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JOINT RESEARCH CENTRE

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European Union Reference Laboratory for Feed Additives

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

Forty five *"micro-organisms used as silage agents"*
(i.e. 39 lactobacilli, 2 lactococci, 2 pediococci, 1 bacillus and 1 saccharomyces)

FAD	CRL
FAD-2010-0033	CRL/100063
FAD-2010-0048	CRL/100068
FAD-2010-0084	CRL/100109
FAD-2010-0085	CRL/100054
FAD-2010-0086	CRL/100055
FAD-2010-0087	CRL/100053
FAD-2010-0102	CRL/100113
FAD-2010-0103	CRL/100105
FAD-2010-0106	CRL/100125
FAD-2010-0108	CRL/100121

FAD	CRL
FAD-2010-0110	CRL/100107
FAD-2010-0169	CRL/090013
FAD-2010-0170	CRL/100110
FAD-2010-0171	CRL/100104
FAD-2010-0172	CRL/100052
FAD-2010-0176	CRL/100154
FAD-2010-0192	CRL/100143
FAD-2010-0240	CRL/100147
FAD-2010-0278	CRL/100333
FAD-2011-0001	CRL/100281

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Date: **02/09/2011**

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Date: **02/09/2011**

EXECUTIVE SUMMARY

This report is on the evaluation of feed additives "*micro-organisms used as silage agents"*, which is related to the application of (1) forty two *micro-organisms* for which authorisation is sought under Article 10(2) and (2) three additional *micro-organisms* for which authorisation is sought under Article 4(1). 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-2011-0001, for which pigs, bovines, sheep, goats and horses are specified.

For identification and characterisation of *Saccharomyces cerevisiae* the EURL recommends for official control Polymerase Chain Reaction (PCR), a generally recognised standard methodology for identification of yeasts. For identification and characterisation of all the other *micro-organisms* of concern (i.e. *lactococci, lactobacilli, pediococci* and *bacilli*) 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:

- Pour plate method using MRS agar (ISO 15214) for *Lactococci;*
- Spread plate method using MRS agar (EN 15787) for *Lactobacilli*;
- Spread plate method using MRS agar (EN 15786) for *Pediococci;*
- Spread plate method using tryptone soya agar (EN 15784) for *Bacilli;* and
- Pour plate method using CGYE agar (EN 15789) for *Saccharomyces.*

None of the Applicants provide 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 forty five *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

Forty five *micro-organisms* (listed in Table 1), technological, silage additives.

1. BACKGROUND

This report is on the evaluation of feed additives "*micro-organisms used as silage agents"*, which is related to the application of (1) forty two *micro-organisms* for which authorisation is sought under Article 10(2) [1] and (2) three additional *micro-organisms* for which authorisation is sought under Article 4(1) [1]. 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 [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];
- the name of the Institute where the original strain is deposited [3]; and
- the minimum activities in the *feed additive* and in *silage* as sought in the authorisation [2].

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-2011-0001, for which pigs, bovines, sheep, goats and horses are specified.

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 European Union Reference Laboratory concerning applications for authorizations of *feed additives*, as last amended by Regulation (EC) No 885/2009, the EURL is requested to submit a full evaluation report to the European Food Safety Authority (EFSA) for each application, or for each group of applications. For this particular dossier, the methods of analysis submitted in connection with the forty five *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)

FAD	micro-ogransim	as found in COM Register	as proposed by Applicant	deposited @	(a) CFU/g	(b) CFU/kg
FAD-2010-0033	Lactococcus lactis	SR 3.54, NCIMB 30117	NCIMB 30117	NCIMB	5.0E+10	1.0E+05
FAD-2010-0048	Lactobacillus plantarum	AK 5106, DSM 20174	AK 5106 - DSM 20174 - DSM 23375	DSMZ	2.0E+10	1.0E+09
	Lactobacillus plantarum	CNCM I-3235 / ATCC 8014	CNCM I-3235 - ATCC 8014	CNCM	5.0E+10	2.0E+07
	Lactobacillus plantarum	IFA96	IFA96 - DSM 19457	DSMZ	1.0E+10	5.0E+07
	Lactobacillus plantarum	DSM 16568	DSM 16568	DSMZ	5.0E+10	1.0E+08
	Lactobacillus plantarum	MiLAB 393, LMG21295	LMG21295	LMG	5.0E+10	1.0E+08
	Lactobacillus plantarum	DSM 16565	DSM 16565	DSMZ	5.0E+10	1.0E+08
	Lactobacillus plantarum	VTT E-78076	VTT E-78076	VTT	1.0E+11	1.0E+09
	Lactobacillus plantarum	DSM 11672 = CNCM MA 18/5U	CNCM MA 18/5U - DSM 11672	CNCM/DSMZ	2.0E+10	1.0E+08
	Lactobacillus plantarum	MBS-LP-01	MBS-LP-01 - NCIMB 30238	NCIMB	2.0E+10	2.0E+07
	Lactobacillus plantarum	24011	24011 - ATCC PTA-6139	ATCC	1.0E+10	2.0E+07
	Lactobacillus plantarum	LP286, DSM 4784, ATCC 53187	LP286 - DSM 18112	DSMZ	1.0E+10	5.0E+06
	Lactobacillus plantarum	LP287, DSM 5257, ATCC 55058	LP287 - ATCC 55058	ATCC	1.0E+10	5.0E+06
	Lactobacillus plantarum	LP318, DSM 4785	LP318 - DSM 18113	DSMZ	1.0E+10	2.0E+07
	Lactobacillus plantarum	LP319, DSM 4786	LP319 - DSM 18114	DSMZ	1.0E+10	2.0E+07
	Lactobacillus plantarum	LP329, DSM 5258, ATCC 55942	LP329 - ATCC 55942	ATCC	1.0E+10	5.0E+06
	Lactobacillus plantarum	LP346, DSM 4787, ATCC 55943	LP346 - ATCC 55943	ATCC	1.0E+10	2.0E+07
	Lactobacillus plantarum	LP347, DSM 5284, ATCC 55944	LP347 - ATCC 55944	ATCC	1.0E+10	5.0E+07
	Lactobacillus plantarum	NCIMB 30094	NCIMB 30094	NCIMB	5.0E+10	1.0E+09
FAD-2010-0084	Lactobacillus buchneri	DSM 16774	DSM 16774	DSMZ	5.0E+11	1.0E+05
FAD-2010-0085	Lactobacillus buchneri	DSM 12856	DSM 12856	DSMZ	5.0E+11	1.0E+05
FAD-2010-0086	Lactobacillus plantarum	DSM 12837	DSM 12837	DSMZ	5.0E+11	1.0E+05
FAD-2010-0087	Lactobacillus paracasei	DSM 16245	DSM 16245	DSMZ	5.0E+11	1.0E+05
FAD-2010-0102	Pediococcus acidilactici	DSM 16243	DSM 16243	DSMZ	5.0E+11	1.0E+05
FAD-2010-0103	Lactobacillus rhamnosus	NCIMB 30121	NCIMB 30121	NCIMB/DSMZ	4.0E+11	1.0E+05
FAD-2010-0106	Lactobacillus salivarius	CNCM I-3238 / ATCC 11741	ATCC 11741 / CNCM I-3238	CNCM	5.0E+08	1.25E+07
	Lactobacillus casei	LC32909	LC32909 - ATCC PTA-6135	ATCC	1.0E+10	1.3E+06
FAD-2010-0108	Lactobacillus brevis	IFA92	IFA92 - DSM 23231	DSMZ	1.0E+10	5.0E+07
	Lactobacillus buchneri	CCM 1819	CCM 1819 - DSM 22501	DSMZ	5.0E+10	1.0E+08
	Lactobacillus buchneri	NCIMB 40788	NCIMB 40788 - CNCM I-4323	CNCM / NCIMB	3.0E+09	1.0E+08
	Lactobacillus buchneri	40177	ATCC PTA-6138	ATCC	1.0E+10	1.0E+08
	Lactobacillus buchneri	LN4637, ATCC PTA-2494	ATCC PTA-2494	ATCC	1.0E+10	1.0E+08

Table 1 *(continued)*

FAD	micro-ogransim	as found in COM Register	as proposed by Applicant	deposited @	(a) CFU/g	(b) CFU/kg
FAD-2010-0110	Lactococcus lactis	NCIMB 30160	NCIMB 30160	NCIMB	4.0E+11	1.0E+05
FAD-2010-0169	Lactobacillus plantarum	DSM 12836	DSM 12836	DSMZ	5.0E+11	1.0E+05
FAD-2010-0170	Lactobacillus brevis	DSM 12835	DSM 12835	DSMZ	5.0E+11	1.0E+05
FAD-2010-0171	Pediococcus pentosaceus	DSM 12834	DSM 12834	DSMZ	4.0E+11	1.0E+05
FAD-2010-0172	Lactobacillus paracasei	DSM 16773	DSM 16773	DSMZ	4.0E+11	1.0E+05
FAD-2010-0176	Lactobacillus pentosus	DSM 14025	DSM 14025	DSMZ	1.0E+11	1.0E+05
FAD-2010-0192	Bacillus	subtilis MBS-BS-01	amyloliquefaciens MBS-BS-01 / NCIMB 30229	NCIMB	2.5E+10	5.0E+07
FAD-2010-0240	Saccharomyces cerevisiae	IFO 0203	NBRC 0203	NBRC	na	na
	Lactobacillus plantarum	ATCC 8014	NBRC 3070	NBRC	na	na
	Lactobacillus casei	ATCC 7469	NBRC 3425	NBRC	na	na
FAD-2010-0278 **(#)**	Lactobacillus brevis		DSMZ 21982	DSMZ	na	na
FAD-2011-0001 **(#)**	Lactobacillus plantarum **and**	DSM 8862	DSM 8862	DSMZ	1.5E+11	na
	Lactobacillus plantarum	DSM 8866	DSM 8866	DSMZ	1.5E+11	na

Grouping of applications as forwarded by the Commission
Major differences in nomenclature highlighted
(#) Art. 4(1)

Culture Collections

ATCC: American Type Culture Collection (US); **BCCM/LMG** Belgian Co-ordinated Collections of Micro-organisms (BE);

CNCM: Collection Nationale De Cultures De Microorganismes (FR); **DSMZ**: Deutsche Sammlung von Mikroorganismen und Zellkulturen (DE);

NBRC: NITE Biological Resource Center (JP); **NCIMB**: The National Collection of Industrial, food and Marine Bacteria (UK) ;

VTT: Technical Research Centre of Finland.

3. EVALUATION

Identification/Characterisation of the feed additive

Qualitative and quantitative composition of the additive

For identification and characterisation of the strain *Saccharomyces cerevisiae* NBRC 0203 the EURL recommends for official control Polymerase Chain Reaction (PCR), a generally recognised standard methodology for identification of yeasts [4].

For identification and characterisation of all the other 44 *micro-organisms* of concern listed in Table 1 (cf. *lactococci, lactobacilli, pediococci* and *bacilli*) the EURL recommends for official control Pulsed Field Gel Electrophoresis (PFGE), a generally recognised standard methodology for microbial identification [4].

Qualitative and quantitative composition of any impurities in the additive

The Applicant 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. Lactococci

For the enumeration of *Lactococcus lactis* NCIMB 30117 and NCIMB 30160 in the *feed additive* the Applicant submitted a pour plate method based on ISO 15214 [5], using de Man, Rogosa and Sharpe agar (MRS) at pH 5.7. The sample is suspended and diluted in a buffer solution and 1 ml of the first decimal dilution is transferred into sterile Petri dishes. Approximately 15 ml of MRS agar is poured into Petri dishes, cooled to 47 °C. The inoculum is carefully mixed with the medium and the mixture allowed to solidify. The dishes are inverted and incubated at 30 °C for 72 h ± 3h. A limit of detection (LOD) of 10^5 CFU/kg is reported in the ISO 7218 standard [6].

The EURL recommends for official control the ISO 15214 method for the determination of *Lactococcus lactis* NCIMB 30117 and NCIMB 30160 in the *feed additive per se*.

b. Lactobacilli

For enumeration of the 39 *lactobacilli* of interest in *feed additive,* Applicants proposed the ring trial validated spread plate method EN 15787 [7]. The sample is suspended and diluted in a phosphate buffered saline (PBS); the appropriate dilutions are then spread on MRS 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 [7]:

- a standard deviation for repeatability (S_r) of 0.24 $\log_{10}$ CFU/g;
- a standard deviation for reproducibility (S_R) ranging from 0.29 to 0.38 $\log_{10}$ CFU/g; and
- a limit of detection (LOD) of 10^5 CFU/kg [6].

Based on the performance characteristics presented the EURL recommends for official control the CEN method (EN 15787) for the determination of the 39 *lactobacilli* of interest in the *feed additive per se*.

c. Pediococci

For enumeration of *Pediococcus acidilactici* DSM 16243 and *Pediococcus pentosaceus* DSM 12834 in the *feed additive* the Applicants proposed the ring-trial validated spread plate method EN 15786 [8]. 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 [8]:

- 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 [6].

Based on the performance characteristics presented the EURL recommends for official control the CEN method (EN 15786) for the determination of the *Pediococcus acidilactici* DSM 16243 and *Pediococcus pentosaceus* DSM 12834 in the *feed additive per se*.

d. Bacillus

For the enumeration of the *Bacillus amyloliquefaciens* MBS-BS-01 in the *feed additive*, the Applicant proposes a ring-trial validated spread plate method EN 15784 [9]. Twenty grams of additive are suspended in PBS. Decimal dilutions are prepared in peptone salt diluent and subjected to a heat treatment at 80 ^{o}C for 10 minutes. Subsequently, appropriate dilutions are spread on tryptone soya agar and plates are incubated at 37 ^{o}C for 16-24 h. The performance characteristics of the CEN method reported after logarithmic transformation are [9]:

- S_r ranging from 0.07 to 0.09 $\log_{10}$ CFU/g;
- S_R ranging from of 0.32 to 0.35 $\log_{10}$ CFU/g; and
- LOD = 10^5 CFU/kg [6].

Based on the performance characteristics presented the EURL recommends for official control the ring trial validated CEN method (EN 15784) for the determination of *Bacillus amyloliquefaciens* MBS-BS-01 in the *feed additive per se.*

e. Saccharomyces

For the enumeration of *Saccharomyces cerevisiae* NBRC 0203 in the *feed additive* the Applicant proposes the ring trial validated pour plate method EN 15789, using yeast extract dextrose chloramphenicol agar (CGYE) [10]. The sample is suspended in PBS and diluted in a peptone salt solution. The appropriate dilutions are transferred to Petri dishes and melted CGYE agar is added. When the agar is solidified, plates are incubated at 35 °C for 48 hours before colony counting. The performance characteristics of the CEN method reported after logarithmic transformation are [10]:

- S_r ranging from 0.17 to 0.36 $\log_{10}$ CFU/g;
- S_R ranging from 0.55 to 0.60 $\log_{10}$ CFU/g; and
- LOD = 10^5 CFU/kg [6].

Based on the performance characteristics presented the EURL recommends, for official control, the CEN method (EN 15789) for the determination of *Saccharomyces cerevisiae* NBRC 0203 in the *feed additive per se*.

None of the Applicants provide 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 forty five *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 Polymerase Chain Reaction (PCR) for identification of *Saccharomyces cerevisiae* NBRC 0203 and Pulsed Field Gel Electrophoresis (PFGE) for the identification of the other 44 *micro-organisms* of concern.

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

- Pour plate method using MRS agar (ISO 15214) for *Lactococci;*
- Spread plate method using MRS agar (EN 15787) for *Lactobacilli;*
- Spread plate method using MRS agar (EN 15786) for *Pediococci;*
- Spread plate method using tryptone soya agar (EN 15784) for *Bacilli;* and
- Pour plate method using CGYE agar (EN 15789) for *Saccharomyces.*

None of the Applicants provide 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 forty five *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

- *Lactococcus lactis* NCIMB 30117 and NCIMB 30160:
  ISO 15214 - Pour plate method using MRS agar
- 39 *Lactobacilli:*
  EN 15787 - Spread plate method using MRS agar
- *Pediococcus acidilactici* DSM 16243 and *Pediococcus pentosaceus* DSM 12834*:*
  EN 15786 - Spread plate method using MRS agar
- *Bacillus amyloliquefaciens* MBS-BS-01:
  EN 15784 - Spread plate method using tryptone soya agar
- *Saccharomyces cerevisiae* NBRC 0203:
  EN 15789 - Pour plate method using CGYE agar

Identification of:

- *Saccharomyces cerevisiae* NBRC 0203: Polymerase Chain Reaction (PCR)
- other 44 *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 forty five *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/D/2:

SANCO Ref.	Dossier
Group Forward Slip ARES(2011)407183	FAD-2010-0033
	FAD-2010-0048
	FAD-2010-0084
	FAD-2010-0085
	FAD-2010-0086
	FAD-2010-0087
	FAD-2010-0102
	FAD-2010-0103
	FAD-2010-0106
	FAD-2010-0108
	FAD-2010-0110

SANCO Ref.	Dossier
(continued)	FAD-2010-0169
	FAD-2010-0170
	FAD-2010-0171
	FAD-2010-0172
	FAD-2010-0176
	FAD-2010-0192
	FAD-2010-0240
Forw.Appl 1831/0137-2010	FAD-2010-0278
Forw.Appl 1831/0003-2011	FAD-2011-0001

[2] *Application, Annex A, Proposal for register entry
[3] *Technical Dossier, Section II.2.1. Identity of the additive
[4] 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)
[5] ISO 15214:1998 - Microbiology of food and animal feeding stuffs -- Horizontal method for the enumeration of mesophilic lactic acid bacteria -- Colony-count technique at 30 degrees C
[6] ISO 7218:1996 - Microbiology of food and animal feedingstuffs – General rules for microbiological examinations
[7] EN 15787:2009 - Animal feeding stuffs. Isolation and enumeration of Lactobacillus spp
[8] EN 15786:2009 - Animal feeding stuffs. Isolation and enumeration of Pediococcus spp
[9] EN 15784:2009 - Animal feeding stuffs. Isolation and enumeration of presumptive Bacillus spp
[10] EN 15789:2009 - Animal feeding stuffs. Isolation and enumeration of yeast probiotic strains

*Refers to all twenty 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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- Państwowy Instytut Weterynaryjny, Puławy (PL)
- Instytut Zootechniki w Krakowie, Krajowe Laboratorium Pasz, Lublin (PL)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Laboratoire de Rennes, SCL L35, Service Commun des Laboratoires, Rennes (FR)
- Foderavdelningen, Statens Veterinärmedicinska Anstalt (SVA), Uppsala (SE)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Sächsische Landesanstalt für Landwirtschaft, Fachbereich 8 — Landwirtschaftliches Untersuchungswesen, Leipzig (DE)