JRC.F.5/CvH/ZE/AS/Ares

Subject: Addendum to the EURL evaluation report

Reference: FAD-2016-0067 – Duddingtonia flagrans IAH 1297
(JRC F.5/CvH/ZE/AS/Ares(2020)379930)

Upon the request from EFSA [1], the EURL evaluated the supplementary information provided [2] in the frame of the dossier FAD-2016-0067 for the enumeration of the active substance (viable spores of *Duddingtonia flagrans IAH 1297*) in *compound feed*.

In the frame of the former evaluation [3], the EURL was not able to recommend for official control the proposed method based on yeast mannitol agar (YMA) (containing the antibiotics streptomycin and chloramphenicol) by using a most probable number (MPN) procedure (YMA-MPN) for the enumeration of the active substance in *compound feed*. The reason, which did not allow recommending this method, was that the dose of the active substance presented by the Applicant in the conditions of use has not been expressed as number of the viable spores per mass fraction of *compound feed*.

However, in the former report [3], the EURL stated that the proposed YMA-MPN method is fit-for-purpose for the enumeration of the active substance at the levels of *Duddingtonia flagrans IAH 1297* in *compound feed* within the range tested in the frame of the validation and verification studies, i.e. 5.5×10^3 to 1.2×10^4 viable spores /g *compound feed*.

Meanwhile, in the frame of the supplementary information [2], the Applicant stated that the conditions of use would correspond to the levels of viable spores of *Duddingtonia flagrans IAH 1297* ranging from 1×10^2 to 3×10^6 viable spores /g *compound feed*.

In order to support the wider applicability range of the method, the Applicant confirmed [2] the linearity of the method at the levels ranging from 4.9×10^1 to 2.4×10^6 viable spores / g *compound feed*, which were already reported in the frame of previous verification studies [4]. Furthermore, the data submitted in the previous verification report [4], allowed establishing the limit of quantification (LOQ) and precision (*repeatability* and *intermediate precision*) of the method to enumerate viable spores of *Duddingtonia flagrans IAH 1297* in *compound feed* at LOQ levels.

After logarithmic transformation of the values corresponding to the LOQ levels of the viable spores in *compound feed*, the EURL derived *repeatability* and *intermediate precision* of the method of 0.32 $\log_{10}$ viable spores/g *compound feed* for the average content of 1.78 $\log_{10}$ viable spores/g *compound feed* [5].

Given all the available data, the EURL recommends for official control the above-mentioned single-laboratory validated and further verified YMA-MPN method for the enumeration of the active substance (viable spores of *Duddingtonia flagrans IAH 1297*) in *compound feed*.

Recommended text for the registry entry (analytical method) (replacing the previous recommendations)

For the identification of *Duddingtonia flagrans IAH 1297*:

- DNA based methods

For the enumeration of viable spores of *Duddingtonia flagrans IAH 1297* in the *feed additive*, *premixtures* and *compound feed*:

- The method using yeast mannitol agar (YMA) with streptomycin and chloramphenicol and a most probable number (MPN) for the enumeration

References

[1] Supplementary Information – FAD-2016-0067 Duddingtonia flagrans IAH 1297_EFSA request (Ares(2023)2194120)

[2] Supplementary Information – FAD-2016-0067 Dudingtonia flagrans_additional information

[3] EURL report - FAD-2016-0067 – Duddingtonia flagrans IAH 1297 (JRC F.5/CvH/ZE/AS/Ares (2020)379930)

[4] Supplementary information – Verification report 17_405-316

[5] Supplementary information – Method precision@LOQ levels

Addendum

- Prepared by Zigmas Ezerskis

- Reviewed and approved by María José González de la Huebra and Christoph von Holst (EURL-FA), respectively, Geel, 29/03/2023

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Directorate F - Health, Consumers & Reference Materials (Geel/Ispra)
European Union Reference Laboratory for Feed Additives

JRC F.5/CvH/ZE/AS/Ares

Evaluation Report on the Analytical Methods submitted in connection with the Application for Authorisation of a Feed Additive according to Regulation (EC) No 1831/2003

Duddingtonia flagrans IAH 1297

(FAD-2016-0067; CRL/160037)

European Union Reference Laboratory
Feed Additives

Evaluation Report on the Analytical Methods submitted in connection with the Application for Authorisation of a Feed Additive according to Regulation (EC) No 1831/2003

Dossier related to:	**FAD-2016-0067 - CRL/160037**
Name of Product:	***Duddingtonia flagrans IAH 1297***
Active Agent (s):	***Duddingtonia flagrans IAH 1297***
Rapporteur Laboratory:	**Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino, Italy**
Report prepared by:	**Stefania Squadrone**
Report checked by: Date:	**Zigmas Ezerskis** **20/01/2020**
Report approved by: Date:	**Christoph von Holst** **20/01/2020**

EXECUTIVE SUMMARY

In the current application authorisation is sought under Article 4(1) for *Duddingtonia flagrans IAH 1297* under the category / functional group 4(d) 'zootechnical additives' /'other zootechnical additives', according to Annex I of Regulation (EC) No 1831/2003. Specifically, the authorisation is sought for the use of *Duddingtonia flagrans IAH 1297* as *feed additive* for all grazing animals.

According to the Applicant, the *feed additive* contains the viable spores of non-genetically modified *Duddingtonia flagrans IAH 1297* as active agent. The *feed additive* is intended to be marketed as a preparation under the trade name of *Bioworma*®, containing a minimum of 5×10^5 spores of *Duddingtonia flagrans IAH 1297* /g *feed additive*.

The *feed additive* is to be used in *premixtures* and *feedingstuffs*. The Applicant proposed the minimum dose of the *feed additive* expressed in terms of 3×10^4 spores of *Duddingtonia flagrans IAH 1297* per kg of body weight of animal per day.

For the identification/characterisation of the *feed additive* the EURL recommends for official control a polymerase chain reaction (PCR) method as specified by the Applicant for the genetic identification of *Duddingtonia flagrans IAH 1297*.

For the enumeration of the active agent (viable spores) of *Duddingtonia flagrans IAH 1297* in the *feed additive*, *premixtures* and *feedingstuffs* the Applicant submitted a single-laboratory validated and further verified method based on yeast mannitol agar (YMA) containing the antibiotics streptomycin and chloramphenicol by using a most probable number (MPN) procedure described in European Pharmacopeia monographs (01/2008:201612 and 20613).

Based on the overall experimental evidence available the EURL recommends for official control the above mentioned single-laboratory validated and further verified YMA-MPN method for the enumeration of the active agent (viable spores) of *Duddingtonia flagrans IAH 1297* in the *feed additive* and *premixtures*.

As the dose of the active agent (viable spores of *Duddingtonia flagrans IAH 1297*) presented by the Applicant was not expressed as number of the spores per mass unit of *feedingstuffs*, the EURL cannot recommend for official control the YMA-MPN method for the enumeration of the active agent (viable spores) of *Duddingtonia flagrans IAH 1297* in *feedingstuffs*. However, the method is fit-for-purpose for the enumeration of the active agent (viable spores) of *Duddingtonia flagrans IAH 1297* in *feedingstuffs* at the validated and verified range of content of *Duddingtonia flagrans IAH 1297*.

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.

KEYWORDS

Duddingtonia flagrans IAH 1297, Bioworma®, zootechnical additives, other zootechnical additives, all grazing animals

1. BACKGROUND

In the current application authorisation is sought under Article 4(1) (authorisation of a feed additive) for *Duddingtonia flagrans IAH 1297* under the category / functional group 4(d) 'zootechnical additives' /'other zootechnical additives', according to Annex I of Regulation (EC) No 1831/2003 [1]. Specifically, the authorisation is sought for the use of *Duddingtonia flagrans IAH 1297* as *feed additive* for all grazing animals [1,2].

According to the Applicant, the *feed additive* contains the viable spores of non-genetically modified *Duddingtonia flagrans IAH 1297* as active agent [3,4]. The strain is deposited at the National Collection of Industrial Food and Marine Bacteria (NCIMB) Ltd., Edinburgh, United Kingdom [4].

The *feed additive* is intended to be marketed as a preparation under the trade name of *Bioworma*® containing a minimum of 5×10^5 of *Duddingtonia flagrans IAH 1297* /g *feed additive* [3,4].

The *feed additive* is to be used in *premixtures* and *feedingstuffs* [3]. The Applicant proposed the minimum dose of the *feed additive* expressed in terms of 3×10^4 spores of *Duddingtonia flagrans IAH 1297* per kg of body weight of animal per day [2,4].

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005, as last amended by Regulation (EU) 2015/1761, on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the duties and the tasks of the European Union Reference Laboratory concerning applications for authorisations of feed additives, the EURL is requested to submit a full evaluation report to the European Food Safety Authority for each application or group of applications. For this particular dossier, the methods of analysis submitted in connection with *Duddingtonia flagrans IAH 1297* and their suitability to be used for official controls in the frame of the authorisation were evaluated.

3. EVALUATION

Description of the analytical methods for the determination of the active substance in the feed additive, premixtures, feedingstuffs and when appropriate water (section 2.6.1 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

For the enumeration of the active agent (viable spores) of *Duddingtonia flagrans IAH 1297* in the *feed additive*, *premixtures* and *feedingstuffs* the Applicant submitted a single-laboratory validated method [5,6] based on yeast mannitol agar (YMA) containing the antibiotics streptomycin and chloramphenicol by using a most probable number (MPN) procedure described in European Pharmacopeia monographs [7,8].

The sample (10 to 50 g of product) is diluted in peptone (PEP) and left for 15 min for rehydration. The rehydrated sample is properly mixed and vortexed. An aliquot of the sample is appropriately diluted with PEP and transferred on the plates containing YMA with the above mentioned antibiotics. A minimum of three ten-fold dilutions are prepared and for each dilution at least five plates are used for the cultivation of the strain. The samples are incubated at 25 °C for 7 days before the enumeration of the spores using the above mentioned MPN procedure [5-8].

In the first validation study [5] for the enumeration of the active agent in ten samples of the *feed additive* (Bioworma®) the Applicant applied the above mentioned YMA-MPN method. For comparative purposes two other classical methods based on the counting of colonies, namely the spread plate method with yeast mannitol agar (YMA) containing the antibiotics streptomycin and chloramphenicol and a pour plate method using sabouraud dextrose agar (SDA) with the above mentioned antibiotics, were applied on the samples [5].

The average content of the viable spores obtained by using the three methods mentioned above was ranging from 5.97 to 6.04 $\log_{10}$ spores/g *feed additive* and the standard deviation for *repeatability* (S_r) was ranging from 0.10 to 0.23 $\log_{10}$ spores/g *feed additive* [5].

The results from this study have demonstrated that the YMA-MPN method is equivalent to the other two above mentioned methods based on the counting of colonies for the enumeration of viable spores of *Duddingtonia flagrans IAH 1297*) in the *feed additive* (Bioworma®).

Furthermore, the Applicant performed the validation and verification studies of the above mentioned YMA-MPN method by using the concentrate of spores of *Duddingtonia flagrans IAH 1297*, the *feed additive* (Bioworma®), *premixtures* (Livamol with Bioworma®) and *feedingstuffs* [6,9-13]. The results of the latter studies are presented in Table 1. In addition, the limit of quantification (LOQ) of 50 and 76 spores /g *feedingstuffs* was reported by the Applicant and the 2nd laboratory, respectively [9].

Table 1 The results of the single-laboratory validated and further verified YMA-MPN method for the enumeration of the active agent (viable spores) of *Duddingtonia flagrans IAH 1297* in the concentrate of spores of *Duddingtonia flagrans IAH 1297*, the *feed additive* (Bioworma®), *premixtures* (Livamol with Bioworma®) and *feedingstuffs*

	Spore concentrate		***Feed additive***		***Premixtures***		***Feedingstuffs***	
	Val.	**Ver.**	**Val.**	**Ver.**	**Val.**	**Ver.**	**Val.**	**Ver.**
Average content, log_{10} MPN spores/g	6.37 – 6.55	5.97 – 6.42	5.63 – 5.77	5.72 – 6.38	4.47 – 4.52	4.5 – 5.10	3.98 – 4.07	3.74 – 3.95
S_r, log_{10} MPN spores/g	0.14 – 0.33	0.28 – 0.36	0.10 – 0.31	0.31 – 0.43	0.09	0.07 – 0.18	0.04 – 0.33	0.13 – 0.33
S_{ip}, log_{10} MPN spores/g	-	0.36	-	0.43	-	0.73	-	0.57
Average R_{Rec}, %	143*	101**	100*	104**	128*	105**	128*	102**
Reference	[6,9]	[10]	[6,9]	[11]	[6,9]	[12]	[6,9]	[13]

S_r and S_{ip}: standard deviations for repeatability and intermediate precision, respectively; R_{Rec} – a recovery rate; * based on spiked samples in the corresponding matrix at the level of 4.11 to 4.36 log_{10} MPN spores/g; ** based on expected value indicated by the Applicant; Val. – validation; Ver. – verification.

Based on the overall experimental evidence available the EURL recommends for official control the above mentioned single-laboratory validated and further verified YMA-MPN method for the enumeration of the active agent (viable spores) of *Duddingtonia flagrans IAH 1297* in the *feed additive* and *premixtures*.

As the dose of the active agent (viable spores of *Duddingtonia flagrans IAH 1297*) presented by the Applicant was not expressed as number of the spores per mass unit of *feedingstuffs*, the EURL cannot recommend for official control the YMA-MPN method for the enumeration of the active agent (viable spores) of *Duddingtonia flagrans IAH 1297* in *feedingstuffs*. However, the method is fit-for-purpose for the enumeration of the active agent (viable spores) of *Duddingtonia flagrans IAH 1297* in *feedingstuffs* at the validated and verified range of content of *Duddingtonia flagrans IAH 1297*.

Methods of analysis for the determination of the residues of the additive in food (section 2.6.2 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

An evaluation of corresponding methods of analysis is not relevant for the present application.

Identification/Characterisation of the feed additive (section 2.6.3 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

For the identification/characterisation of the *feed additive*, the Applicant used a polymerase chain reaction (PCR) method and DNA sequencing of the specific internal transcribed spacer (ITS) region of the ribosomal RNA gene of *Duddingtonia flagrans IAH 1297* [4].

The EURL recommends for official control the PCR method as specified by the Applicant for the genetic identification of *Duddingtonia flagrans IAH 1297*.

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.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation the EURL recommends for official control:

- the polymerase chain reaction (PCR) method as specified by the Applicant for the genetic identification of *Duddingtonia flagrans IAH 1297*; and
- the single-laboratory validated and further verified method based on yeast mannitol agar (YMA) with the antibiotics streptomycin and chloramphenicol by using a most probable number (MPN) for the enumeration of the active agent (viable spores) of *Duddingtonia flagrans IAH 1297* in the *feed additive* and *premixtures*.

As the dose of the active agent (viable spores of *Duddingtonia flagrans IAH 1297*) presented by the Applicant was not expressed as number of the spores per mass unit of *feedingstuffs*, the EURL cannot recommend for official control the YMA-MPN method for the enumeration of the active agent (viable spores) of *Duddingtonia flagrans IAH 1297* in *feedingstuffs*. However, the method is fit-for-purpose for the enumeration of the active agent (viable spores) of *Duddingtonia flagrans IAH 1297* in *feedingstuffs* at the validated and verified range of content of *Duddingtonia flagrans IAH 1297*.

Recommended text for the register entry (analytical method)

For the identification of *Duddingtonia flagrans IAH 1297*:

- polymerase chain reaction (PCR) method (as specified by the Applicant)

For the enumeration of viable spores of *Duddingtonia flagrans IAH 1297* in the *feed additive* and *premixtures*:

- the method using yeast mannitol agar (YMA) with streptomycin and chloramphenicol and a most probable number (MPN) for the enumeration

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Duddingtonia flagrans IAH 1297* have been sent to the European Union Reference Laboratory for Feed Additives. The dossier has been made available to the EURL by EFSA.

6. REFERENCES

[1] *Application, Reference SANTE_E5_FWD. APPL. 1831-0056-2016

[2] *Application, Annex I – 1473148876504-1998

[3] *Application, Proposal for Register Entry – Annex A

[4] *Technical dossier, Section II: Identity, characterisation and conditions of use of the feed additive; methods of analysis

[5] *Technical dossier, Section II – Annex_2.6.1_02_dossier

[6] *Technical dossier, Section II – Annex_2.6.1_01_dossier

[7] European Pharmacopeia monograph 01/2008:20612

[8] European Pharmacopeia monograph 01/2008:20613

[9] *Supplementary information – IAH Bioworma Applicant summary - Method verification studies

[10] *Supplementary information – Verification report 17_402-316

[11] *Supplementary information – Verification report 17_403-316

[12] *Supplementary information – Verification report 17_404-316

[13] *Supplementary information – Verification report 17_405-316

*Refers to Dossier no: FAD-2016-0067

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation is Centro di Referenza Nazionale per la sorveglianza ed il controllo degli Alimenti per gli Animali (CReAA), Torino, Italy. This report is in accordance with the opinion of the consortium of National Reference Laboratories as referred to in Article 6(2) of Commission Regulation (EC) No 378/2005, as last amended by Regulation (EU) 2015/1761.

8. ACKNOWLEDGEMENTS

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- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Centre wallon de Recherches agronomiques (CRA-W), Gembloux (BE)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia, PESCA, Alimentació i Medi Natural. Generalitat de Catalunya, Cabrils (ES)