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

Fifteen *"micro-organisms used as silage agents"*
(i.e. 6 enterococci, 6 lactobacilli and 3 pediococci)

FAD	CRL
FAD-2010-0135	CRL/100122
FAD-2010-0302	CRL/100258
FAD-2010-0387	CRL/100250
FAD-2010-0388	CRL/100151
FAD-2010-0389	CRL/100155
FAD-2010-0395	CRL/100241

Rapporteur Laboratory: **European Union Reference Laboratory for Feed Additives (EURL-FA)**

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Date: **02/04/2012**

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Date: **20/04/2011**

EXECUTIVE SUMMARY

This report is on the evaluation of feed additives "*micro-organisms used as silage agents"*, which is related to the application of fifteen *micro-organisms* for which authorisation is sought under Article 10(7). Authorisation is sought for all the above mentioned *micro-organisms* under category/functional group 1(k), "technological additives/silage additives", according to Annex I of Regulation (EC) No 1831/2003. The list of *micro-organisms* of interest and the minimum activities in the *feed additives* and in *silage*, as sought in the authorisation, are presented in Table 1(*). The intended use of the current applications is for all animal species, except for FAD-2010-0387, for which bovines, ovines, pigs, poultry, rabbits, horses and goats are specified.

For identification and characterisation of all fifteen *micro-organisms* of concern (i.e. *enterococci, lactobacilli* and *pediococci*) the EURL recommends for official control Pulsed Field Gel Electrophoresis (PFGE), a generally recognised standard methodology for microbial identification.

The EURL recommends for enumeration in the *feed additives* the following ring-trial validated methods:

- Spread plate method using Bile Esculin Azide agar (EN 15788) for *enterococci*;
- Spread plate method using MRS agar (EN 15787) for *lactobacilli*; and
- Spread plate method using MRS agar (EN 15786) for *pediococci.*

None of the Applicants provided experimental data for the determination of *micro-organisms* in *silage*. Furthermore, the unambiguous determination of the content of *micro-organisms* added to *silage* is not achievable by analysis. Therefore the EURL cannot evaluate nor recommend any method for official control to determine any of the fifteen *micro-organisms* of concern in *silage*.

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.

(*)Full list provided in EURL evaluation report, available from the EURL website.

KEYWORDS

fifteen *micro-organisms* (listed in Table 1), technological additives, silage additives, all animal species and categories.

1. BACKGROUND

In the current application authorisation is sought under Article 10(7) (re-evaluation of already authorised additives) for fifteen *"micro-organisms used as silage agents"*, under the category/functional group 1(k), "technological additives/silage additives", according to Annex I of Regulation (EC) No 1831/2003 [1].

The list of *micro-organisms* of interest is presented in Table 1 which includes:

- the species as currently specified in the Community register;
- the species as proposed by the Applicant [2, 3];
- the name of the Institute where the original strain is deposited [4]; and
- the minimum activities in the *feed additive* [2,3] and in *silage* [2,5] as sought in the authorisation.

Specifically, authorisation is sought for the *feed additive* to be placed on the market in the form of powder [3]. The intended use of the current applications is for all animal species, except for FAD-2010-0387, for which bovines, ovines, pigs, poultry, rabbits, horses and goats are specified.

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 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 fifteen *micro-organisms* listed in Table 1 and their suitability to be used for official controls in the frame of the authorisation were evaluated.

Table 1: List of *micro-organisms* with the corresponding minimum activities in the *feed additive* (a) and in *silage* (b) as proposed in Annex A [2] and/or Technical Dossier, Section II [3, 5]

FAD	micro-organism	as found in COM Register	as proposed by Applicant	deposited @	(a) CFU/g	(b) CFU/kg
FAD-2010-0135	Enterococcus faecium	NCIMB 10415	NCIMB 10415	NCIMB	1.0E+10	1.0E+08
	Enterococcus faecium	BIO 34	BIO 34 – DSM 3530	DSMZ	1.0E+10	5.0E+07
	Enterococcus faecium	(*) M74 NCIMB 11181 or M74 CCM 6226	CCM 6226 – NCIMB 11181 – DSM 22502	DSMZ	1.0E+11	1.0E+08
	Enterococcus faecium	SF202 – DSM 4788 – ATCC 53519	ATCC 53519	ATCC	1.0E+10	1.0E+07
	Enterococcus faecium	SF301 – DSM 4789 – ATCC 55593	ATCC 55593	ATCC	1.0E+10	5.0E+06
FAD-2010-0302	Lactobacillus buchneri	NCIMB 30139	NCIMB 301398	NCIMB	5.0E+10	1.0E+08
FAD-2010-0387	Lactobacillus plantarum	DSM 3676	DSM 3676	DSMZ	6.0E+11	1.0E+08#
	Lactobacillus plantarum	DSM 3677	DSM 3677	DSMZ	4.0E+11	1.0E+08#
	Lactobacillus buchneri	DSM 13573	DSM 13573	DSMZ	2.0E+11	1.0E+08
FAD-2010-0388	Lactobacillus plantarum	LSI NCIMB 30083	NCIMB 30083	NCIMB	5.0E+10	1.0E+05
	Lactobacillus plantarum	L-256 NCIMB 30084	NCIMB 30084	NCIMB	5.0E+10	1.0E+05
FAD-2010-0389	Pediococcus pentosaceus	DSM 14021	DSM 14021	DSMZ	1.0E+11	1.0E+05
	Pediococcus	acidilactici 33-06 NCIMB 30086	pentosaceus DSM 23688	DSMZ	1.0E+11	1.0E+05
	Pediococcus	acidilactici 33-11 NCIMB 30085	pentosaceus DSM 23689	DSMZ	1.0E+11	1.0E+05
FAD-2010-0395	Enterococcus faecium	CNCM I-3236 / ATCC 19434	CNCM I-3236	CNCM	2.0E+10	1.0E+08

Major differences in nomenclature highlighted

**According to the Applicant, two strains of Enterococcus faecium are synonymous; they are currently listed as two separate entries in Community Register of Feed Additives*

 # A minimum of 5.0E+7 CFU/kg fresh matter for each strain, when Lactobacillae plantarum DSM 3676 and DSM 3677 are combined

Culture Collections

ATCC: American Type Culture Collection (US);

CNCM: Collection Nationale de Cultures de Microorganismes (FR);

DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen (DE);

NCIMB: The National Collection of Industrial, food and Marine Bacteria (UK).

3. EVALUATION

Identification/Characterisation of the feed additive

Qualitative and quantitative composition of the additive

For identification and characterisation of all 15 *micro-organisms* of concern listed in Table 1 (cf. *enterococci, lactobacilli* and *pediococci*) the EURL recommends for official control Pulsed Field Gel Electrophoresis (PFGE), a generally recognised standard methodology for microbial identification [6].

Qualitative and quantitative composition of any impurities in the additive

The Applicants analysed the *feed additive* for microbial contaminants (such as Enterobacteria, *Escherichia coli*, Salmonella spp. and yeasts) by using appropriate EN ISO tests. For undesirable substances (i.e. arsenic, cadmium, mercury, lead, selenium, copper, zinc, chrome, aflatoxins) internationally recognised standard methods are available at the respective European Union Reference Laboratory, 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

a. Enterococci

For enumeration of the 6 *enterococci* of interest in the *feed additive,* the Applicants proposed the ring-trial validated spread plate method EN 15788 [7]. The sample is suspended in phosphate buffered saline (PBS) and diluted in a peptone salt solution; the appropriate dilutions are then spread on Bile Esculin Azide agar. The agar plates are incubated at 37 °C for 24 hours before colony counting. The performance characteristics of the CEN method reported after logarithmic transformation are [7]:

- a standard deviation for *repeatability* (s_r) ranging from 0.12 to 0.2 $\log_{10}$ CFU/g;
- a standard deviation of *reproducibility* (s_R) ranging from 0.23 to 0.41 $\log_{10}$ CFU/g; and
- a limit of detection (LOD) of $1x10^5$ CFU/kg *feedingstuffs* [8].

Based on the performance characteristics presented the EURL recommends for official control the CEN method (EN 15788) for the determination of the 6 *enterococci* of interest in the *feed additive per se*.

b. Lactobacilli

For enumeration of the 6 *lactobacilli* of interest in the *feed additive,* the Applicants proposed the ring-trial validated spread plate method EN 15787 [9]. The sample is suspended and diluted in a phosphate buffered saline (PBS); the appropriate dilutions are then spread on MRS (de Man, Rogosa, Sharp) agar plates. The agar plates are incubated at 37 °C for 48 to 72 hours. The performance characteristics of the CEN method reported after logarithmic transformation are [9]:

- s_r = 0.24 $\log_{10}$ CFU/g;
- s_R ranging from 0.29 to 0.38 $\log_{10}$ CFU/g; and
- LOD = 10^5 CFU/kg *feedingstuffs* [8].

Based on the performance characteristics presented the EURL recommends for official control the CEN method (EN 15787) for the determination of the 6 *lactobacilli* of interest in the *feed additive per se*.

c. Pediococci

For enumeration of the 3 *pediococci* of interest in the *feed additive*, the Applicants proposed the ring-trial validated spread plate method EN 15786 [10]. The sample is suspended and diluted in PBS; the appropriated dilutions are then spread on MRS agar plates. The agar plates are incubated at 37 °C for 48 hours. The performance characteristics of the CEN method reported after logarithmic transformation are [10]:

- s_r ranging from 0.01 to 0.17 $\log_{10}$ CFU/g;
- s_R ranging from 0.10 to 0.26 $\log_{10}$ CFU/g; and
- LOD = 10^5 CFU/kg *feedingstuffs* [8].

Based on the performance characteristics presented the EURL recommends for official control the CEN method (EN 15786) for the determination of the 3 *pediococci* of interest in the *feed additive per se*.

None of the Applicants provided experimental data for the determination of *micro-organisms* in *silage*. Furthermore, the unambiguous determination of the content of *micro-organisms* added to *silage* is not achievable by analysis. Therefore the EURL cannot evaluate nor recommend any method for official control to determine any of the fifteen *micro-organisms* of concern in *silage*.

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.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation the EURL recommends Pulsed Field Gel Electrophoresis (PFGE) for the identification of the 15 *micro-organisms* of concern.

The EURL recommends for enumeration in the *feed additives* the following methods:

- Spread plate method using Bile Esculin Azide agar (EN 15788) for *enterococci*;
- Spread plate method using MRS agar (EN 15787) for *lactobacilli;* and
- Spread plate method using MRS agar (EN 15786) for *pediococci.*

None of the Applicants provided experimental data for the determination of *micro-organisms* in *silage*. Furthermore, the unambiguous determination of the content of *micro-organisms* added to *silage* is not achievable by analysis. Therefore the EURL cannot evaluate nor recommend any method for official control to determine any of the fifteen *micro-organisms* of concern in *silage*.

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

Enumeration in the *feed additive* of

- 6 *enterococci*: EN 15788 - Spread plate method using Bile Esculin Azide agar
- *6 lactobacilli:* EN 15787 - Spread plate method using MRS agar
- *3 pediococci:* EN 15786 - Spread plate method using MRS agar

Identification of fifteen *micro-organisms* of concern:

- Pulsed Field Gel Electrophoresis (PFGE)

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, samples of the fifteen *micro-organisms* of concern, have been sent to the European Union Reference Laboratory for Feed Additives Authorisation. The dossier has been made available to the EURL by EFSA.

6. REFERENCES

[1] *Application/Ref: SANCO/G/1: Group Forward Slip ARES(2011)1369341

SANCO Ref.	Dossier
Forw.Appl 1831/0099-2011	FAD-2010-0135
Forw.Appl 1831/0090-2011	FAD-2010-0302
Forw.Appl 1831/0089-2011 Forw.Appl 1831/0088-2011 Forw.Appl 1831/0087-2011	FAD-2010-0387
Forw.Appl 1831/0103-2011 Forw.Appl 1831/0104-2011	FAD-2010-0388
Forw.Appl 1831/0102-2011 Forw.Appl 1831/0100-2011 Forw.Appl 1831/0101-2011	FAD-2010-0389
Forw.Appl 1831/0091-2011	FAD-2010-0395

[2] *Application, Annex A, Proposal for register entry

[3] *Technical Dossier, Section II.2.1. Identity of the additive

[4] *Technical Dossier, Section II.2.2. Characterisation of the active substance(s)/agent(s)

[5] *Technical Dossier, Section II.2.5. Conditions of use of the additive

[6] European Community Project SMT4-CT98-2235.'Methods for the Official Control of Probiotics Used as Feed Additives, Report 20873/1 EN (2002) ISBN 92-894-6250-7 (Vol. I)

[7] EN 15788:2009 - Animal feeding stuffs - Isolation and enumeration of Enterococcus *(E. faecium*) spp

[8] ISO 7218:1996 - Microbiology of food and animal feedingstuffs – General rules for microbiological examinations

[9] EN 15787:2009 - Animal feeding stuffs. Isolation and enumeration of Lactobacillus spp

[10] EN 15786:2009 - Animal feeding stuffs. Isolation and enumeration of Pediococcus spp

*Refers to all six FAD Dossiers, listed under [1]

7. RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was European Union 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.

8. ACKNOWLEDGEMENTS

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- Schwerpunktlabor Futtermittel des Bayerischen Landesamtes für Gesundheit und Lebensmittelsicherheit (LGL), Oberschleißheim (DE)
- Staatliche Betriebsgesellschaft für Umwelt und Landwirtschaft, Geschäftsbereich 6 – Labore Landwirtschaft, Leipzig (DE)
- Thüringer Landesanstalt für Landwirtschaft (TLL), Abteilung Untersuchungswesen. Jena (DE)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)