



D08/FSQ/CVH/RL/D(2008) 25871

CRL Evaluation Report on the Analytical Methods submitted in
connection with Section II, 2.5 (Control Methods) of the Application
for Authorisation as a Feed Additive
according to Regulation (EC) No 1831/2003

Dossier related to: EFSA-Q-2008-303
FAD-2008-0012

Name of Additive: Miya-Gold[®] S

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

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

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Date: 06/10/2008

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Date: 06/10/2008

EXECUTIVE SUMMARY

In the current application authorisation is sought for the microbial product Miya-Gold[®] S as a feed additive under the category 'zootechnical additives', functional group 'gut flora stabilisers' according to Annex I of Regulation (EC) No 1831/2003. Specifically, the use of Miya-Gold[®] S for chickens for fattening is requested. Miya-Gold[®] S contains 5.0×10^8 viable cells (c.f.u., colony-forming units) of *Clostridium butyricum* MIYAIRI 588 (as active agent) per gram. The feed additive is intended to be mixed into complete feedingstuffs at final concentrations of 5×10^8 c.f.u./kg.

For the quantification of the active agent, *Clostridium butyricum* MIYAIRI 588, in the *feed additive* and *premixtures* an enumeration method using iron sulfite agar as described in ISO Standard 15213 (2003) is proposed by the applicant. For *feedingstuffs* the applicant proposes a selective CMB588 agar (Microbiol. Immunol. 1997, 41(9), 665-671). A three-laboratory validation study demonstrated satisfactory performance of the iron sulfite agar and selective CMB588 agar using samples of the additive, premixtures and feedingstuffs. The method performance characteristics were standard deviations for repeatability (s_r) and reproducibility (s_R) of below $0.10 \log_{10}$ and of between $0.09 - 0.31 \log_{10}$ calculated from the base 10 logarithms of the measured c.f.u./g, respectively. Presumptive colonies of *Clostridium butyricum* MIYAIRI 588 shall be confirmed microscopically, for absence of growth under aerobic conditions and for formation of butyric acid on plate count agar.

For official controls of the active agent *Clostridium butyricum* MIYAIRI 588 in the *feed additive* and *premixtures* iron sulfite agar according to ISO 15213 and in *feedingstuffs* the selective CMB588 agar (Microbiol. Immunol. 1997, 41(9), 665-671) are recommended followed by confirmation of presumptive *Clostridium butyricum* MIYAIRI 588 colonies on plate count agar. The CRL-FA recommends using for analysis of premixtures 20 g samples for feedingstuffs 50 g.

The limits of quantification (LOQ) of the spread plate methods are around 10^4 colony forming units (c.f.u) per gram (g) feed additive or premixture and around 10^7 c.f.u./kg feedingstuff which is well below the anticipated target concentrations

The applicant applied a wide range of biochemical and molecular techniques for strain identification of *Clostridium butyricum* MIYAIRI 588 including pulsed field gel electrophoresis (PFGE) [Jpn. Pharmacol. Ther. 2000, 28(12), 999-1004]. PFGE is widely

applied for microbial strain identification and is considered suitable for official controls in the frame of the authorisation.

On the basis of the supplied documentation, no supplementary experimental work (testing or method validation) is required.

KEYWORDS

Miya-Gold[®] S, zootechnical, *Clostridium butyricum* MIYAIRI 588, chickens for fattening

1. BACKGROUND

Miya-Gold[®] S is a product for which authorisation as a feed additive is sought under the category 'zootechnical additives', functional groups 'gut flora stabilisers' according to Annex I of Regulation (EC) No 1831/2003. Miya-Gold[®] S contains 5×10^8 c.f.u. viable cells of *Clostridium butyricum* MIYAIRI 588 per gram as active agent. The strain is deposited at the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan [1]. The intended use of the current application are feedingstuffs for chickens for fattening, by mixing the feed additive into complete feedingstuffs at final concentrations of 5×10^8 c.f.u./kg [2].

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005 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. For this particular dossier, the methods of analysis submitted in connection with the Miya-Gold[®] S dossier (EFSA-Q-2008-303) and their suitability to be used for official controls in the frame of the authorisation, were evaluated.

EVALUATION

The numbering system under this point refers to the 'Guidelines for the assessment of additives in feedingstuffs, part II: Enzymes and Micro-organisms' (2.5. Control methods), in the following referred to as 'the Guidelines'.

Description of some of the methods listed under item 2.5.1. of the Guidelines

Qualitative and quantitative composition of the additive

The method for determination of the quantitative composition of the active agent in the additive is provided by the applicant [3, 5]. The applicant provided further a supplementary protocol in which the iron sulfite agar according to ISO 15213 was suggested for an enumeration of the feed additive and validation data for the applicability of the method [4, 5]. For official controls this enumeration method is recommended.

The active agent is a strain of *Clostridium butyricum* MIYAIRI 588. A range of techniques are applied by the applicant for identification of *Clostridium butyricum* MIYAIRI 588 comprising microscopical, biochemical and molecular methods [6]. The applicant uses pulsed field gel electrophoresis (PFGE) which is considered as an appropriate method for official controls [7].

Description of qualitative and quantitative methods for routine control of the active agent in premixtures and feedingstuffs (cf. requirements of Guidelines section 2.5.2)

The applicant proposes the same method as above [3, 5] to analyse premixtures for *Clostridium butyricum* MIYAIRI 588. Iron sulfite agar according to ISO 15213 is applicable for the analysis of premixtures followed by confirmatory procedures such as absence of growth under aerobic conditions and formation of butyric acid of presumptive colonies on plate count agar [4, 5]. For the analysis of feedingstuffs a selective CMB588 agar is recommended followed by the same confirmatory procedure [5, 8]. The applicant provided validation data for the proposed methods obtained from three-laboratory ring-trials using premixtures and for feedingstuffs and a method protocol detailing the methods and media compositions [5]. A heat treatment of samples as described in ISO 15213 is not necessary.

Performance characteristics of the methods obtained in a three-laboratory study were expressed in terms of standard deviations for repeatability (s_r) and reproducibility (s_R) calculated from the base 10 logarithms of the measured c.f.u./g. The method's performance

characteristics of the enumeration method are standard deviations for repeatability (s_r) and reproducibility (s_R) below $0.10 \log_{10}$ and between $0.09 - 0.31 \log_{10}$ calculated from the base 10 logarithms of the measured c.f.u./g, respectively [5]. The limits of quantification (LOQ) of this method are around 10^4 colony forming units (c.f.u) per gram (g) feed additive or premixture and around 10^7 c.f.u./kg feedingstuff which is well below the anticipated target concentrations. Iron sulfite agar according to ISO 15213 for an analysis of premixtures and selective CMB588 agar for an analysis of feedingstuffs is recommended for official controls. The CRL-FA recommends using for analysis of premixtures 20 g samples for feedingstuffs 50 g.

Concerning the unambiguous identification of the specific strain *Clostridium butyricum* MIYAIRI 588 in the feed additive, premixtures and feedingstuffs PFGE is recommended as outlined above [7]. This methodology is widely accepted and used for microbial identifications and is therefore considered suitable for official controls in the frame of this authorisation.

3. CONCLUSIONS AND RECOMMENDATIONS

The applicant provided methods for the enumeration and identification of the active agent *Clostridium butyricum* MIYAIRI 588 in the feed additive, premixtures and feedingstuffs. Determination of colony forming units on iron sulfite agar according to ISO 15213 are recommended for official controls of the additive and premixtures and selective CBM588 agar (Microbiol. Immunol. 1997, 41(9), 665-671) for feedingstuffs. PFGE (Jpn Pharmacol. Ther. 28(12) 999-1004) is recommended for strain identification

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

Quantification: Iron sulfite agar for the additive and premixtures and selective CMB588 agar for feedingstuffs

Identification: Pulsed-field gel electrophoresis (PFGE)

4. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

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

5. REFERENCES

- [1] Technical dossier. Section II. 2.2.2. Biological origin, strain deposit
- [2] Proposal of Register entry Annex III
- [3] Technical dossier. Section II. Annexe 2.5.2. Determination of *Clostridium butyricum* MIYAIRI 588 (CMB) in Miya-Gold® S products, premixtures, feeds and faeces
- [4] ISO 15213:2003. Microbiology of food and feedingstuffs – Horizontal method for the enumeration of sulfite-reducing bacteria growing under anaerobic conditions. ISO, Geneva, Switzerland
- [5] Technical dossier. Section II. Updated Annex 2.5.2. of 23 September 2008 entitled 'ISO 15213 based analysis of *Clostridium butyricum* MIYAIRI (CMB 588 – Miya-Gold®) Rev3' (Rev3_CBM588_ISO15213.pdf)
- [6] Technical dossier. Section II. 2.2.2. Biological origin. Strain identification procedures
- [7] Wang X., Karasawa T., Maegawa T., Kozaki S., Tsukamoto K., Nakamura S. 2000. Comparative analysis of nontoxigenic and neurotoxigenic *Clostridium butyricum* by molecular typing methods. Jpn Pharmacol. Ther. 28(12) 999-1004
- [8] Sato R., Tanaka, M. 1997. Intestinal distribution and intraluminal localization of orally administered *Clostridium butyricum* in rats. Microbiol. Immunol. 41(9) 665-671

6. RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was the Community Reference Laboratory for Feed Additives (CRL-FA), Geel, Belgium. The initial evaluation report was made available for commenting to the consortium of National Reference Laboratories.

8. ACKNOWLEDGEMENTS

The following National Reference Laboratories contributed to this report:

- Sächsische Landesanstalt für Landwirtschaft, Leipzig, Germany
- National Feed Laboratory, Lublin, Poland
- National Veterinary Research Institute, Pulawy, Poland
- National Veterinary Institute, Ljubljana, Slovenia