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# Safety and efficacy of a feed additive consisting of endo-1,4-β-xylanase produced by *Komagataella phaffii* DSM 33574, and viable spores of *Bacillus velezensis* DSM 21836 and *Bacillus licheniformis* ATCC 53757 (EnzaPro) for chickens for fattening, chickens reared for laying/breeding, turkeys for fattening, turkeys reared for breeding and growing minor poultry species (BioResource International (BRI), Inc.)

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## Abstract

Following a request from the European Commission, the EFSA 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 the product EnzaPro containing viable cells/spores of strains of *Bacillus velezensis* (DSM 21836) and *Bacillus licheniformis* (ATCC 53757) and an endo-1,4-β-xylanase produced by a genetically modified strain of *Komagataella phaffii* (DSM 33574) as a zootechnical additive in chickens for fattening, chickens reared for laying/breeding, turkeys for fattening, turkeys reared for breeding and minor poultry species for fattening or raised to the point of lay. The strains *B. velezensis* DSM 21836 and *B. licheniformis* ATCC 53757 were considered to meet the qualified presumption of safety (QPS) requirements. The *K. phaffii* xylanase production strain is genetically modified. No viable cells and no recombinant DNA of the genetically modified production strain were detected in the final product. Therefore, the Panel concluded that the additive does not pose any safety concern regarding the xylanase production strain. EnzaPro is safe for all poultry species for fattening or reared to the point of lay at the proposed conditions of use. The FEEDAP Panel concluded that the use of EnzaPro in animal nutrition is safe for the consumers and the environment. EnzaPro is not a skin irritant but should be considered an eye irritant and a respiratory sensitiser. No conclusions could be drawn on the potential of the additive to cause skin sensitisation. Due to the lack of data, the FEEDAP Panel could not conclude on the efficacy of EnzaPro for the target species. EnzaPro is compatible with diclazuril, halofuginone and nicarbazin.



**Keywords:** zootechnical additives, digestibility enhancers, gut flora stabiliser, EnzaPro, safety, efficacy, poultry

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## Table of contents

# 1. Introduction

## 1.1. Background and Terms of Reference

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

The European Commission received a request from BioResource International, Inc. (BRI) represented in the EU by Pen & Tec consulting S.L.U.[2] for the authorisation of the additive consisting of endo-1,4-β-xylanase produced by *Komagataella phaffii* DSM 33574, and viable spores of *Bacillus velezensis* DSM 21836[3] and *Bacillus licheniformis* ATCC 53757 (EnzaPro), when used as a feed additive for chickens for fattening, chickens reared for laying/breeding, turkeys for fattening, turkeys reared for breeding and growing minor poultry species[4] (category: zootechnical additive; functional groups: digestibility enhancer and gut flora stabiliser).

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 5 August 2021.

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 feed additive consisting of endo-1,4-β-xylanase produced by *K. phaffii* DSM 33574, and viable spores of *B. velezensis* DSM 21836 and *B. licheniformis* ATCC 53757 (EnzaPro), when used under the proposed conditions of use (see **Section 3.1.6**).

## 1.2. Additional information

The feed additive consisting of endo-1,4-β-xylanase produced by *K. phaffii* DSM 33574, and viable spores of *B. velezensis* DSM 21836 and *B. licheniformis* ATCC 53757 (EnzaPro) is not authorised as a feed additive in the European Union.

EFSA issued an opinion on the safety and efficacy of a feed additive consisting of endo-1,4-β-xylanase produced by *K. phaffii* DSM 33574 for chickens and turkeys for fattening, chickens reared for laying/breeding, turkeys reared for breeding and minor poultry species for fattening or raised to the point of lay (EFSA FEEDAP Panel, 2022) and another opinion on the safety and efficacy of a preparation of *B. licheniformis* ATCC 53757 and its protease (Cibenza® EP150) as a feed additive for chickens for fattening, chickens reared for laying and minor avian species for fattening and to point of lay and ornamental birds (EFSA FEEDAP Panel, 2015).

The active agent *B. licheniformis* ATCC 53757, in combination with its protease is authorised as a feed additive for chickens for fattening, chickens reared for laying and minor avian species for fattening and to point of lay and ornamental birds according to Regulation (EU) 2016/898.[5]

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

[2] BioResource International, Inc. (BRI) 4,222 Emperor Blvd Suite 460 Durham NC 27703 USA, represented in the EU by Pen & Tec consulting S.L.U, Plaza Ausias March 1, 08195 Sant Cugat del Vallès Barcelona Spain.

[3] Originally designated as *Bacillus amyloliquefaciens* DSM 21836.

[4] Following the request for Supplementary information dated 18 November 2021, the applicant clarified the target species for which the application was made, restricting it to minor poultry species.

[5] Commission Implementing Regulation (EU) 2016/898 of 8 June 2016 concerning the authorisation of a preparation of *Bacillus licheniformis* (ATCC 53757) and its protease (EC 3.4.21.19) as a feed additive for chickens for fattening, chickens reared for laying and minor poultry species for fattening and reared for laying and ornamental birds (holder of authorisation Novus Europe SA/NV). OJ L 152, 9.6.2016, p. 11–14.

# 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[6] in support of the authorisation request for the use of endo-1,4-β-xylanase (produced by *K. phaffii* DSM 33574), and viable spores of *B. velezensis* DSM 21836 and *B. licheniformis* ATCC 53757 (EnzaPro) as a feed additive.

EFSA has verified the European Union Reference Laboratory (EURL) report as it relates to the methods used for the control of the endo-1,4-β-xylanase and active agents in animal feed. The Executive Summary of the EURL report can be found in Annex A.[7]

## 2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of EnzaPro (endo-1,4-β-xylanase produced by *K. phaffii* DSM 33574, and viable spores of *B. velezensis* DSM 21836 and *B. licheniformis* ATCC 53757) is in line with the principles laid down in Regulation (EC) No 429/2008[8] and the relevant guidance documents: Guidance on studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012), Guidance on the assessment of the safety of feed additives for the consumer (EFSA FEEDAP Panel, 2017a), Guidance on the identity, characterisation and conditions of use of feed additives (EFSA FEEDAP Panel, 2017b), Guidance on the assessment of the safety of feed additives for the target species (EFSA FEEDAP Panel, 2017c), Guidance on the assessment of the efficacy of feed additives (EFSA FEEDAP Panel, 2018a), Guidance on the characterisation of microorganisms used as feed additives or as production organisms (EFSA FEEDAP Panel, 2018b) and Guidance on the assessment of the safety of feed additives for the environment (EFSA FEEDAP Panel, 2019).

# 3. Assessment

The additive under assessment contains endo-1,4-β-xylanase produced by *K. phaffii* DSM 33574, and viable cells/spores of *B. velezensis* DSM 21836 and *B. licheniformis* ATCC 53757 and is intended to be used as a zootechnical additive (functional groups: digestibility enhancers and gut flora stabilisers) in feed for chickens for fattening, chickens reared for laying/breeding, turkeys for fattening, turkeys reared for breeding and minor poultry species for fattening or raised to the point of lay. The additive under assessment will be hereafter referred to as EnzaPro.

## 3.1. Characterisation

### 3.1.1. Characterisation of the xylanase production organism

The xylanase is produced by a genetically modified strain of *K. phaffii* which is deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) with deposition number DSM 33574.[9]

The identity of the production strain and the characterisation of its genetic modification were established in a previous assessment (EFSA FEEDAP Panel, 2022). [redacted]

### 3.1.2. Characterisation of the active agents

The active agents *B. velezensis* DSM 21836 and *B. licheniformis* ATCC 53757 were isolated from soil and from a poultry waste digester, respectively. The *B. velezensis* strain is deposited at the DSMZ with

[6] FEED dossier reference: FAD-2021-0056.
[7] The full report is available on the EURL website: https://joint-research-centre.ec.europa.eu/document/download/5c3de7ce-c7e3-43db-adf5-08189d470d43_en?filename=finrep-fad-2021-0056-enzapro.pdf.
[8] 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.
[9] Technical dossier/Section II/Annexes_Sect_II/Annex_II_2_1c.

the accession number DSM 21836,[10] while the *B. licheniformis* strain is deposited in the American Type Culture Collection (ATCC) with the accession number ATCC 53757.[11]

Taxonomic identification of the active agents DSM 21836 and ATCC 53757 as *B. velezensis* and *B. licheniformis*, respectively, was established [redacted][12] [redacted]

The toxigenic potential of the active agents DSM 21836 and ATCC 53757 was assessed according to the Guidance on the characterisation of microorganisms used as feed additives or as production organisms (EFSA FEEDAP Panel, 2018b).[13] No lysis of Vero cells was detected with any of the active agents. Therefore, *B. velezensis* DSM 21836 and *B. licheniformis* ATCC 53757 are considered to be non-toxigenic.

The susceptibility of *B. velezensis* DSM 21836 and *B. licheniformis* ATCC 53757 to the antibiotics recommended by the FEEDAP Guidance (EFSA FEEDAP Panel, 2018b) was tested [redacted] and all of the minimum inhibitory concentration (MIC) values determined were equal or fell below the FEEDAP cut-off values except [redacted] for *B. licheniformis* ATCC 53757, which was exceeded by one dilution [redacted][14] Exceedance of the cut-off value by one dilution is considered to be within the normal range of variation and thus, not a matter of concern. Therefore, *B. velezensis* DSM 21836 and *B. licheniformis* ATCC 53757 are susceptible to all relevant antibiotics.

The WGS data of both active agents was interrogated for the presence of antimicrobial resistance (AMR) genes [redacted].[12] No genes of concern were identified.

The production of aminoglycosides by *B. velezensis* DSM 21836 was analysed [redacted][15] None of the reference strains was inhibited, demonstrating the absence of antimicrobial activity in the supernatant of *B. velezensis* DSM 21836.

### 3.1.3. Manufacturing process

The additive contains xylanase produced by *K. phaffii* DSM 33574 and viable spores of *B. velezensis* DSM 21836 and *B. licheniformis* ATCC 53757. The xylanase is produced by fermentation with *K. phaffii* DSM 33574 [redacted].[16] [redacted].

The manufacturing process of the xylanase is the same as the one assessed in a previous assessment (EFSA FEEDAP Panel, 2022).

[10] Technical dossier/Supplementary information April 2022/Annex_II_2_1a.
[11] Technical dossier/Supplementary information April 2022/Annex_II_2_1b.
12 Technical dossier/Section II/Annexes_Sect_II/Annex_II_2_1d and e.
[13] Technical dossier/Section II/Annex_II_2_2f and Annex_II_2_2g.
[14] Technical dossier/Section II/Annex_II_2_2a, Annex_II_2_2b, Annex_II_2_2c and Annex_II_2_2d.
[15] Technical dossier/Section II/Annexes_Sect_II/Annex_II_2_2e.
[16] Technical dossier/Section II/Annexes_Sect_II/Annex_II_3_1a.

[redacted][17] [redacted][18]

The applicant declared that no antibiotics are used during the manufacturing process of the additive.

### 3.1.4. Characterisation of the additive

EnzaPro is available in solid form and ensures a guaranteed minimum xylanase activity of 100,000 XU[19]/g of additive. The guaranteed minimum total content of viable spores of *Bacillus* spp. in the product is $1 \times 10^9$ colony forming units (CFU)/g additive (*B. velezensis* DSM 21836 and *B. licheniformis* ATCC 53757 are present in a 1:1 ratio, i.e., $5 \times 10^8$ CFU/g each strain). [redacted].[20]

The batch-to-batch variation was studied in five batches of the product and the mean values were 121,623 XU/g product (range 106,275–139,499 XU/g) and $1.8 \times 10^9$ total bacilli CFU/g product (range $1.1 \times 10^9$–$3.4 \times 10^9$ CFU/g).[21] Individual counts of *B. velezensis* DSM 21836 and *B. licheniformis* ATCC 53757 were analysed by identifying colonies via specific polymerase chain reaction (PCR) after culturing. Results supported the declared qualitative and quantitative (1:1 ratio) composition of the product: $9.7 \times 10^8$ CFU/g product (range $5.5 \times 10^8$–$1.9 \times 10^9$ CFU/g) for *B. velezensis* DSM 21836 and $8.7 \times 10^8$ CFU/g product (range $5.5 \times 10^8$–$1.5 \times 10^9$ CFU/g) for *B. licheniformis* ATCC 53757.

*B. licheniformis* ATCC 53757 is known to produce a protease (EFSA FEEDAP Panel, 2015); the applicant investigated the potential presence of protease activity in the final product.[22] Analysis of three batches of EnzaPro showed that protease activity was not detected.

Three batches of the additive were analysed for chemical contamination.[23] The analysis included arsenic (range 0.43–0.57 mg/kg), cadmium (range 0.46–0.55 mg/kg), lead (range 0.21–0.23 mg/kg), mercury (range 0.020–0.024 mg/kg), and mycotoxins, including aflatoxins (B1, B2, G1, G2, M1 and M2), deoxynivalenol, fumonisins (B1 and B2), HT-2 toxin, T-2 toxin, ochratoxin A and zearalenone which showed values below the corresponding limits of quantification (LOQs). Polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and coplanar dioxin-like polychlorinated biphenyls (co-planar PCBs) were found below the corresponding LOQ.[24] The calculated (upper bound) levels of dioxins were < 0.06 ng WHO-PCDD/F-TEQ/kg and the sum of dioxins and dioxin-like-PCBs ranged between 0.0945 and 0.0976 ng WHO-PCDD/F-PCB-TEQ/kg.

Three additional batches of the additive were analysed for the presence of residues of methanol, isopropanol and ethanol and found to be below the LOQ (< 10 mg/kg).[25] Further, one batch of the additive was analysed for the presence of potential residues (diethylene glycol, ethylene glycol and propylene glycol) [redacted] and showed values < 0.01%.[26]

The applicant set specifications for microbiological contamination which include moulds ($\leq 10^3$ CFU/g), *Escherichia coli* (≤ 10 CFU/g), coliforms (≤ 10 CFU/g) and *Salmonella* (not detected in 25 g). These specifications were confirmed in the analysis of three batches: moulds (< 10 CFU/g), coliforms

[17] Technical dossier/Section II/Annexes_Sect_II/Annex_II_3_1b and Supplementary information April 2022/Annexes/Annex_II_1_3a.
[18] Technical dossier/Section II/Annexes_Sect_II/Annex_II_3b.
[19] One unit of endo-1,4-β-xylanase activity (XU) is defined as the amount of enzyme needed for the release of 1 nanomole of reducing sugars (xylose equivalents) per second from 0.5% xylan at 50°C in 50 mM trisodium citrate buffer pH 6.0.
[20] Technical dossier/Supplementary information April 2022/Annexes/Annex_II_1_3a.
[21] Technical dossier/Section II/Annexes_Sect_II/Annex_II_1_3b.
[22] Technical dossier/Supplementary information April 2022/Annexes/Annex_II_1_4g.
[23] Technical dossier/Section II/Annexes_Sect_II/Annex_II_1_4a and Supplementary information April 2022/Annexes/Annex_II_1_4a. Limit of quantification (LOQ) in mg/kg was 0.02 for arsenic and lead, 0.01 cadmium and mercury and in μg/kg was 0.5 for aflatoxins (B1, G1, B2, G2, M1 and M2), 100 for deoxynivalenol and HT-2 toxin, 25 for fumonisins (B1 and B2), 10 for T-2 toxin, 1 for ochratoxin A, 30 for zearalenone.
[24] Technical dossier/Annexes_Sect_II/Annex_II_1_4a.
[25] Technical dossier/Supplementary information April 2022/Annexes/Annex_II_1_4f.
[26] Technical dossier/Section II/Annexes_Sect_II/Annex_II_1_4b.

(< 10 CFU/g), *E. coli* (< 10 CFU/g) and *Salmonella* spp. (not detected in 25 g). *Bacillus cereus*[27] and Enterobacteriaceae[28] were not detected (< 10 CFU/g) in three batches of the additive.

The detected amounts of the above-described impurities do not raise safety concerns.

The presence of viable cells of the xylanase production strain in the final additive was investigated in three batches of EnzaPro, analysed in triplicate.[29] [redacted]. Therefore, no viable cells of *K. phaffii* DSM 33574 were found in the final product.

The presence of recombinant DNA of the production strain of the xylanase was tested in three batches of EnzaPro, each tested in triplicate.[30] [redacted] No DNA of *K. phaffii* DSM 33574 was detected.

The additive appears as a grey to yellowish brown powder and it has a bulk density of 950 kg/m$^3$.[31] The dusting potential of three batches of the additive was determined using the Stauber–Heubach method and showed values on average of 3.302 g/m$^3$ (range 1.466–5.787 g/m$^3$).[32] The same three batches were analysed for particle size distribution by laser-diffraction method; the results showed that particles < 100 μm, < 50 μm, < 10 μm and < 1 μm were on average 90.5% (range 88.3–92.6%), 66.6% (range 61.7–69.3%), 9.8% (range 6.9–12.5%) and 0.2% (range 0.0–0.3%), respectively.

### 3.1.5. Stability and homogeneity

The shelf life of the additive (three batches analysed each in triplicate) was studied in samples stored at 25°C in aluminium bags for up to 15 months. After 15 months, no losses in total bacilli counts were detected and the losses of enzyme activity were on average 1.3%.[33]

Stability in complete feed was investigated using one batch of the additive (10 subsamples analysed in triplicate) when added at 100 mg/kg to a mash soy/corn-based feed for poultry (with choline chloride). The mash feed supplemented with the additive was pelleted at 80°C in order to study the effect of the temperature. The heat treatment resulted in no losses in total bacilli counts while a loss in xylanase activity of 28% was observed. Samples of the mash and pelleted feed were stored for 4 months at 25°C and 60% relative humidity (in paper bags). Xylanase activity and counts of total bacilli were evaluated at the start and after 1, 2, 3 and 4 months. At the end of the storage period, negligible losses in total bacilli counts (< 0.5 Log) were detected while losses in xylanase activity were 26.1 and 18.8% in mash and pelleted feed, respectively.

The homogeneous distribution of the additive in feed was studied in 10 subsamples of the mash and pelleted feeds described above and the coefficients of variation (CVs) were 0.6% (total bacilli counts) and 7.5% (xylanase activity) in mash feed and 0.4% (total bacilli counts) and 3.6% (xylanase activity) in pelleted feed.[34]

[27] Technical dossier/Section II/Annexes_Sect_II/Annex_II_1_4a. LOQ 10 CFU/g for yeasts, moulds, coliforms, *E. coli* and *B. cereus*.
[28] Technical dossier/Supplementary information April 2022/Annexes/Annex II_1_4f.
[29] Technical dossier/Section II/Annexes_Sect_II/Annex_II_1_4c and Annex_II_1_4e.
[30] Technical dossier/Section II/Annexes_Sect_II/Annex_II_1_4d.
[31] Technical dossier/Section II/Annexes_Sect_II/Annex_II_1_5b.
[32] Technical dossier/Section II/Annexes_Sect_II/Annex_II_1_5a.
[33] Technical dossier/Section II/Annexes_Sect_II/Annex_II_4_1.
[34] Technical dossier/Section II/Annexes_Sect_II/Annex_II_4_2.

### 3.1.6. Conditions of use

The additive is intended for use in feed for chickens for fattening, chickens reared for laying/breeding, turkeys for fattening, turkeys reared for breeding and minor poultry species for fattening or raised to the point of lay at a proposed minimum level of 10,000 XU and $1 \times 10^8$ total bacilli CFU/kg feed. The applicant requests for the simultaneous use of the additive with the following coccidiostats: diclazuril, halofuginone and nicarbazin. The additive is not to be used in premixtures.

## 3.2. Safety

### 3.2.1. Safety of the microorganisms

The production strain of the xylanase present in the product belongs to *K. phaffii*, a species considered by EFSA to be suitable for the qualified presumption of safety (QPS) approach to safety assessment when used for enzyme production (EFSA BIOHAZ Panel, 2007; EFSA BIOHAZ Panel, 2020). The safety aspects of its genetic modification were evaluated in a previous assessment (EFSA FEEDAP Panel, 2022). In the context of the current assessment, the applicant provided data excluding the presence of viable cells and recombinant DNA of *K. phaffii* DSM 33574 in EnzaPro final product. Therefore, the FEEDAP Panel concludes that the additive does not pose any safety concern regarding the xylanase production strain.

*B. velezensis* DSM 21836 and *B. licheniformis* ATCC 53757 belong to species considered by EFSA to be suitable for the QPS approach to safety assessment (EFSA, 2007; EFSA BIOHAZ Panel, 2020). This approach requires the identity of the strains to be conclusively established, evidence that the strains lack toxigenic potential and do not show acquired resistance to antibiotics of human and veterinary importance, and for *B. velezensis* absence of aminoglycoside production ability. The identity of the strains has been unambiguously established and the compliance with the other qualifications confirmed. Therefore, the strains qualify for the QPS approach.

### 3.2.2. Toxicological studies

The applicant submitted a bacterial reverse mutation test,[35] an *in vitro* mammalian micronucleus assay[36] and a sub-chronic oral toxicity study[37] performed with the intermediate concentrate form of xylanase to support the safety of the xylanase contained in EnzaPro. Those studies were assessed in a previous opinion (EFSA FEEDAP Panel, 2022) and the results showed that the xylanase intermediate product used for the formulation of EnzaPro did not show any genotoxicity potential. Moreover, the results obtained in the sub-chronic oral toxicity study raised no concerns regarding the product and allowed to derive a no observed adverse effect level (NOAEL) of 1,292,410 XU/kg body weight (bw) per day (highest dose tested).

### 3.2.3. Safety for the target species

No tolerance studies in relevant target species were submitted. *B. velezensis* DSM 21836 and *B. licheniformis* ATCC 53757 are considered to be eligible for the QPS approach. Therefore, the active agents do not raise safety concerns for the target species.

In order to support the safety of the xylanase present in the additive for the target species, the applicant referred to the 90-day toxicity study that was already assessed in a previous opinion (EFSA FEEDAP Panel, 2022). The NOAEL identified (1,292,410 XU/kg bw per day) was used to calculate the maximum safe level in feed for chickens and turkeys for fattening in accordance with the procedure described in the Guidance on the safety for the target species (EFSA FEEDAP Panel, 2017c). The calculated maximum safe concentration in feed was 143,964 XU/kg complete feed for chickens for fattening and 192,766 XU/kg complete feed for turkeys for fattening. These values are higher than the recommended use level for all target species of 10,000 XU/kg feed. Therefore, the Panel concludes that the use of the xylanase present in the additive is safe for chickens for fattening, chickens reared for laying/breeding, turkeys for fattening, turkeys reared for breeding and minor poultry species for fattening or raised to the point of lay at the recommended level of 10,000 XU/kg feed.

[35] Technical dossier/Supplementary information April 2022/Annexes/Annex_II_2_2_2a.
[36] Technical dossier/Supplementary information April 2022/Annexes/Annex_II_2_2_2b.
[37] Technical dossier/Supplementary information April 2022/Annexes/Annex_II_2_2_3.

Since the other components of the additive do not give rise to safety concerns, EnzaPro is considered safe for the target species at the proposed conditions of use.

### 3.2.4. Safety for the consumer

*B. velezensis* DSM 21836 and *B. licheniformis* ATCC 53757 are considered to be eligible for the QPS approach. Therefore, the active agents do not raise safety concerns for consumers of products derived from animals fed the additive.

The enzyme is produced by a genetically modified strain of *K. phaffii* (DSM 33574); this species is considered to qualify for the QPS approach to safety assessment when used for enzyme production. The identity of the strain was established, and the genetic modification introduced [redacted]; however, this is not expected to have an impact on the toxicological profile of the production strain. Therefore, the production strain is presumed safe for production purposes and no concerns would raise for the consumer from the fermentation product obtained from this strain. The results obtained in the genotoxicity studies and the 90-day study support this conclusion. The FEEDAP Panel concludes that the use of EnzaPro in animal nutrition under the proposed conditions of use is safe for the consumers.

### 3.2.5. Safety for the user

#### 3.2.5.1. Effect on respiratory system

The highest dusting potential of the additive measured was 5.787 g/m$^3$. Therefore, exposure of the respiratory system to the additive is possible. Due to the proteinaceous nature of the active substances of the additive, the additive is considered to be a respiratory sensitiser.

#### 3.2.5.2. Effect on skin and eyes

The skin irritation potential of EnzaPro was investigated in an *in vitro* skin irritation study according to OECD TG 439 and following Good Laboratory Practice (GLP).[38] Based on the results the additive is classified as non-irritant to the skin (UN GHS 'No Category').

The eye irritation potential of EnzaPro was investigated in an *in vitro* eye irritation study according to OECD TG 492 and following GLP.[39] Based on the results of the study, the additive is considered an eye irritant.

No information on skin sensitisation potential was provided, therefore the FEEDAP Panel cannot conclude on the skin sensitisation potential of the additive.

#### 3.2.5.3. Conclusions on safety for the user

EnzaPro is not a skin irritant but should be considered an eye irritant and a respiratory sensitiser. No conclusions can be drawn on the potential of the additive to cause skin sensitisation.

### 3.2.6. Safety for the environment

The active agents *B. velezensis* DSM 21836 and *B. licheniformis* ATCC 53757 are considered to be eligible for the QPS approach. Therefore, the active agents do not raise safety concerns for the environment. No cells nor DNA of *K. phaffii* DSM 33574 were detected in the product and therefore, no concerns would rise from the production strain of the xylanase.

Regarding the xylanase, it is a protein, and as such will be degraded/inactivated during the passage through the digestive tract of animals or in the environment. Therefore, the FEEDAP Panel concludes that EnzaPro is safe for the environment.

## 3.3. Efficacy

To support the efficacy as a zootechnical additive (functional groups: digestibility enhancer and gut flora stabiliser), the applicant submitted four long-term trials in chickens for fattening and five publications.

The four trials submitted aimed at studying the effect of the additive on the zootechnical performance and on the apparent metabolisable energy content of the diets. The Panel notes that the

[38] Technical dossier/Section III/Annexes_Sect_III/Annex_III_3_1_2a.
[39] Technical dossier/Section III/Annexes_Sect_III/Annex_III_3_1_2b.

husbandry conditions applied in all trials did not reflect those in which the birds are raised in the EU and were not in line with Directive 2007/43/EC[40] (regarding the lighting program and/or bedding used). Therefore, the performance parameters measured were not considered for the assessment of efficacy.

The Panel considers that the husbandry conditions would have lower impact on the energy utilisation data. However, trials 1[41], 3[42] and 4[43] also showed limitations that would prevent considering the energy utilisation data for the assessment of efficacy. In trial 1, the excreta collection period was too short. In trial 3, the litter was spiked with avian pathogens. In trial 4, a very low growth rate (ca. 60% of expected according to breed standards) and high mortality in control birds (> 6%) were observed.

In trial 2[44], 648 one-day-old male chickens for fattening (Ross 308) were distributed in 36 pens and allocated to 3 dietary treatments (12 replicates per treatment, 18 birds per pen). Three basal diets (starter, from day 1 to 10; grower, from 11 to 21; finisher, from 22 to 42) based on maize and soya bean meal were either not supplemented (control) or supplemented with EnzaPro to provide 10,000 XU/kg feed and $1 \times 10^8$ CFU/kg feed of *Bacillus* spp. spores (confirmed by analysis). A positive control diet with higher energy content was also considered in the trial. The experimental diets were offered *ad libitum* in mashed form for 42 days. The diets contained an external marker for the digestibility analysis. From days 19 to 21, excreta samples were collected by spot sampling and pooled per pen. The diets and excreta were analysed for the content of gross energy, external marker and nitrogen contents to determine apparent metabolisable energy adjusted for nitrogen (AMEn). The experimental data were analysed by one-way analysis of variance (ANOVA), with the treatment as factor. The method used to compare the group means was Tukey's and the significance level applied was of 0.05. The supplementation of the diets with EnzaPro at the minimum recommended level showed significantly higher AMEn (2,883 kcal/kg) in comparison with the control diet (2,693 kcal/kg). No statistical difference on AMEn was observed between the treatment and the positive control diet.

In addition to the *in vivo* trials, the applicant submitted five publications in which the effect of the additive was studied (Flores et al., 2017; Nusairat et al., 2018; Duarte et al., 2019, 2020; Nusairat and Wang, 2020). Two of them (Duarte et al., 2019, 2020) reported studies in pigs, which are out of the scope of the current assessment. The other three publications studied the effect of the additive mainly on zootechnical endpoints in chickens for fattening. However, as above, the husbandry conditions applied in the three trials did not reflect the conditions in which the birds are raised in the EU and were not in line with Directive 2007/63/EC (i.e. under continuous light[45] or receiving a higher number of hours of light[46] or exposed to reused bedding[47]). Therefore, the Panel did not consider the results of the zootechnical parameters further in the assessment. However, the study from Nusairat and Wang (2020) included data on the utilisation of the energy of the diets at two different time points. The results showed that the supplementation of a combination of xylanase (10,000 XU/kg feed) with the same two *Bacillus* spp. strains ($1 \times 10^8$ CFU/kg feed) improved, compared to the control, the AMEn on days 21 (2,902 vs 2,857 kcal/kg feed) and 42 (3,102 vs 3,054 kcal/kg feed).

Overall, the supplementation of the additive at the minimum use level showed an improvement in dietary AMEn in two studies. These results could support the efficacy of the additive, given the presence of xylanase. However, considering the additive under assessment and the functional groups claimed in the application, the Panel deems that data on the zootechnical performance are needed. Therefore, due to the lack of adequate data, the Panel is not in a position to conclude on the efficacy of the additive.

### 3.3.1. Compatibility with coccidiostats

The applicant provided *in vitro* studies to support the compatibility of *B. velezensis* DSM 21836 and *B. licheniformis* ATCC 53757 with diclazuril, halofuginone and nicarbazin.[48]

[40] Council Directive 2007/43/EC of 28 June 2007 laying down minimum rules for the protection of chickens kept for meat production, OJ L 182 12.7.2007, p. 19.
[41] Technical dossier/Section IV/Annexes_Sect_IV/Annex 3_1.
[42] Technical dossier/Section IV/Annexes_Sect_IV/Annex 3_3.
[43] Technical dossier/Section IV/Annexes_Sect_IV/Annex 3_4.
[44] Technical dossier/Section IV/Annexes_Sect_IV/Annex 3_2.
[45] Nusairat et al., 2018 and Nusairat and Wang, 2020.
[46] Flores et al., 2017.
[47] Flores et al., 2017, Nusairat et al., 2018 and Nusairat and Wang, 2020.
[48] Technical dossier/Section II/Annexes_Sect_II/Annex_II_4_4.

[redacted] The MIC values[49] for diclazuril [redacted], halofuginone [redacted] and nicarbazin [redacted] were greater than four times their maximum authorised level (1.2, 3 and 125 mg/kg, respectively).[50]

The results indicate that the active agents present in EnzaPro are compatible with diclazuril, halofuginone and nicarbazin.

#### 3.3.1.1. Conclusions on efficacy

Due to the lack of adequate data, the FEEDAP Panel is not in the position to conclude on the efficacy of EnzaPro for the target species.

The FEEDAP Panel concludes that EnzaPro is compatible with diclazuril, halofuginone and nicarbazin.

### 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[51] and Good Manufacturing Practice.

## 4. Conclusions

The production strain of the xylanase is a genetically modified strain of *K. phaffii* (DSM 33574). No viable cells nor DNA of the production strain were detected in the final product. Therefore, the additive does not pose any safety concern regarding the xylanase production strain.

EnzaPro is safe for all poultry species for fattening or reared to the point of lay at the recommended level of 10,000 XU/kg and $1 \times 10^8$ total bacilli CFU/kg feed.

The additive is safe for the consumers of food derived from animals fed with the additive.

EnzaPro is not a skin irritant but should be considered an eye irritant and a respiratory sensitiser. No conclusions can be drawn on the potential of the additive to cause skin sensitisation.

The use of the product as a feed additive is of no concern for the environment.

The FEEDAP Panel cannot conclude on the efficacy of EnzaPro for the target species. EnzaPro is compatible with diclazuril, halofuginone and nicarbazin.

## 5. Documentation provided to EFSA/Chronology

| Date | Event |
|---|---|
| **25/03/2021** | Dossier received by EFSA. EnzaPro (preparation of endo-1,4-beta-xylanase (EC 3.2.1.8) produced by *Komagaetella phaffii* xyl-2 (DSM 33574), *Bacillus amyloliquefaciens* Ba-BPD1 (DSM 21836) and *Bacillus licheniformis* PWD-1 (ATCC 53757)) for chickens for fattening, chickens reared for laying, turkeys for fattening, turkeys reared for breeding and minor avian species. Submitted by BioResource International, Inc (BRI). |
| **21/04/2021** | Reception mandate from the European Commission |
| **05/08/2021** | Application validated by EFSA – Start of the scientific assessment |
| **08/11/2021** | Comments received from Member States |
| **18/11/2021** | Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. *Issues: characterisation* |
| **19/11/2021** | Reception of the Evaluation report of the European Union Reference Laboratory for Feed Additives |
| **21/12/2021** | Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. *Issues: characterisation, efficacy* |
| **12/01/2022** | Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. *Issues: target species safety* |

[49] Data shown is for the most sensitive strain, *B. velezensis* DSM 21836.

[50] Maximum authorised levels: diclazuril 1.2 mg/kg (chickens for fattening, turkeys for fattening, guinea fowl for fattening and for breeding) and 1 mg/kg (chickens reared for laying); halofuginone 3 mg/kg (chickens for fattening and turkeys); nicarbazin 125 mg/kg (chickens for fattening).

[51] Regulation (EC) No 183/2005 of the European Parliament and of the Council of 12 January 2005 laying down requirements for feed hygiene. OJ L 35, 8.2.2005, p. 1.

| Date | Event |
|---|---|
| **16/03/2022** | Clarification teleconference during risk assessment |
| **13/04/2022** | Reception of supplementary information from the applicant - Scientific assessment re-started |
| **27/09/2022** | Opinion adopted by the FEEDAP Panel. End of the Scientific assessment |

## References


Duarte M, Zhou F, Wilson D Jr and Sung K, 2019. Dietary supplementation of xylanase and protease on growth performance, digesta viscosity, nutrient digestibility, immune and oxidative stress status, and gut health of newly weaned pigs. Animal Nutrition, 5(2019), 351–358.

Duarte ME, Tyus J and Kim SW, 2020. Synbiotic effects of enzyme and probiotics on intestinal health and growth of newly weaned pigs challenged with enterotoxigenic F18$^{+}$ *Escherichia coli*. Frontiers in Veterinary Science, 7, 573. https://doi.org/10.3389/fvets.2020.00573

EFSA (European Food Safety Authority), 2007. Opinion of the Scientific Committee on a request from EFSA on the introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA. EFSA Journal 2007;5(12):587, 16 pp. https://doi.org/10.2903/j.efsa.2007.587

EFSA BIOHAZ Panel (EFSA Panel on Biological Hazards), Koutsoumanis K, Allende A, Alvarez-Ordóñez A, Bolton D, Bover-Cid S, Chemaly M, Davies R, De Cesare A, Hilbert F, Lindqvist R, Nauta M, Peixe L, Ru G, Simmons M, Skandamis P, Suffredini E, Cocconcelli PS, Fernández Escámez PS, Maradona MP, Querol A, Suarez JE, Sundh I, Vlak J, Barizzone F, Correia S and Herman L, 2020. Scientific Opinion on the update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA (2017–2019). EFSA Journal 2020;18(2):5966, 56 pp. https://doi.org/10.2903/j.efsa.2020.5966

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2012. Guidance on studies concerning the safety of use of the additive for users/workers. EFSA Journal 2012;10(1):2539, 5 pp. https://doi.org/10.2903/j.efsa.2012.2539

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2015. Scientific Opinion on the safety and efficacy of Cibenza® EP150 (a preparation of *Bacillus licheniformis* (ATCC 53757) and its protease (EC 3.4.21.19)) as a feed additive for chickens for fattening, chickens reared for laying and minor avian species for fattening and to point of lay and ornamental birds. EFSA Journal 2015;13(3):4055, 16 pp. https://doi.org/10.2903/j.efsa.2015.4055

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), Bampidis V, Bastos M, Christensen H, Dusemund B, Kouba M, Kos Durjava M, López-Alonso M, López Puente S, Marcon F, Mayo B, Pechová A, Petkova M, Ramos F, Sanz Y, Villa RE, Woutersen R, Brock T, de Knecht J, Kolar B, van Beelen P, Padovani L, Tarres-Call J, Vettori MV and Azimonti G, 2019. Guidance on the assessment of the safety of feed additives for the environment. EFSA Journal 2019;17(4):5648, 78 pp. https://doi.org/10.2903/j.efsa.2019.5648

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), Rychen G, Aquilina G, Azimonti G, Bampidis V, Bastos ML, Bories G, Chesson A, Cocconcelli PS, Flachowsky G, Gropp J, Kolar B, Kouba M, López-Alonso M, López Puente S, Mantovani A, Mayo B, Ramos F, Saarela M, Villa RE, Wallace RJ, Wester P, Anguita M, Dujardin B, Galobart J and Innocenti ML, 2017a. Guidance on the assessment of the safety of feed additives for the consumer. EFSA Journal 2017;15(10):5022, 17 pp. https://doi.org/10.2903/j.efsa.2017.5022

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), Rychen G, Aquilina G, Azimonti G, Bampidis V, Bastos ML, Bories G, Chesson A, Cocconcelli PS, Flachowsky G, Gropp J, Kolar B, Kouba M, López-Alonso M, López Puente S, Mantovani A, Mayo B, Ramos F, Saarela M, Villa RE, Wallace RJ, Wester P, Anguita M, Galobart J and Innocenti ML, 2017b. Guidance on the identity, characterisation and conditions of use of feed additives. EFSA Journal 2017;15(10):5023, 12 pp. https://doi.org/10.2903/j.efsa.2017.5023

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), Rychen G, Aquilina G, Azimonti G, Bampidis V, Bastos ML, Bories G, Chesson A, Cocconcelli PS, Flachowsky G, Gropp J, Kolar B, Kouba M, López-Alonso M, López Puente S, Mantovani A, Mayo B, Ramos F, Saarela M, Villa RE, Wallace RJ, Wester P, Anguita M, Galobart J, Innocenti ML and Martino L, 2017c. Guidance on the assessment of the safety of feed additives for the target species. EFSA Journal 2017;15(10):5021, 19 pp. https://doi.org/10.2903/j.efsa.2017.5021

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), Rychen G, Aquilina G, Azimonti G, Bampidis V, Bastos ML, Bories G, Chesson A, Cocconcelli PS, Flachowsky G, Gropp J, Kolar B, Kouba M, López-Alonso M, López Puente S, Mantovani A, Mayo B, Ramos F, Saarela M, Villa RE, Wallace RJ, Wester P, Anguita M, Galobart J, Innocenti ML and Martino L, 2018a. Guidance on the assessment of the efficacy of feed additives. EFSA Journal 2018;16(5):5274, 25 pp. https://doi.org/10.2903/j.efsa.2018.5274

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), Rychen G, Aquilina G, Azimonti G, Bampidis V, Bastos ML, Bories G, Chesson A, Cocconcelli PS, Flachowsky G, Gropp J, Kolar B, Kouba M, López-Alonso M, López Puente S, Mantovani A, Mayo B, Ramos F, Saarela M, Villa RE, Wallace RJ, Wester P, Glandorf B, Herman L, Kärenlampi S, Aguilera J, Anguita M, Brozzi R and Galobart J, 2018b. Guidance on the characterisation of microorganisms used as feed additives or as production organisms. EFSA Journal 2018;16(3):5206, 24 pp. https://doi.org/10.2903/j.efsa.2018.5206

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances usedin Animal Feed), Bampidis V, Azimonti G, Bastos ML, Christensen H, Dusemund B, Fašmon Durjava M, Kouba M, López-Alonso M, López Puente S, Marcon F, Mayo B, Pechová A, Petkova M, Ramos F, Sanz Y, Villa RE, Woutersen R, Glandorf B, Svensson K, Zeljezic D, Anguita M, Brozzi R, Galobart J, Ortuño J, Revez J, Tarrés-Call J and Pettenati E, 2022. Safety and efficacy of a feed additive consisting of endo-1,4-b-xylanase produced by *Komagataella phaffii* DSM 33574 (Xylamax) for chickens and turkeys for fattening, chickens reared for laying/breeding, turkeys reared for breeding and minor poultry species for fattening or raised to the point of lay (BioResource international, Inc.). EFSA Journal 2022;20(7):7428, 15 pp. https://doi.org/10.2903/j.efsa.2022.7428

Flores CA, Williams MP, Smith K, Pieniazek J, Latham R, Wang JJ, Tyus J and Lee JT, 2017. Evaluation of a thermotolerant xylanase on broiler growth performance and dietary ileal digestible energy value. Journal of Applied Poultry Research, 26(1), 60–71.

Nusairat B, McNaughton J, Tyus J and Wang JJ, 2018. Combination of xylanase and Bacillus direct-fed microbials, as an alternative to antibiotic growth promoters, improves live performance and gut health in sub-clinical challenged broilers. International Journal of Poultry Science, 17, 362–366.

Nusairat B and Wang JJ, 2020. Xylanase and direct-fed microbials (DFM) potential for improvement of live performance, energy digestibility, and reduction of environmental microbial load of broilers. Frontiers in Veterinary Science, 7, 606415. https://doi.org/10.3389/fvets.2020.606415

## Abbreviations

| | |
|---|---|
| AMEn | apparent metabolisable energy adjusted for nitrogen |
| AMR | antimicrobial resistance genes |
| ANOVA | analysis of variance |
| ATCC | American Type Culture Collection |
| bw | body weight |
| CFU | colony forming unit |
| CV | coefficient of variation |
| DSMZ | Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH |
| EURL | European Union Reference Laboratory |
| FEEDAP | EFSA Panel on Additives and Products or Substances used in Animal Feed |
| GLP | Good Laboratory Practice |
| LOQ | limit of quantification |
| MIC | minimum inhibitory concentration |
| NOAEL | no observed adverse effect level |
| OECD | Organisation for Economic Co-operation and Development |
| PCB | polychlorinated biphenyls |
| PCDD | polychlorinated dibenzodioxin |
| PCDF | polychlorinated dibenzofurans |
| PCR | polymerase chain reaction |
| PFGE | pulsed-field gel electrophoresis |
| QPS | qualified presumption of safety |
| TEQ | toxic equivalents |
| WGS | Whole genome sequencing |
| WHO | World Health Organization |

# Annex A – Executive Summary of the Evaluation Report of the European Union Reference Laboratory for Feed Additives on the Method(s) of the Analysis for the preparation of endo-1,4-beta-xylanase (EC 3.2.1.8) produced by *Komagaetella phaffi* xyl-2 (DSM 33574) and viable spores of *Bacillus amyloliquefaciens*[52] Ba-BPD1 (DSM 21836) and of *Bacillus licheniformis* PWD-1 (ATCC 53757) (EnzaPro)

In the current application, an authorisation of a preparation of endo-1,4-beta-xylanase (EC 3.1.3.28) produced by Komagaetella phaffi xyl-2 (DSM 33574) and viable spores of Bacillus amyloliquefaciens Ba-BPD1 (DSM 21836) and of Bacillus licheniformis PWD-1 (ATCC 53757) (EnzaPro) is sought under Article 4 (1) for poultry for fattening, chickens reared for laying, turkeys for fattening, turkeys reared for breeding and minor avian species (including also sporting, ornamental and exotic birds) under the category/functional group 4 (a,b) "zootechnical additives"/"digestibility enhancers", "gut flora stabilisers" according to Annex I of Regulation (EC) No 1831/2003.

According to the Applicant, the active substances of the feed additive are endo-1,4-beta-xylanase produced by genetically modified strain Komagaetella phaffi xyl-2 (DSM 33574) and viable spores of Bacillus amyloliquefaciens Ba-BPD1 (DSM 21836) and of Bacillus licheniformis PWD-1 (ATCC 53757).

The activity of the endo-1,4-beta-xylanase is expressed in xylanase units (XU), where one unit (XU) is defined as the amount of the enzyme needed for the release of 1 nanomole of reducing sugars (xylose equivalents) per second from 0.5% xylan from beachwood or from wheat arabinoxylan at 50°C in 50 mM trisodium citrate buffer pH 6.0.

The feed additive is intended to be marketed as a solid preparation, having a guaranteed minimum content of the following active substances per gram of the preparation: 100000 XU of endo-1,4-beta-xylanase, $1 \times 10^9$ Colony Forming Units (CFU) of total Bacilli spp. Bacillus amyloliquefaciens Ba-BPD1 (DSM 21836) and Bacillus licheniformis PWD-1 (ATCC 53757).

The feed additive is intended to be incorporated directly into complete feedingstuffs to obtain a minimum xylanase activity of 10,000 XU/kg feedingstuffs and a minimum of $1x10^8$ CFU of the Bacilli spp. Bacillus amyloliquefaciens Ba-BPD1 (DSM 21836) and Bacillus licheniformis PWD-1 (ATCC 53757)/ kg feedingstuffs.

For the quantification of endo-1,4-beta-xylanase activity in the feed additive the Applicant submitted a single-laboratory validated and further verified method based on the enzymatic hydrolysis of wheat arabinoxylan substrate at pH 6.0 and 50 °C, and the colour formation of the released reducing sugar (xylose) with 3,5-dinitrosalicylic acid (DNS).

For the quantification of endo-1,4-beta-xylanase activity in feedingstuffs the Applicant submitted another single-laboratory validated and further verified colorimetric method, based on the quantification of the 4-nitrophenol group produced by the action of xylanase on a commercially available XylX6 substrate (Megazyme) at pH 6.0 and 50 °C.

Based on the performance characteristics available, the EURL recommends for official control the above mentioned two single-laboratory validated and further verified colorimetric methods for the quantification of endo-1,4-beta-xylanase activity in the feed additive and feedingstuffs.

For the identification of Bacillus amyloliquefaciens Ba-BPD1 (DSM 21836) and of Bacillus licheniformis PWD-1 (ATCC 53757) the Applicant proposed the pulsed-field gel electrophoresis (PFGE), a generally recognised methodology for the genetic identification of bacterial strains. The EURL recommends for official control the pulsed-field gel electrophoresis (PFGE) for the genetic identification of the bacterial strains.

For the enumeration of total Bacilli spp. Bacillus amyloliquefaciens Ba-BPD1 (DSM 21836) and Bacillus licheniformis PWD-1 (ATCC 53757) in the feed additive the Applicant proposed the ring-trial validated VDLUFA method 28.2.2. This method is applicable to mineral feeds composed mainly of minerals and containing at least 40% crude ash.

Based on the performance characteristics and experimental data available, the EURL recommends for official control the ring-trial validated VDLUFA method 28.2.2 for the enumeration of total Bacilli spp. Bacillus amyloliquefaciens Ba-BPD1 (DSM 21836) and Bacillus licheniformis PWD-1 (ATCC 53757) in the feed additive.

For the enumeration of total Bacilli spp. Bacillus amyloliquefaciens Ba-BPD1 (DSM 21836) and Bacillus licheniformis PWD-1 (ATCC 53757) in feedingstuffs the Applicant proposed the ring-trial

[52] During the assessment the strain was identified as *Bacillus velezensis*.

validated spread plate CEN method EN 15784, which has been already evaluated and recommended by the EURL at least in several recent Bacillus spp. dossiers.

Based on the performance characteristics and experimental data available, the EURL recommends for official control the ring-trial validated 15,784 method for the enumeration of total Bacilli spp. Bacillus amyloliquefaciens Ba-BPD1 (DSM 21836) and Bacillus licheniformis PWD-1 (ATCC 53757) in feedingstuffs.

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.