents in support of the application were considered valid by EFSA as of 12 October 2017.

According to Article 8 of Regulation (EC) No 1831/2003, EFSA, after verifying the particulars and documents submitted by the applicant, shall undertake an assessment in order to determine whether the feed additive complies with the conditions laid down in Article 5. EFSA shall deliver an opinion on the safety for the target animals, consumer, user and the environment and on the efficacy of the product muramidase produced by *Trichoderma reesei* DSM 32338 when used under the proposed conditions of use (see Section 3.1.5).

## 2. Data and methodologies

### 2.1. Data

The present assessment is based on data submitted by the applicant in the form of a technical dossier<sup>3</sup> in support of the authorisation request for the use of muramidase produced by *Trichoderma reesei* DSM 32338 as a feed additive. The technical dossier was prepared following the provisions of Article 7 of Regulation (EC) No 1831/2003, Regulation (EC) No 429/2008<sup>4</sup> and the applicable EFSA guidance documents.

EFSA has verified the European Union Reference Laboratory (EURL) report as it relates to the methods used for the control of the active substance in animal feed. The Executive Summary of the EURL report can be found in Annex A.<sup>5</sup>

### 2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of muramidase produced by *Trichoderma reesei* DSM 32338 is in line with the principles laid down in Regulation (EC) No 429/2008 and the relevant guidance documents: Guidance on zootechnical additives (EFSA FEEDAP Panel, 2012a), Technical guidance: Tolerance and efficacy studies in target animals (EFSA FEEDAP Panel, 2011), Technical Guidance for assessing the safety of feed additives for the environment (EFSA, 2008a), Guidance for establishing the safety of additives for the consumer (EFSA FEEDAP Panel, 2012b), Guidance on studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012c), Technical Guidance: Microbial Studies (EFSA, 2008b), Technical Guidance: Extrapolation of data from major species to minor species regarding the assessment of additives for use in animal nutrition (EFSA, 2008c), Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance (EFSA FEEDAP Panel, 2012d), and Guidance on the risk assessment of genetically modified microorganisms and their products intended for food and feed use (EFSA GMO Panel, 2011).

<sup>1</sup> Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. OJ L 268, 18.10.2003, p. 29.

<sup>2</sup> DSM Nutritional Products Ltd., Switzerland represented in EU by Novozymes A/S, Krogshoejvej 37, 2880 Bagsvaerd, Denmark.

<sup>3</sup> FEED dossier reference: FAD-2017-0046.

<sup>4</sup> Commission Regulation (EC) No 429/2008 of 25 April 2008 on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the preparation and the presentation of applications and the assessment and the authorisation of feed additives. OJ L 133, 22.5.2008, p. 1.

<sup>5</sup> The full report is available on the EURL website: <https://ec.europa.eu/jrc/en/eurl/feed-additives/evaluation-reports/fad-2017-0046?search&form-return>

### 3. Assessment

The present opinion deals with the assessment of the safety and efficacy of the enzyme preparation containing muramidase produced by *Trichoderma reesei* DSM 32338 as a zootechnical feed additive (functional group: other zootechnical additives) for chickens for fattening and minor poultry species.

#### 3.1. Characterisation

##### 3.1.1. Characterisation of active substance

The additive is a muramidase enzyme containing product (Enzyme Commission Number 3.2.1.17, lysozyme or *N*-acetylmuramidase) which is produced by a genetically modified strain of *T. reesei* deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen with the accession number DSM 32338.

##### 3.1.1.1. Information relating to the genetically modified microorganism

*Characteristics of the recipient microorganism*

[REDACTED]

*Characteristics of the introduced sequences*

[REDACTED]

*Description of the genetic modification process*

[REDACTED]

[REDACTED]

### 3.1.2. Manufacturing process

The product is obtained by submerged fermentation. [REDACTED]

[REDACTED] The resulting filtrate is used to formulate the additive which is available in solid (GT form) or liquid form (L form). For the solid form (GT), the liquid concentrate is mixed with [REDACTED], dried [REDACTED] using [REDACTED] as coating agent and the coated granules are finally sieved to the desired particle size. For the L form, the filtrate is mixed with [REDACTED]. The applicant states that no antibiotics are used in the manufacturing process.<sup>12</sup>

### 3.1.3. Characterisation of the additive

The solid and liquid forms ensure a guaranteed minimum activity of 60,000 LSU(F)<sup>13</sup> per gram. The product also contains endo-1,4- $\beta$ -glucanase, endo-1,3(4)- $\beta$ -glucanase and endo-1,4- $\beta$ -xy lanase activity as side activities.

The batch-to-batch variation of the solid formulation was studied in five batches and the mean value was 73,600 LSU(F)/g, ranging from 70,800 to 76,800 LSU(F)/g (coefficient of variation (CV) of 2.8%).<sup>14</sup> This formulation contains the fermentation extract (18%), sodium sulfate (70%), cellulose (6%), kaolin (4%), sucrose (1%) and water (1%). This formulation is a fine-granular powder that shows a mean particle size of 550  $\mu$ m and contains no particles below 100  $\mu$ m (measurement done with laser diffraction) and it is dust free (< 0.010 g dust/m<sup>3</sup>, measured with the Stauber–Heubach test).<sup>15</sup> The tapped density is 1.3 kg/L.

The batch-to-batch variation of the liquid formulation was studied in five batches and the mean value was 87,300 LSU(F)/g, ranging from 80,000 to 95,000 LSU(F)/g (CV of 7.0%).<sup>16</sup> This formulation contains the fermentation extract (19%), potassium sorbate (0.05%), sodium benzoate (0.15%), sorbitol (22%) and water (58.8%). It is a solution with a pH that ranges from 4.1 to 4.5 with a density of 1.1 kg/L and a viscosity of 15 cP at 25°C.<sup>17</sup>

The applicant set specifications for microbial and chemical contamination of the additive as follows: total viable counts < 5  $\times$  10<sup>4</sup> colony forming units (CFU)/g, coliform bacteria < 30 CFU/g, the absence of *Escherichia coli* and *Salmonella* spp. in 25 g of the additive, arsenic < 3 mg/kg, cadmium < 0.5 mg/kg, lead < 5 mg/kg and mercury < 0.5 mg/kg. Data on five batches of each formulation of the additive showed compliance with these specifications.<sup>18</sup> No specifications were set for mycotoxins. [REDACTED]

<sup>12</sup> Technical dossier/Section II/Annex 2.32.

<sup>13</sup> LSU(F) is defined as the amount of enzyme that increases the fluorescence of 12.5  $\mu$ g/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.

<sup>14</sup> Technical dossier/Section II/Annex II.1 and II.2 and supplementary information March 2018/Annex 9.

<sup>15</sup> Technical dossier/Section II/Annex II.14 and supplementary information March 2018/Annex 9.

<sup>16</sup> Technical dossier/Section II/Annex II.3 and II.4.

<sup>17</sup> Technical dossier/Section II/Annex II.4 and II.15.

<sup>18</sup> Technical dossier/Section II/Annexes II.2 and II.3.

[REDACTED]

The intermediate enzyme product was tested for the presence of antimicrobial activity<sup>21</sup> against the five strains recommended by EFSA (EFSA FEEDAP Panel 2012d; *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212 and *Bacillus subtilis* ATCC 6633), two more reference strains commonly present in poultry faeces (*Campylobacter jejuni* ATCC 33560 and *Clostridium perfringens* ATCC 13124) and a total of 30 field strains from poultry origin (including five *Salmonella* Enteritidis, five *C. jejuni*, five commensal *E. coli*, five *Enterococcus faecium*, five *E. faecalis* and five *C. perfringens*). In all cases, the minimum inhibitory concentration (MIC) values were equal or higher than 400 mg/L against all strains tested. A MIC of 400 mg/L corresponds to an enzymatic activity of about 500,000 LSU(F)/L. The next dilution tested of 200 mg/L would be the no effect concentration, and this would represent six times the maximum dose of the additive in feed. Considering that muramidase catalyzes the hydrolysis of 1,4-glycosidic linkages between *N*-acetylmuramic acid and *N*-acetylglucosamine in cell wall peptidoglycan, it could be expected an effect of the additive especially against gram positive bacteria, however, this effect was not seen in the species tested at the use level of the product.

The production strain was not detected in a test volume of 1 g of three batches [REDACTED]

The absence of recombinant DNA in three batches of the final product (liquid form), was demonstrated [REDACTED]

### 3.1.4. Stability and homogeneity

The shelf-life of the solid and liquid formulations was studied in samples of at least three batches of each formulation stored for 12 months in closed containers at 10, 25, 35 or 40°C.<sup>24</sup> Recovery of the initial enzyme activity for the GT formulation were 100%, 92%, 73% and 56% at 10, 25, 35 and 40°C, respectively; the corresponding values for the liquid formulation were 82%, 68%, 50% and 36%.

For the solid formulation, the stability was studied in premixtures and feed, including the effect of heat treatment. Three batches of the solid formulation were mixed to two different vitamin–mineral premixtures for chickens for fattening (one containing choline chloride) at a dose rate of 2,500 LSU(F)/g premixture. Samples were stored in closed containers at 25°C for 6 months. Recovery of the initial enzyme activity after 6 months was 75%.<sup>25</sup> Three batches of the solid formulation were added to a complete feed for chickens for fattening (at 40,000 LSU(F)/kg feed), based on maize and wheat, which was pelleted at 80 or 90°C. The enzyme recovery after pelleting was approximately 80% at either temperature.<sup>25</sup> Three batches of the solid formulation were added to two different complete feeds for chickens for fattening (at 40,000 LSU(F)/kg feed), based on maize and wheat, which was subject to pelleting 80°C.<sup>25</sup> Samples of the mash and pelleted feed were stored in closed containers at room temperature for 3 months. The enzyme recovery after the 3-month storage was close to 86% for the mash and 92% for the pelleted feed compared to the initial activity (after pelleting for the pelleted feed).

The stability of the liquid formulation was studied in three batches that were sprayed onto two different complete and pelleted feeds for chickens for fattening, based on maize and wheat.<sup>26</sup> The intended dosage was 40,000 LSU(F) per kg feed (but the analysed dosage was below 30,000). Samples of the pelleted feeds were stored in closed containers at room temperature for 3 months. The enzyme recovery after the 3-month storage was close to 94%.

The capacity of the additive to homogeneously distribute was studied in samples of the feeds used in the stability studies. Ten subsamples of each feed under study were analysed and showed a

<sup>21</sup> Technical dossier/Section II/Annex II.30.

<sup>24</sup> Technical dossier/Section II/Annex II.13 and II.14 and supplementary information March 2018/Annexes 14 and 15.

<sup>25</sup> Technical dossier/Section II/Annex II.14.

<sup>26</sup> Technical dossier/Section II/Annex II.15.



coefficient of variation < 11% (enzyme activity close to 35,000 LSU(F)/kg feed) for the solid formulation and < 6.5% (enzyme activity close to 28,000 LSU(F)/kg feed) for the liquid formulation.

### 3.1.5. Conditions of use

The additive is to be added to feed for chickens for fattening and minor poultry species to provide between 25,000 and 45,000 LSU(F) per kg feed. The GT form should be incorporated directly to feed or via premixture. The L form is designed to be sprayed directly to the compound feed, in case of pelleting the liquid should be incorporated post-pelleting.

## 3.2. Safety

### 3.2.1. Safety of the genetic modification

The parental organism [REDACTED] is considered safe as a production organism. The introduced sequences do not give rise to safety concerns. There are no antimicrobial resistance genes in the production strain remaining from the genetic modification process. No viable cells and no DNA of the production strain were detected in the additive. The product does not give rise to any safety concerns with regard to the genetic modification.

### 3.2.2. Safety for the target species

#### 3.2.2.1. Safety for chickens for fattening

A total of 576 one-day-old male and female chickens for fattening (Ross 308) were penned in groups of 18 birds of the same sex and allocated to four dietary treatments (representing eight replicates per treatment).<sup>27</sup> Two basal diets (starter and grower) based on maize, wheat and soya bean meal were either not supplemented (control) or supplemented with the muramidase to provide 45,000 (1×, highest recommended level), 225,000 (5×) or 450,000 (10×) LSU(F) per kg feed. Confirmation by analysis showed 2,420 in the control, 39,800 in the 1×, 201,723 in the 5× and 414,663 for the 10×. Diets were offered in pellet form and on ad libitum basis for 42 days. Mortality was checked every day and dead animals were necropsied. Animals were weighed at the start and on days 21, 35 and 42. Feed intake was measured on days 21, 35 and 42, and feed to weight gain ratio was calculated. On day 35, two birds per pen, selected at the start of the study, were removed in order to reduce the bird density; the weight of the birds in the pen was measured before and after the removal. Litter was visually examined twice a week; the wetness and structure were scored on a 1–5 scale. On day 25 of study, blood spots were found in the excreta and a daily examination of the excreta for the presence/absence of blood was done until the end of the study. On the last day of study, blood samples were collected from two birds per pen, selected at the start of the study, and analysed for haematological<sup>28</sup> and biochemical<sup>29</sup> parameters. Also, on the last day of study gross pathological examination was done on four birds per pen, selected at the start of the study. Samples of ileum were collected for microscopic examination. Finally, contents of the caecum of two birds per pen, selected at the beginning of the study, were collected and analysed for total aerobic/anaerobic microorganisms, clostridia, enterobacteria, coliforms, lactobacilli, *Salmonella* spp. and *Campylobacter*.

An analysis of variance (ANOVA) was done with the data considering the treatment and the sex and their interaction, pen basis with the exception of necropsy and microbial analysis of the caecal contents. Effects were considered significant at  $p < 0.05$ . If the overall treatment effect was significant, contrasts between treatments were further considered using a least significant difference (LSD) test.

Mortality of the chickens during the study was 1.7%; no differences were found between the treatments (2.1%, 0.7%, 3.5% and 0.7% for control, 1×, 5× and 10×). The mean daily feed intake was 104 g/bird and was not different in the four groups. The daily mean body weight gain of the birds was 66.5, 68.9, 69.6 and 70.0 g/bird for control, 1×, 5× and 10×, the corresponding values for feed to gain ratio were 1.55, 1.51, 1.52 and 1.51. Daily weight gain and feed to gain ratio were significantly improved in the groups fed the muramidase compared to control. No differences were identified on the haematological or biochemical parameters studied with the exception of the enzyme amylase. The

<sup>27</sup> Technical dossier/Section III/Annex III.1.

<sup>28</sup> Including: total blood cells, total white blood cells, mean corpuscular volume, haemoglobin and haematocrit.

<sup>29</sup> Including: alkaline phosphatase, gamma-glutamyl transferase, glutamate dehydrogenase, aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, albumin, amylase, bilirubin, calcium, cholesterol, creatinine, glucose, phosphorus, potassium, total protein, sodium, chloride, uric acid.

amylase results were 607, 695, 573 and 664 U/L for control, 1×, 5× and 10× groups, respectively. The values in 1× group were significantly higher than in the control, but no differences were observed between the other muramidase groups and the control. No differences were identified on the litter wetness and structure, but data were not statistically analysed. The results of the macroscopic and microscopic examination of the necropsied animals showed no gross lesions in any of the organs examined. Histopathological evaluation of ileal tissue did not reveal differences on the alterations between the groups. The study of the microbial populations of the caecal digesta showed, in general, no effects of the additive. Lactobacilli counts showed higher values in the muramidase groups compared to control, which reached significance only in the females. These results show no effects of the additive on the microbial populations from the gastrointestinal tract that were studied.

The results of this tolerance trial in chickens showed no negative effects on the performance, blood parameters and gross pathology examination of the birds when fed 10× the maximum recommended dose.

### 3.2.2.2. Safety for minor poultry species

The margin of safety identified in chickens for fattening (10-fold) allows the Panel to extrapolate the conclusion to minor poultry species in the growing-fattening phase. However, no conclusion can be drawn for minor poultry species in the laying phase.

### 3.2.2.3. Conclusions on safety for the target species

The FEEDAP Panel concludes that the additive is safe for chickens for fattening up to the maximum recommended dose of 45,000 LSU(F) per kg feed and this conclusion can be extrapolated to minor poultry species for fattening purposes.

## 3.2.3. Safety for the consumer

### 3.2.3.1. Genotoxicity studies

#### *Bacterial reverse mutation test*

The fermentation filtrate, (10.8% total organic solids (TOS)) was tested for mutagenicity in the bacterial reverse mutation test in the histidine auxotroph *Salmonella* Typhimurium strains TA1535, TA100, TA1537, TA98 and in the tryptophan auxotroph *E. coli* WP2 *uvrA* with and without metabolic activation (liver S9 mix from induced rats) according to OECD guideline 471.<sup>30</sup> As the test substance contains several nutrients including the amino acids histidine and tryptophan that could interfere with the experimental system, the 'treat and wash' assay was applied: bacteria were exposed to the test substance in a phosphate-buffered nutrient broth for 3 h and then the test substance was removed by centrifugation prior to plating. Nonetheless, some stimulation of bacterial growth was sporadically reported. The top concentration tested was 5,000 µg TOS per mL. Two independent tests were done.

In the first experiment with *Salmonella* strain TA1535 in the absence of S9 mix, a doubling in the number of revertant colonies compared to the solvent control was seen at the two highest dose levels (2,500 and 5,000 µg TOS/mL). Although growth stimulation was observed at these two concentrations, no real dose relationship was present and the increase was not reproduced in the second experiment; therefore, this observation was not considered biologically relevant. No other increase in the number of revertants was reported in any other experimental condition. The positive controls performed as expected.

#### *In vitro chromosomal aberration test*

The same test item, dissolved in purified water, was assessed for its potential to induce structural chromosome aberrations in human lymphocytes with and without metabolic activation, up to a maximum concentration of 5,000 µg TOS/mL in compliance with OECD guideline 473.<sup>31</sup> Cells were exposed to the test item, vehicle or positive controls for 3 h followed by a recovery period of 17 h (with and without metabolic activation) or for 20 h without recovery (only without metabolic activation).

Cytotoxicity higher than 50% was observed only after the 20-h treatment with 5,000 µg/mL without metabolic activation. Neither precipitation nor relevant influence on osmolarity or pH value was observed. At least 1,000 metaphases per culture were evaluated for structural chromosome

<sup>30</sup> Technical dossier/Section III/Annex III.6.

<sup>31</sup> Technical dossier/Section III/Annex III.7.

aberrations. No increases in the frequency of cells with numerical aberrations, exceeding the normal range, were observed in the cultures treated with the test item in the absence and presence of metabolic activation, while the positive controls performed as expected.

### 3.2.3.2. Sub-chronic oral toxicity study

The applicant provided a 90-day study in rats conducted on the fermentation filtrate (10.8% total organic solids (TOS)), according to OECD guideline 408.<sup>32</sup> Groups of ten RccHan™ WIST strain rats of each sex received by gavage 0, 38,462, 126,923 or 384,616 LSU(F) per kg body weight and day for 13 weeks. The volume received in all groups was kept constant to 10 mL per kg body weight.

No animals died during the study. No effects were observed on the clinical and behavioural observations, body weight, food and water consumption or in the ophthalmic examination. Haematological analysis showed an increase in the activated partial thromboplastin time in males receiving the highest dose; a similar difference was not found in females. A decrease was seen in the monocyte counts in females receiving the two highest dosages; however, the result was not dose dependent. Biochemical parameters analysed in blood showed a reduction of plasma alanine aminotransferase in all treated groups, except the lowest dose in females. All individual values of the treated animals were within background control ranges. Also, a lower aspartate aminotransferase activity was found in males given the highest dose, and a reduction of albumin to globulin ratio in females given the highest dose. Relative kidney weight was slightly higher than controls for males given the highest dose, and relative adrenal gland weights were slightly higher than controls at all doses in males, but there was no dose–response relationship, and the majority of individual absolute values were within background control ranges. These differences were not found in females. The incidence and distribution of the macroscopic and microscopic findings at necropsy and histological examination, respectively, showed no relation of such findings to the treatment.

In summary, the results showed few differences in the parameters evaluated. Those identified were generally small (with values within the background control ranges), confined to one sex or not dose-related, consequently are not considered to be related to the treatment. Therefore, the dose of 384,616 LSU(F) per kg body weight is retained as the no-observed-adverse-effect-level (NOAEL) for the test substance.

The applicant calculated the maximum safe concentration for chickens for fattening in feed according to the guidance on the safety for the target species (EFSA FEEDAP Panel, 2018). The result of the calculation was 43,000 LSU/kg feed, and this would support the results obtained in the tolerance study done in chickens for fattening.

### 3.2.3.3. Conclusions on safety for the consumer

Based on the results of the genotoxicity and oral toxicity studies, the FEEDAP Panel considers that there are no risks for consumers of the products derived from chickens for fattening and other minor poultry species for fattening receiving feed containing muramidase as a feed additive at the proposed levels.

## 3.2.4. Safety for the user

### 3.2.4.1. Effects on the respiratory system

No specific tests were provided. However, considering the proteinaceous nature of the active substance of the additive, it is to be considered as a potential respiratory sensitiser. The dusting potential of the solid formulation is negligible and the exposure of the user is unlikely.

### 3.2.4.2. Effects on the skin and eyes

The fermentation product was tested in an *in vitro* skin irritation test following OECD guideline 439 and in an *in vitro* eye irritation test following OECD guideline 438.<sup>33</sup> The results of the two tests showed that the fermentation product is not irritant to the skin or eyes. However, the test item showed a lower enzyme activity than the additive and therefore the Panel cannot draw conclusions on the irritancy potential of the additive.

No specific studies investigating the skin sensitisation potential of the additive were submitted.

<sup>32</sup> Technical dossier/Section III/Annex III.10.

<sup>33</sup> Technical dossier/Section III/Annexes III.11 and III.12.

### 3.2.4.3. Conclusions on safety for the user

The Panel cannot conclude on the potential of the additive for skin/eye irritancy or for its skin sensitisation potential. Owing to the proteinaceous nature of the active substance, the additive is considered to have potential for respiratory sensitisation, it is noted that the solid formulation is dust-free and therefore the exposure is unlikely.

### 3.2.5. Safety for the environment

The production strain and its DNA were not detected in the filtrate and the final additive, respectively. The final product does not pose any environmental safety concern associated with the genetic modification of the production strain.

The active substance of the additive is a protein and, as such, will be degraded/inactivated during passage through the digestive tract of animals or in the environment. Therefore, no risks to the environment are expected and no further environmental risk assessment is required.

## 3.3. Efficacy

The additive is to be used in chickens for fattening and minor poultry species as a zootechnical additive, functional group of other additives as an additive that supports the digestive function that improves the feed to gain ratio.

### 3.3.1. Efficacy for chickens for fattening

The applicant provided three efficacy trials which shared a similar design, and were performed in two experimental sites. The details on the study design are provided in Table 1 and the main results in Table 2.

In the three trials, 1-day-old male chickens for fattening were used and were fed either a non-supplemented diet (control) or diets containing the muramidase at different levels. All trials included the minimum recommended dose of 25,000 LSU(F) per kg feed. The enzyme activities were confirmed by analysis (Table 1). The diets were administered on *ad libitum* basis from day 1 of life and until day 35. The health and mortality were monitored throughout the study. Body weight and feed intake were measured and feed to gain ratio was calculated. In trial 1, ileal digestibility of dry matter, crude protein, fat and ash, calcium and phosphorus was measured. In trials 2 and 3, the quality of litter was recorded (visually and dry matter content); at the end of the study birds were assessed for the presence of foot pad dermatitis (0 (no lesions) to 2 scale) and carcass parameters (carcass weight, dressing percentage, breast muscle and abdominal fat).

An ANOVA was done with the data; for performance data, the pen was considered the experimental unit except body weight in trial 1. Group means comparison was done with Tukey test in trial 1 or Duncan in trials 2 and 3.

**Table 1:** Trial design and dosages of the efficacy trials performed in laying hens

Trial	Total no animals (birds/replicate) replicates/treatment	Breed (age at start) duration	Diet (composition) form	Enzyme activity (LSU(F)/kg feed)	
				Intended	Analysed
1 <sup>(a)</sup>	960 (20) 12	Cobb 1 day 35	Starter-grower (Maize, wheat, soya bean meal) mash	0	1,860
				25,000	25,612
				35,000	33,955
				45,000	40,664
2 <sup>(b)</sup>	990 (10) 33	Ross 308 1 day 35	Starter-grower (wheat, maize, soya bean meal) pelleted	0	1,141
				25,000	27,215
				35,000	35,072
3 <sup>(c)</sup>	990 (10) 33	Ross 308 1 day 35	Starter-grower (wheat, maize, soya bean meal) pelleted	0	4,219
				15,000	12,178
				25,000	26,234

(a): Technical dossier/Section IV/Annex IV.4.

(b): Technical dossier/Section IV/Annex IV.5.

(c): Technical dossier/Section IV/Annex IV.6.

Mortality was low and not treatment related. Birds that received muramidase at any dose showed a significantly better feed to gain ratio in the three trials. In trial 3, no significant effects were found in any of the other parameters evaluated. The results on the ileal apparent digestibility in trial 1 indicated improvements on the digestibility of crude protein and crude fat with the addition of muramidase in the diets, reaching significance at 45,000 LSU(F) per kg feed. In trial 2, the dry matter content of the litter was higher in the control group compared to groups with the muramidase (53%, 45% and 44% for control, 25,000 and 35,000 LSU per kg feed) and the foot pad dermatitis score was lower in the groups receiving muramidase (1.33, 1.07 and 1.13).

**Table 2:** Effect of muramidase on the performance of chickens for fattening

Trial	Treatments LSU(F)/kg	Feed intake <sup>1</sup> (g)	Final body weight (g)	Feed to gain ratio	Mortality/culls (n)
1	0	3,030	2,212	1.40 <sup>a</sup>	11/0
	25,000	3,017	2,254	1.36 <sup>b</sup>	6/0
	35,000	3,006	2,257	1.36 <sup>b</sup>	5/0
	45,000	2,998	2,256	1.35 <sup>b</sup>	6/0
2	0	106	2,481	1.52 <sup>a</sup>	10/5
	25,000	106	2,512	1.49 <sup>b</sup>	12/6
	35,000	106	2,520	1.49 <sup>b</sup>	9/5
3	0	109	2,584	1.49 <sup>a</sup>	9/8
	15,000	110	2,608	1.47 <sup>b</sup>	19/7
	25,000	108	2,622	1.45 <sup>b</sup>	14/3

1: In trial 1, values are total feed intake per bird and in trials 2 and 3 values are daily feed intake per bird.

a,b: Mean values within a trial and within a column with different superscripts are significantly different  $p < 0.05$ .

### 3.3.1.1. Efficacy for minor poultry species

Considering the results obtained in the chickens for fattening, the Panel extrapolates this conclusion to minor poultry species in the growing-fattening phase, but not for the laying phases.

### 3.3.1.2. Conclusions on efficacy for the target species

The results of three studies showed improvements of the feed to gain ratio in birds receiving 25,000 LSU(F)/kg feed. Therefore, the Panel concludes that the additive has a potential to be efficacious as a zootechnical additive in chickens for fattening when added to feed at 25,000 LSU(F) per kg feed. The Panel considers that these conclusions can be extrapolated to minor poultry species for fattening.

## 3.4. Post-market monitoring

The FEEDAP Panel considers that there is no need for specific requirements for a post-market monitoring plan other than those established in the Feed Hygiene Regulation<sup>34</sup> and Good Manufacturing Practice.

## 4. Conclusions

The production strain and its recombinant DNA were not detected in the additive. The additive does not give rise to safety concerns with regard to the genetic modification of the production strain.

The FEEDAP Panel concludes that the additive is safe for chickens for fattening at a level of 45,000 LSU(F)/kg feed. The margin of safety established (10-fold) allows the extrapolation of this conclusion to minor poultry species for fattening.

The use of the product as a feed additive does not give rise to concerns for consumers of the food products obtained from animals fed with it.

The FEEDAP Panel cannot conclude on the skin/eye irritancy potential of the additive nor on its dermal sensitisation potential due to the lack of studies. Owing to the proteinaceous nature of the active substance the additive is considered to be a potential respiratory sensitiser.

The use of the product as a feed additive does not raise safety concerns for the environment.

<sup>34</sup> Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 laying down requirements for feed hygiene. OJ L 35, 8.2.2005, p. 1.

The product has a potential to be efficacious as a zootechnical additive in chickens for fattening when added at 25,000 LSU(F)/kg complete feed. This conclusion can be extrapolated to minor poultry species for fattening.

## Documentation provided to EFSA

- 1) Muramidase produced by *Trichoderma reesei* DSM 32338 for chickens for fattening and minor poultry species. August 2017. Submitted by Novozymes A/S on behalf of DSM Nutritional Products Ltd.
- 2) Muramidase produced by *Trichoderma reesei* DSM 32338 for chickens for fattening and minor poultry species. Supplementary information. March 2018. Submitted by Novozymes A/S on behalf of DSM Nutritional Products Ltd.
- 3) Evaluation report of the European Union Reference Laboratory for Feed Additives on the Methods(s) of Analysis for Muramidase produced by *Trichoderma reesei* DSM 32338 for chickens for fattening and minor poultry species.
- 4) Comments from Member States.

Date	Event
14/8/2017	Dossier received by EFSA
31/8/2017	Reception mandate from the European Commission
12/10/2017	Application validated by EFSA – Start of the scientific assessment
17/1/2018	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003
12/1/2018	Comments received from Member States
12/2/2018	Reception of the Evaluation report of the European Union Reference Laboratory for Feed Additives
28/3/2018	Reception of supplementary information from the applicant - Scientific assessment re-started
14/6/2018	Opinion adopted by the FEEDAP Panel. End of the Scientific assessment

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- EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2012d. Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. EFSA Journal 2012;10(6):2740, 10 pp. <https://doi.org/10.2903/j.efsa.2012.2740>
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EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms), 2011. Scientific Opinion on Guidance on the risk assessment of genetically modified microorganisms and their products intended for food and feed use. EFSA Journal 2011;9(6):2193, 54 pp. <https://doi.org/10.2903/j.efsa.2011.2193>

## Abbreviations

ANOVA	analysis of variance
CFU	colony forming unit
CV	coefficient of variation
EURL	European Union Reference Laboratory
FEEDAP	EFSA Panel on Additives and Products or Substances used in Animal Feed
LSD	least significant difference
MIC	minimum inhibitory concentration
NOAEL	no-observed-adverse-effect-level
OECD	Organisation for Economic Co-operation and Development
PCR	polymerase chain reaction
TOS	total organic solids

## **Appendix A – Executive Summary of the Evaluation Report of the European Union Reference Laboratory for Feed Additives on the Methods of Analysis for Muramidase produced by *Trichoderma reesei* DSM 32338**

In the current application authorisation is sought under Article 4(1) of Regulation (EC) No 1831/2003 for a preparation of muramidase under the category/functional group 4(d) 'zootechnical additives'/other zootechnical additives'. Specifically, authorisation is sought for chickens for fattening and minor poultry species for fattening.

According to the Applicant the active substance in the preparation is muramidase (lysozyme) produced by *Trichoderma reesei* DSM 32338. The activity of muramidase is expressed in LSU(F) units. 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 product is intended to be marketed as solid and liquid formulation having a guaranteed minimum muramidase activity of 60,000 LSU(F)/g or mL. Muramidase is intended to be used directly in feedingstuffs or through premixtures to obtain a minimum activity of 25,000 LSU(F)/kg feedingstuffs.

For the quantification of muramidase activity in the feed additive, premixtures and feedingstuffs the Applicant submitted a single-laboratory validated and further verified fluorometric enzyme assay.

Based on the performance characteristics available, the EURL recommends for official control this method.

Further testing or validation of the methods to be performed through the consortium of National Reference Laboratories as specified by article 10 (Commission Regulation (EC) No 378/2005, as last amended by Regulation (EU) 2015/1761) is not considered necessary.