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

Dossier related to: FAD-2010-0005
CRL/090048

Name of Additive: Miya-Gold

Active Agent (s): *Clostridium butyricum* MIYARI 588

Rapporteur Laboratory: Community Reference Laboratory for
Feed Additives (CRL-FA)
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EXECUTIVE SUMMARY

In the current application authorisation is sought for the microbial feed additive *Clostridium butyricum* MIYARI 588 under the category of 'zootechnical additives' functional group 4(b), 'gut flora stabilisers' according to Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought for the use of *Miya-Gold* for weaned piglets, minor weaned porcine species, chickens for fattening, chickens for rearing and minor avian species. *Miya-Gold* is a fine dry grayish-white powder, available in two forms as *Miya-Gold* EU (with a concentration of 1×10^{10} CFU/g *Clostridium butyricum* MIYARI 588) and *Miya-Gold* S (concentration of 5×10^8 CFU/g *Clostridium butyricum* MIYARI 588). The product is intended to be mixed at a final concentration of 2.5×10^8 CFU/kg of complete *feedingstuffs* for weaned piglets and minor weaned porcine species and 5×10^8 CFU/kg of complete *feedingstuffs* for chickens for fattening and rearing and minor avian species.

For the enumeration of *Clostridium butyricum* MIYARI 588 in *feed additive*, *premixtures* and *feedingstuffs*, the applicant proposes the ISO 15213 standard pour plate method, used for enumeration of sulfite-reducing bacteria growing under anaerobic conditions. This method was ring-trial validated and verified by four laboratories. The performance characteristics obtained for a concentration ranging between 10^4 CFU/g and 10^{11} CFU/g and reported after logarithmic transformation (CFU) are:

- for *feed additive*:

- a repeatability standard deviation (s_t) ranging from 0.05 to 0.07 \log_{10} CFU/g,
- a reproducibility standard deviation (s_R) of 0.09 \log_{10} CFU/g, and
- a recovery ranging from 98-110 %

- for *premixtures* and *feedingstuffs*:

- s_t ranging from 0.04 to 0.13 \log_{10} CFU/g,
- s_R ranging from 0.11 to 0.31 \log_{10} CFU/g
- a recovery ranging from 97-113 %, and
- a limit of quantification (LOQ) of 5×10^4 CFU/kg, well below the minimum dose proposed by the applicant (2.5×10^8 CFU/kg of *feedingstuffs*).

Based on these acceptable performance characteristics the CRL recommends, for official control, the ring-trial validated pour plate method, based on the ISO 15213 standard for enumeration of *Clostridium butyricum* MIYARI 588 in *feed additives, premixtures* and *feedingstuffs*.

Molecular methods were used by the applicant for identification of the active agent. The CRL recommends for official control Pulsed Field Gel Electrophoresis (PFGE), a generally recognised standard methodology for microbial identification.

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) is not considered necessary.

KEYWORDS

Clostridium butyricum MIYARI 588, zootechnical additives, gut flora stabilisers, weaned piglets, minor weaned porcine species, chickens for fattening, chickens for rearing, minor avian species

1. BACKGROUND

Clostridium butyricum MIYARI 588 is a feed additive for which authorisation under Article 4(1) (new use) and 13(3) (modification of the existing authorisation) is sought under the category of 'zootechnical additives' functional group 4(b), 'gut flora stabilisers' according to Annex I of Regulation (EC) No 1831/2003 [1]. Miya-Gold S is already authorised under the Commission Regulation (EC) 903/2009 as a feed additive (EC number 4b1830) for chickens for fattening. The applicant requests a modification of the strain number, due to a change in the strain deposit number from FERM-P N° 1467 to FERM BP-2789 after submission for an international patent [2]. The strain is deposited at the 'National Institute of Advanced Industrial Science and Technology: International Organism Depository (AIST)' in Japan [2]. Specifically, authorisation is sought for the use of Miya-Gold for weaned piglets, minor weaned porcine species, chickens for fattening, chickens for rearing and minor avian species. Miya-Gold is a fine dry grayish-white powder with a *Clostridium butyricum* MIYARI 588 concentration of 1×10^{10} CFU/g (Miya-Gold EU) and 5×10^8 CFU/g (Miya-Gold S) [3]. The

product is intended to be mixed at a final concentration of 2.5×10^8 CFU/kg of complete *feedingstuffs* for weaned piglets and minor weaned porcine species, or 5×10^8 CFU/kg of complete *feedingstuffs* for chickens for fattening and rearing and minor avian species [3].

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005, as last amended by Regulation (EC) No 885/2009, 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 tasks of the Community Reference Laboratory concerning applications for authorisations of feed additives, the CRL is requested to submit a full evaluation report to the European Food Safety Authority (EFSA) for each application or group of applications. For this dossier, the methods of analysis submitted in connection with the *Miya-Gold* dossier and their suitability to be used for official controls in the frame of the authorisation were evaluated.

3. EVALUATION

Identification/Characterisation of the feed additive

Qualitative and quantitative composition of the additive

For identification and characterisation of the strain *Clostridium butyricum* MIYARI 588 the applicant used routine microbiological methods (microscopy and biochemical characteristics) as well as molecular methods such as Random Amplified Polymorphic DNA (RAPD) analysis. These methods are suitable for the purpose of analysis [4, 5].

However, the CRL recommends for official control, Pulsed Field Gel Electrophoresis (PFGE), a generally recognised standard methodology for genetic identification [6].

Qualitative and quantitative composition of any impurities in the additive

The applicant analysed the *feed additive* for microbial contaminants (such as *Escherichia coli*, *Salmonella*, yeasts and moulds) by using appropriate tests [7]. For undesirable substances (i.e. arsenic, cadmium, mercury, lead, selenium, copper, zinc, chrome, aflatoxins) internationally

recognised standard methods are available at the respective Community Reference Laboratories, in accordance with Commission Regulation (EC) No 776/2006.

Description of the analytical methods for the determination of active agent(s) in feed additive, premixtures and feedingstuffs

For the enumeration of *Clostridium butyricum* MIYARI 588 in *feed additive, premixtures* and *feedingstuffs*, the applicant proposes the ISO 15213 standard pour plate method [8], used for enumeration of sulfite-reducing bacteria growing under anaerobic conditions. The applicant adapted the standard to be used for the enumeration of *Clostridium* spp. The applicant uses iron sulfite agar for the analysis of *feed additive* and *premixtures* and CBM 588 selective blood agar for the *feedingstuffs* [9]. The sample is suspended and diluted in peptone water; the appropriate dilutions are then transferred into petri dishes and molten agar is added. When the agar is solidified, plates are incubated at 37°C for 48 hours before colony counting. This method was ring-trial validated (and verified) by four laboratories. The performance characteristics obtained for a concentration ranging between 10⁴ CFU/g and 10¹¹ CFU/g are reported after logarithmic transformation (CFU) [9]:

- for *feed additive*:

- a repeatability standard deviation (s_r) ranging from 0.05 to 0.07 log₁₀ CFU/g,
- a reproducibility standard deviation (s_R) of 0.09 log₁₀ CFU/g, and
- a recovery ranging from 98-110 %

- and for *premixtures* and *feedingstuffs*:

- s_r ranging from 0.04 to 0.13 log₁₀ CFU/g,
- s_R ranging from 0.11 to 0.31 log₁₀ CFU/g,
- a recovery ranging from 97-113 %, and
- a limit of quantification (LOQ) of 5x10⁴ CFU/kg, well below the minimum dose proposed by the applicant (2.5x10⁸ CFU/kg of *feedingstuffs*).

Based on these acceptable performance characteristics the CRL recommends, for official control, the ring-trial validated pour plate method, based on ISO 15213 for enumeration of *Clostridium butyricum* MIYARI 588 in *feed additives, premixtures and feedingstuffs*.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation the CRL recommends for official control the ring-trial validated pour plate method, based on the ISO 15213 standard method, for the enumeration of the active agent *Clostridium butyricum* MIYARI 588 in *feed additive, premixtures and feedingstuffs*.

For the analysis of the identity of the bacterial strain, *Clostridium butyricum* MIYARI 588 the CRL recommends Pulsed Field Gel Electrophoresis (PFGE) 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) is not considered necessary.

Recommended text for the register entry, fourth column (Composition, chemical formula, description, analytical method)

- Enumeration: Pour plate method based on ISO 15213 standard
- Identification: Pulsed Field Gel Electrophoresis (PFGE)

5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, samples of *Miya-Gold* have been sent to the Community Reference Laboratory for Feed Additives Authorisation. The dossier has been made available to the CRL by EFSA.

6. REFERENCES

- [1] *Application/Ref:SANCO/D/2: Forw.Appl.1831/006-2010
- [2] *Technical dossier, section II: Identity, Characterisation and conditions of use of the additive; methods of analysis
- [3] *Application, Proposal for Register Entry, Annex A

- [4] *Technical dossier: Annex_II_2_1_2_2
- [5] *Technical dossier: Annex_II_2_1_2_3
- [6] European Community Project SMT4-CT98-2235.'Methods for the Official Control of Probiotics Used as Feed Additives, Volume 1. 2002. Report 20873-1. Office for official Publications of the European Communities. ISBN 92-894-6250-7 (Vol. I)
- [7] *Technical dossier: Annex_II_1_4
- [8] ISO 15213 'Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of sulfite-reducing bacteria growing under anaerobic conditions'
- [9] *Technical dossier: Annex II_6_1_0

*Refers to Dossier no: FAD-2010-0005

7. RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was Community Reference Laboratory for Feed Additives, IRMM, Geel, Belgium. 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 (EC) No 885/2009.

8. ACKNOWLEDGEMENTS

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