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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

Preparation of bacteriophages (3sent1, 8sent65, 8sent1748 & 5sent1) (BAFASAL®)

(FAD-2017-0039; CRL/170007)

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-2017-0039 - CRL/170007**
Name of Product:	***Preparation of bacteriophages (3sent1, 8sent65, 8sent1748 & 5sent1)***
Active Agent (s):	**3sent1, 8sent65, 8sent1748 & 5sent1**
Rapporteur Laboratory:	**RIKILT, Wageningen University and Research, Wageningen, Netherlands**
Report prepared by:	**Mariël Pikkemaat**
Report checked by: Date:	**María José González de la Huebra** **01/10/2018**
Report approved by: Date:	**Christoph von Holst** **05/10/2018**

EXECUTIVE SUMMARY

In the current application authorisation is sought under Article 4(1) for a preparation of bacteriophages (BAFASAL®) under the category / functional group 4(d) 'zootechnical additives' / 'other zootechnical additives', according to Annex I of Regulation (EC) No 1831/2003. Authorisation is sought for the use of the *feed additive* for all avian species.

According to the Applicant, the *feed additive* contains a preparation of four bacteriophages: 3sent1, 8sent65, 8sent1748 and 5sent1, obtained from *Salmonella enterica* ser. Gallinarum 1 (accession number B/0011, Polish Collection of Microorganisms (PCM)). The *feed additive* is to be marketed in liquid form, containing equivalent amounts of the four bacteriophages, with a minimum concentration of each phage of 1.25×10^7 Plaque Forming Units (PFU)/ml, leading to a total concentration $\geq 5 \times 10^7$ PFU/ml. The *feed additive* is intended to be used directly in *water* and *liquid complementary feeds* at a minimum dose of 2×10^6 PFU/bird/day.

For the identification of the four bacteriophages 3sent1, 8sent65, 8sent1748 and 5sent1, the EURL recommends for official control the Phage-specific PCR method (BF-PCR) proposed by the Applicant.

For the enumeration of the four bacteriophages 3sent1, 8sent65, 8sent1748 and 5sent1 in the *feed additive*, *water* and *liquid complementary feeds*, the Applicant submitted a single-laboratory validated and further verified method based on a double agar overlay plaque assay. Based on the performance characteristics available, the EURL recommends this method for official control.

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

Bacteriophages 3sent1, 8sent65, 8sent1748 and 5sent1, zootechnical additives, other zootechnical additives, all avian species

1. BACKGROUND

In the current application authorisation is sought under Article 4(1) for a preparation of bacteriophages (3sent1, 8sent65, 8sent1748, 5sent1) under the category / functional group 4(d) 'zootechnical additives' / 'other zootechnical additives', according to Annex I of Regulation (EC) No 1831/2003 [1].

Authorisation is sought for the use of the *feed additive* for all avian species [1, 2]. According to the Applicant, the *feed additive* contains a preparation of four bacteriophages: 3sent1, 8sent65, 8sent1748 and 5sent1 [3], obtained from *Salmonella enterica* ser. Gallinarum 1 which is deposited at the Polish Collection of Microorganisms (PCM) under the deposit number B/0011 [4].

The *feed additive* is to be marketed in liquid form, containing equivalent amounts of the four bacteriophages, with a minimum concentration of each phage of $1.25x10^7$ Plaque Forming Units (PFU)/ml, leading to a total concentration $\geq 5x10^7$ PFU/ml [5]. The *feed additive* is intended to be used directly in *water* and *liquid complementary feeds* at a minimum dose of $2x10^6$ PFU/bird/day [6].

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 the *Preparation of bacteriophages (3sent1, 8sent65, 8sent1748 & 5sent)* 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 bacteriophages in the *feed additive*, *liquid complementary feed* and *water* the Applicant submitted a single-laboratory validated and further verified method (in the *feed additive*) based on a double agar overlay plaque assay [7]. According to the Applicant, the method, based on a suspension-dilution technique, can also be applied to the other matrices, i.e. *water* and *liquid complementary feed*.

The assay is based on the infection of a susceptible *Salmonella enterica* ser. Enteritidis (65/S/10) strain, available from the Applicant upon request, by the bacteriophages using solid and semi-solid culture media [8]. After an incubation period, the plaques i.e. areas of lysed cells within the plate, are observed and counted as PFU. The titer of bacteriophages is calculated taking into account serial dilutions of the analysed suspension. Each analysis

should be performed on three independent dilution series and comprises a range of at least three effective dilutions so that, on the middle dilution, the plaque number is in the range between 5 and 150. The titer represents the number of active bacteriophages per ml, considering each plaque as derived from a single infectious virus particle.

In the frame of the validation and verification studies the Applicant reported recoveries ranging from 99 to 102 % and a *limit of quantification* (LOQ) of $1x10^3$ PFU/ml.

Additionally, the EURL recalculated, based on the validation and further verification data reported by the Applicant for the *feed additive* after logarithmic transformation of the PFU values [7], the following precision performance characteristics for the enumeration of bacteriophages in the *feed additive*:

- a maximum standard deviation for *repeatability* (S_r) of 0.102 $\log_{10}$ PFU/ml;
- a maximum standard deviation for *intermediate precision* (S_{ip}) of 0.107 $\log_{10}$ PFU/ml;

The Applicant applied the proposed method in the frame of the stability and homogeneity studies [9] to BAFASAL® in *water* and in different *liquid complementary feeds* leading to similar performance characteristics, thus confirming the applicability of the proposed method to *water* and *liquid complementary feeds*.

Based on the performance characteristics presented, the EURL recommends for official control the single-laboratory validated and further verified method based on a double agar overlay plaque assay for the enumeration of bacteriophages in the *feed additive*, *liquid complementary feed* and *water*.

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 of the bacteriophages in BAFASAL® (3sent1, 8sent65, 8sent1748 and 5sent1), the Applicant provided a phage-specific PCR method [10]. This BF-PCR identification method is based on the selective amplification of DNA fragments unique for each of the four target phages (3sent1, 8sent65, 8sent1748 and 5sent1).

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 double agar overlay plaque assay for the enumeration of bacteriophages in the *feed additive*, *liquid complementary feed* and *water*. For the identification of the bacteriophages the EURL recommends a phage-specific PCR method for official control.

Recommended text for the register entry (analytical method)

- Identification: phage-specific PCR method
- Enumeration in the *feed additive*, *liquid complementary feed* and *water*: double agar overlay plaque assay

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of the *Preparation of bacteriophages (3sent1, 8sent65, 8sent1748 & 5sent1)* 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: FORW. APPL. 1831/0045-2017 & Annex 1, Application form

[2] *Application, Proposal for Register Entry, Annex A

[3] *Technical dossier, Section II: Annex II.2.1.2.4: Analysis of bacteriophages based on sequence data

[4] *Technical dossier, Section II: Annex II.2.1.2.1: Certificate of deposition of production host strain *Salmonella* Gallinarum 1

[5] *Technical dossier, Section II: 2.1.3 Qualitative and quantitative composition

[6] *Technical dossier, Section II: 2.5 Conditions of use of the additive

[7] *Technical dossier, Section II: Annex II.6.1.1: Method validation report

[8] *Supplementary information, Annex II.6.1.4: Enumeration of bacteriophages by agar overlay plate method

[9] *Technical dossier, Section II: Annexes II.4.3.1- II.4.2.3

[10] *Supplementary information, Annex II.6.1.5: Phage-specific PCR (BF-PCR) – Method description

*Refers to Dossier no: FAD-2017-0039

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation is the RIKILT Wageningen UR, Wageningen, the Netherlands. 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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