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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**

Lavipan®

(FAD-2013-0048; CRL/130014)



**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-2013-0048 - CRL/130014**

Name of Product: **Lavipan®**

Active Agent (s): ***Lactococcus lactis IBB500***
Carnobacterium divergens S1
Lactobacillus casei LOCK 0915
Lactobacillus plantarum LOCK 0862
Saccharomyces cerevisiae LOCK 0141

Rapporteur Laboratory: **Centre wallon de Recherches
agronomiques (CRA-W), Gembloux,
Belgium**

Report prepared by: **Véronique Ninane**

Report checked by: **Jone Omar and Piotr Robouch (EURL-FA)**
Date: **03/03/2014**

Report approved by: **Christoph von Holst**
Date: **06/03/2014**

EXECUTIVE SUMMARY

In the current application authorisation is sought under Article 4(1) for *Lavipan®* under the category / functional group 4(b) 'zootechnical additives' / 'gut flora stabilisers', according to Annex I of Regulation (EC) No 1831/2003. Specifically, authorization is sought for the use of the *feed additive* for piglets (weaned), chickens for fattening and turkeys for fattening.

According to the Applicant, the *feed additive* contains five non-genetically modified microorganisms: *Saccharomyces cerevisiae* LOCK 0141, *Lactococcus lactis* IBB500, *Carnobacterium divergens* S1, *Lactobacillus casei* LOCK 0915 and *Lactobacillus plantarum* LOCK 0862. The strain *Lactococcus lactis* IBB500 is deposited at the Polish Collection of Microorganisms (PCM, Wroclaw, Poland), the strain *Carnobacterium divergens* S1 is deposited at the Collection of Industrial Microorganisms (IAFB, Warsaw) while the other three strains are deposited at the Centre of Industrial Microorganisms Collection (LOCK, Lodz, Poland).

The product is intended to be marketed as a light cream to light brown powder containing at least 1×10^5 Colony Forming Units (CFU) *Saccharomyces cerevisiae* LOCK 0141 per gram and a minimum of 1×10^7 CFU bacterial active substances per gram. [3]. The feed additive is intended to be used in *feedingstuffs* at a minimum dose of 5×10^6 CFU of *Saccharomyces cerevisiae* LOCK 0141 per kg and 5×10^8 CFU of bacterial active substances per kg.

The Applicant used 16S rRNA gene sequence analysis for the identification and characterisation of the bacterial strains and internal transcribed spacer rRNA gene sequence analysis for the identification and characterisation of *Saccharomyces cerevisiae* LOCK 014. In addition, the Applicant referred to Pulsed Field Gel Electrophoresis (PFGE), a generally recognised standard methodology for the genetic identification of the bacterial strains and to Polymerase Chain Reaction (PCR) for the identification of *Saccharomyces cerevisiae* LOCK 0141. The EURL recommends for official control these two methods (PFGE and PCR) for the identification of the bacterial and yeast strains.

For the enumeration of *Lactococcus lactis* IBB500, *Carnobacterium divergens* S1, *Lactobacillus casei* LOCK 0915 and *Lactobacillus plantarum* LOCK 0862 in the *feed additive* and *feedingstuffs* the Applicant submitted the ISO 15214 poured-plate method and demonstrated its suitability by providing experimental data obtained in the frame of the stability study.

The EURL identified instead, for the enumeration of the *Lactobacilli* in the *feed additive* and *feedingstuffs*, the ring-trial validated spread plate method EN 15787 for the enumeration in the *feed additive* and *feedingstuffs*.

For the enumeration of *Saccharomyces cerevisiae* LOCK 0141 in *feed additive* and *feedingstuffs* the Applicant submitted the EN 15789 poured-plate method.

Based on the performance characteristics available the EURL recommends for official control the five ring trial validated CEN and ISO methods mentioned above.

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

Lavipan®, *Lactococcus lactis* IBB500, *Carnobacterium divergens* S1, *Lactobacillus casei* LOCK 0915, *Lactobacillus plantarum* LOCK 0862, *Saccharomyces cerevisiae* LOCK 0141, zootechnical additives, gut flora stabilisers, piglets (weaned), chickens for fattening and turkeys for fattening.

1. BACKGROUND

In the current application authorisation is sought under Article 4(1) for *Lavipan®* under the category / functional group 4(b) 'zootechnical additives' / 'gut flora stabilisers', according to Annex I of Regulation (EC) No 1831/2003 [1]. Specifically, authorization is sought for the use of the *feed additive* for piglets (weaned), chickens for fattening and turkeys for fattening.

According to the Applicant, the *feed additive* contains five non-genetically modified microorganisms: *Saccharomyces cerevisiae* LOCK 0141, *Lactococcus lactis* IBB500, *Carnobacterium divergens* S1, *Lactobacillus casei* LOCK 0915 and *Lactobacillus plantarum* LOCK 0862 [2,3,4]. The strain *Lactococcus lactis* IBB500 is deposited at the Polish Collection of Microorganisms (PCM, Wroclaw, Poland) and the strain *Carnobacterium divergens* S1 is deposited at the Collection of Industrial Microorganisms (IAFB, Warsaw) while the other three strains are deposited at the Centre of Industrial Microorganisms Collection (LOCK, Lodz, Poland) [5].

Note: The genus *Carnobacterium divergens* is not listed in the European Union Register of Feed Additives [6]. This genus was proposed by Collins et al. (1987) to accommodate the species of *Lactobacillus divergens* and *Lactobacillus piscicola* and other atypical lactobacilli isolated from poultry meat [7].

The product is intended to be marketed as a light cream to light brown powder containing at least 1×10^5 Colony Forming Units (CFU) *Saccharomyces cerevisiae* LOCK 0141 per gram and a minimum of 1×10^7 CFU bacterial active substances per gram. [3]. The feed additive is intended to be used in *feedingstuffs* at a minimum dose of 5×10^6 CFU of *Saccharomyces cerevisiae* LOCK 0141 per kg and 5×10^8 CFU of bacterial active substances per kg [2, 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 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 *Lavipan®* 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 impurities in the additive

When required by EU legislation, analytical methods for official control of undesirable substances in the *feed additive* (e.g. arsenic, cadmium, lead, mercury and mycotoxins) are available from the respective European Union Reference Laboratories [8].

Furthermore, the Applicant analysed the *feed additive* for microbial contaminants including *Escherichia coli*, *Salmonella* yeasts and moulds, by using the methods described in the technical dossier [3, 9].

Qualitative and quantitative composition of the additive

For the identification and characterisation of the yeast strain *Saccharomyces cerevisiae* LOCK 0141 the Applicant applied internal transcribed spacer rRNA gene sequence analysis [4]. For the identification and characterization of the bacterial strains *Lactococcus lactis* IBB500, *Carnobacterium divergens* S1, *Lactobacillus casei* LOCK 0915 and *Lactobacillus plantarum* LOCK 0862 the Applicant applied 16S rRNA gene sequence analysis. For phenotypic characterisation, the Applicant used API 50 CHL [4] for the *Lactobacillus* spp. and observed genus specific growth response as well as lactic acid conformation and peptidoglycan type of the cell wall for *Carnobacterium divergens* S1 [7]. In addition, the Applicant proposed (i) Pulsed Field Gel Electrophoresis (PFGE), a generally recognised standard methodology for the genetic identification of the bacterial strains [10] and (ii) Polymerase Chain Reaction (PCR) for the identification of *Saccharomyces cerevisiae* LOCK 0141.

The EURL recommends for official control the PFGE and PCR methods proposed by the Applicant for the identification of the bacterial and yeast strains. Both methods are currently being evaluated by the CEN Technical Committee 327 to become European Standards.

Description of the analytical methods for the determination of the active substances in feed additive and feedingstuffs

The Applicant does not specify the concentration of the individual strains in *Lavipan®* but provides the overall concentration (sum of) of bacterial active substances and the concentration of *Saccharomyces cerevisiae* LOCK 0141 [3].

For the enumeration of *Lactococcus lactis* IBB500, *Carnobacterium divergens* S1, *Lactobacillus casei* LOCK 0915 and *Lactobacillus plantarum* LOCK 0862 in the *feed additive* and *feedingstuffs* the Applicant submitted the ISO 15214 poured-plate method [9]

and demonstrated its suitability by providing experimental data obtained in the frame of the stability study [11]. The ISO 15214 method is internationally recognised for the enumeration of mesophilic lactic acid bacteria in *feedingstuffs*. The sample is suspended and diluted in a buffer solution; the appropriated dilutions are then spread in liquefied MRS (de Man, Rogosa, Sharp) agar plates. The agar plates are incubated at 30°C for 72 hours [12]. The ISO 15214 method was validated for *Lactobacilli* spp. in feed matrices. R. Leuschner *et al.* reported the following performance characteristics after logarithmic transformation (CFU) [13]:

- a *repeatability* standard deviation (s_r) of 0.24 log₁₀ CFU/g;
- a *reproducibility* standard deviation (s_R) ranging from 0.29 to 0.39 log₁₀ CFU/g; and
- a limit of detection (LOD) of 1x10⁴ CFU/kg [14], well below the minimum dose proposed by the Applicant (1x10⁸ CFU/kg *feedingstuffs*).

The EURL identified instead for the enumeration of the *Lactobacilli* in the *feed additive* and *feedingstuffs* the ring-trial validated spread plate method EN 15787 [15]. 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 following performance characteristics were reported after logarithmic transformation: s_r of 0.24 log₁₀ CFU/g and s_R ranging from 0.29 to 0.38 log₁₀ CFU/g.

For enumeration of *Saccharomyces cerevisiae* LOCK 0141 in *feed additive* and *feedingstuffs* the Applicant submitted the CEN method (EN 15789), an internationally recognised poured-plate method. The sample is suspended and diluted in a buffer solution; the appropriated dilutions are then spread in liquefied CGYE (yeast extract dextrose chloramphenicol) agar plates. The agar plates are incubated at 35°C for 48 hours [16]. The following performance characteristics were reported after logarithmic transformation (CFU):

- s_r ranging from 0.17 to 0.36 log₁₀ CFU/g,
- s_R ranging from 0.55 to 0.60 log₁₀ CFU/g, and
- LOD = 1x10⁴ CFU/kg, well below the minimum dose proposed by the Applicant (5x10⁶ CFU/kg *feedingstuffs*).

Based on the performance characteristics available the EURL recommends for official control the five ring-trial validated ISO and CEN methods mentioned above.

Note: The two CEN methods (EN 15787 for the enumeration of *Lactobacilli* and EN 15789 for the enumeration of *Saccharomyces cerevisiae*) are not applicable to mineral feeds composed mainly of minerals and containing at least 40 % crude ash. For these matrices laboratories may consider using the ring trial validated VDLUFA methods 28.2.4 [17] and 28.2.6 [18] instead.

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 for the enumeration in the *feed additive* and *feedingstuffs* the ISO 15214 method for *Lactococcus lactis* and *Carnobacterium divergens* and two CEN methods (EN 15787 and EN 15789) for *Lactobacilli* and *Saccharomyces cerevisiae*, respectively.

For the identification of the bacterial strains and of *Saccharomyces cerevisiae* LOCK 0141 the EURL recommends for official control Pulsed Field Gel Electrophoresis (PFGE) and Polymerase Chain Reaction (PCR), respectively. Both methods are currently evaluated by the CEN technical Committee 327 to become European Standards.

Recommended text for the register entry (analytical method)

For the enumeration of *Lactococcus lactis* and *Carnobacterium divergens* in the *feed additive* and *feedingstuffs*:

- Poured-plate method using MRS agar - ISO 15214

For the enumeration of *Lactobacilli* in the *feed additive* and *feedingstuffs* :

- Poured-plate method using MRS agar - EN 15787

For the enumeration of *Saccharomyces cerevisiae* in the *feed additive* and *feedingstuffs*:

- Poured-plate method using CGYE agar - EN 15789

For the identification of *Lactobacilli*, *Lactococcus lactis* and *Carnobacterium divergens*:

- Identification: Pulsed Field Gel Electrophoresis (PFGE)

For the identification of *Saccharomyces cerevisiae*

- Polymerase Chain Reaction (PCR)

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Lavipan®* 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 SANCO/G1: Forw. Appl. 1831/0050-2013
- [2] *Application, Proposal for Register Entry, Annex A
- [3] *Technical dossier, Section II, 2.1. Identity of the additive
- [4] *Technical dossier, Section II, 2. Characterisation of the active substance(s)/agent(s)
- [5] * Technical dossier, Section II, Annexes – Annex_II_19, 20, 21 & 22
- [6] European Union Register of Feed Additives pursuant to Regulation (EC) No 1831/2003-Annex I: List of additives, Edition 185
- [7] *Supplementary information, SIN-FAD-2013-0048

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- [8] Commission Regulation (EC) No 776/2006 amending Annex VII to Regulation (EC) No 882/2004 of the European Parliament and of the Council as regards to Community Reference Laboratories
- [9] *Technical dossier, Section II, 6. Method of analysis and reference samples
- [10] 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).
- [11] * Technical dossier, Section II, Annexes – Annex_II_41 & 42.
- [12] 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 °C*
- [13] Leuschner R.G.K.*et al.* Food Microbiol. 20 (2003) 57-66
- [14] ISO 7218:2007 - *Microbiology of food and animal feedingstuffs – General requirements and guidance for microbiological examinations*
- [15] EN 15787:2009 - *Animal feeding stuffs - Isolation and enumeration of Lactobacillus spp*
- [16] EN 15789:2009 - *Animal feeding stuffs - Isolation and enumeration of yeast probiotic strains*
- [17] VDLUFA method – 28.2.4 Determination of Enterococcus faecium and Lactobacillus rhamnosus (Method book III, 8 Suppl. 2012, VDLUFA , Darmstadt)
- [18] VDLUFA method – 28.2.6 Determination of Saccharomyces cerevisiae (Method book III, 8 Suppl. 2012, VDLUFA , Darmstadt)

*Refers to Dossier no: FAD-2013-0048

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation was Centre wallon de Recherches agronomiques (CRA-W), Gembloux, 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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- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT).
- Laboratoire de Rennes, SCL L35, Service Commun des Laboratoires, Rennes (FR).
- Państwowy Instytut Weterynaryjny, Puławy (PL).
- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)