



D08/FSQ/CVH/RL/D(2008)1697

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-2006-135  
FAD-2006-0029

Name of Additive: Probiotic LACTINA® for chickens, piglets and pigs for fattening

Active Agent(s): *Lactobacillus acidophilus* NBIMCC 8242,  
*Lactobacillus helveticus* NBIMCC 8268,  
*Lactobacillus bulgaricus* NBIMCC 8244,  
*Lactobacillus lactis* NBIMCC 8250,  
*Enterococcus faecium* NBIMCC 8270,  
*Streptococcus thermophilus* NBIMCC 8253,

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

Report prepared by: Renata Leuschner (CRL-FA)

Report revised by: Giuseppe Simone, Piotr Robouch, Renata Leuschner (CRL-FA)

Date: 14/01/2008

Report approved by: Christoph von Holst (CRL-FA)

Date: 15/01/2008

## EXECUTIVE SUMMARY

In the current application authorisation is sought for the microbial feed additive Probiotic LACTINA® under the category 'zootechnical additives', functional group 'gut flora stabilisers' according to Annex I of Regulation (EC) No 1831/2003. Specifically, the use of Probiotic LACTINA® for chickens for fattening, piglets and pigs is requested. Probiotic LACTINA® consists of a minimum of  $5 \times 10^9$  of viable cells (colony-forming units, c.f.u.) of lactic acid bacteria (LAB) per gram which comprise six strains as active agents, *Lactobacillus acidophilus* NBIMCC 8242, *Lactobacillus helveticus* NBIMCC 8269, *Lactobacillus bulgaricus* NBIMCC 8244, *Lactobacillus lactis* NBIMCC 8250, *Streptococcus thermophilus* NBIMCC 8253, *Enterococcus faecium* NBIMCC 8270. The feed additive is intended to be mixed into complete feedingstuffs at final concentrations of  $5 \times 10^8$  to  $9 \times 10^9$  c.f.u./kg for chickens for fattening, of  $5 \times 10^9$  to  $1 \times 10^{10}$  c.f.u./kg for piglets and of  $9 \times 10^8$  to  $5 \times 10^9$  c.f.u./kg for pigs.

For the determination of the active agents (LAB), in the *feed additive*, identification and control methods for lactic acid bacteria monocultures in accordance to International Dairy Federation Standard Methods IDF 146:1991 and IDF 149A:1997 are used by the applicant. For enumeration of the active agents de Man, Rogosa, Sharp (MRS) agar is used whereby for *Streptococcus thermophilus* M17 agar is suggested. The incubation temperature used is 37 °C. These methods are considered appropriate. ISO 4833 is used for the enumeration of the active agents in *premixtures* and *feedingstuffs*.

For official controls of the active agents (LAB) in the *feed additive*, *premixtures* and *feedingstuffs* a spread plate method using MRS agar is suggested by the CRL-FA. The enumeration method was validated in a collaborative study [Food Microbiol., (2003), 20, 57-66]. The method's performance characteristics of the enumeration method using MRS, acidified MRS or MRS supplemented with triphenyl tetrazolium chloride (TTC) agar and an incubation temperature of 37 °C revealed standard deviations for repeatability ( $s_r$ ) and reproducibility ( $s_R$ ) of around  $0.10 - 0.26 \log_{10}$  and  $0.18 - 0.39 \log_{10}$  calculated from the base 10 logarithms of the measured c.f.u./g in feedingstuffs, respectively.

The limit of quantification (LOQ) of this method is 100 colony forming units (c.f.u) per gram (g) feed additive or premixture and 10000 c.f.u./g feedingstuff. These performance characteristics are considered acceptable.

For identification of the active agents, methods suitable for the purpose of analysis were used by the applicant. For official controls pulsed-field gel electrophoresis (PFGE) is

recommended in principle, however - as the concentrations for individual strains in the product are not provided - it may not be applicable.

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

## KEYWORDS

Probiotic LACTINA<sup>®</sup>, zootechnical, *Lactobacillus acidophilus*, *Lactobacillus helveticus*, *Lactobacillus bulgaricus*, *Lactobacillus lactis*, *Streptococcus thermophilus*, *Enterococcus faecium*

## 1. BACKGROUND

Probiotic LACTINA<sup>®</sup> is a feed additive for which authorisation is sought under the category 'zootechnical additives', functional group 'gut flora stabilisers' according to Annex I of Regulation (EC) No 1831/2003. Probiotic LACTINA<sup>®</sup> is provided in form of a powder containing at least  $5 \times 10^9$  colony forming units (c.f.u.)/g of *Lactobacillus acidophilus* NBIMCC 8242, *Lactobacillus helveticus* NBIMCC 8269, *Lactobacillus bulgaricus* NBIMCC 8244, *Lactobacillus lactis* NBIMCC 8250, *Streptococcus thermophilus* NBIMCC 8253, *Enterococcus faecium* NBIMCC 8270 as active agents. The strains are deposited at the Bulgarian National Bank of Industrial Microorganisms and Cell Cultures (NBIMCC) in Sofia, Bulgaria. The intended use of the current application is chickens for fattening, pigs and piglets, by mixing the feed additive into complete feedingstuffs at a final concentration of  $5 \times 10^8$  to  $9 \times 10^9$  c.f.u./kg for chickens for fattening, of  $5 \times 10^9$  to  $1 \times 10^{10}$  c.f.u./kg for piglets and of  $9 \times 10^8$  to  $5 \times 10^9$  c.f.u./kg for pigs c.f.u./kg [1, 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 Probiotic

LACTINA<sup>®</sup> dossier (EFSA-Q-2006-135) and their suitability to be used for official controls in the frame of the authorisation, were evaluated.

### 3. 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 methods for determination of the quantitative composition of the active agents in the additive are provided by the applicant. The applicant uses a method for enumeration of the active agents in the additive which is based on the International Dairy Federation (IDF) 146:1991 and IDF 149:1997 Standards [3, 4]. The applicant propose MRS medium for all active agents with an exception of M17 medium for *Streptococcus thermophilus* in combination with an incubation temperature of 37 °C. The number of viable microorganisms is given in colony forming units (c.f.u.) per g and was provided by the applicant. The proposed methods are suitable for the intended purpose. However, a fully ring-trial validated method is recommended for official controls in the frame of the authorisation [5]. MRS medium is used in the validated method which is considered appropriate for all active agents in the feed additive including *Streptococcus thermophilus* together with an incubation temperature of 37 °C. The possibility that M17 might result in slightly higher cell counts than MRS medium when used for *Streptococcus thermophilus* is considered acceptable based on reports in the literature [6]. For *Lactobacillus bulgaricus* the use of acidified MRS medium (pH 5.4) that was validated in the same study [5] might result in slightly higher counts than MRS medium according to reference [6]. A selective enumeration of enterococci in the feed additive can be carried out with a validated method using bile esculin azid (BEA) agar [7]. The applicant does not specify the concentrations of the individual strains of the active agents in the feed additive, however this method might be useful to examine the presence of that species if required.

For identification of the six lactic acid bacteria strains of *Lactobacillus acidophilus* NBIMCC 8242, *Lactobacillus helveticus* NBIMCC 8269, *Lactobacillus bulgaricus* NBIMCC 8244,

*Lactobacillus lactis* NBIMCC 8250, *Streptococcus thermophilus* NBIMCC 8253, *Enterococcus faecium* NBIMCC 8270 identity cards were established by the applicant for the first four strains, whereby one for the enterococci strains appears to be missing [8]. The identity cards represent morphological, physiological and molecular profiles of each strain. Some results were shown however the method protocols for the analysis were not provided. For official controls the use of pulsed-field gel electrophoresis (PFGE) is recommended in principal, whereby the applicant does not provide the concentrations of the strains present in the additive and therefore the method may not be applicable.

#### *Qualitative and quantitative composition of any impurities in the additive*

The applicant analyses the feed additive for microbial contaminants such as *Enterobacteriaceae*, *Salmonella* species, *Staphylococcus aureus*, yeast and moulds by using appropriate methods. Heavy metals including cadmium, mercury, copper, and lead are analysed using atomic absorption spectroscopy [9]. The methods are considered suitable for the proposed application. Internationally recognised standardised methods such as ISO/CEN standards where available are recommended for official controls in line with current EU legislation.

#### *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)*

To analyse feedingstuffs for the active agents in Probiotic LACTINA<sup>®</sup>, the applicant uses ISO 4833 [10]. The ISO method is a general method for the quantification of viable counts in food and feed.

The enumeration methods that were mentioned above [5, 7] were validated using feed matrices. The validated method for probiotic lactobacilli uses de Man, Rogosa, Sharp (MRS) medium, acidified MRS and MRS supplemented with 0.01% triphenyl tetrazolium chloride (TTC) [5]. MRS is a generally accepted medium for the enumeration of lactobacilli. Therefore it is considered suitable for the active agents *Lactobacillus acidophilus* NBIMCC 8242, *Lactobacillus helveticus* NBIMCC 8269, *Lactobacillus bulgaricus* NBIMCC 8244, *Lactobacillus lactis* NBIMCC 8250. MRS is as well suitable to enumerate *Streptococcus thermophilus* NBIMCC 8253 and *Enterococcus faecium* NBIMCC 8270 [6]. It may be

possible that slightly higher counts would be obtained when using M17 medium for *Streptococcus thermophilus* NBIMCC 8253. The applicant does not specify the concentrations of each individual strain in Probiotic LACTINA<sup>®</sup> and provides the overall concentration of lactic acid bacteria instead. Therefore, the enumeration method that was validated for feedingstuffs is recommended for official controls in the frame of the authorisation [5]. The performance characteristics of the enumeration method (using MRS, acidified MRS or MRS supplemented with triphenyl tetrazolium chloride, TTC, agar) are calculated from the base 10 logarithms of the measured c.f.u./g in feedingstuffs. The corresponding standard deviations for repeatability ( $s_r$ ) and reproducibility ( $s_R$ ) are around 0.10 – 0.26  $\log_{10}$  and 0.18 – 0.39  $\log_{10}$ , respectively. The method is recommended for official controls.

For the selective enumeration of *Enterococcus faecium* NBIMCC 8270 a second validated plate count method is recommended [7] however this would only allow the quantification of enterococci for which a concentration in Probiotic LACTINA<sup>®</sup> is not provided by the applicant. For the selective enumeration of enterococci bile esculin azide (BEA) agar is recommended. The performance characteristics of the enumeration method are standard deviations for repeatability ( $s_r$ ) and reproducibility ( $s_R$ ) of around 0.12 – 0.20  $\log_{10}$  and 0.23 – 0.41  $\log_{10}$  calculated from the base 10 logarithms of the measured c.f.u./g in feedingstuffs, respectively.

The limit of quantification (LOQ) of the enumeration methods is 100 c.f.u./g additive or premixture and 10000 c.f.u./g for feedingstuffs.

Concerning the unambiguous identification of the specific strains the applicant provides colony morphologies, fermentation spectra and molecular characteristics. Some results and analytical protocols are kept confidential on request of the applicant. For identification of the strains of the active agents suitable methods for the purpose of analysis were used by the applicant. For official controls pulsed-field gel electrophoresis (PFGE) is recommended in principle with the constraints as explained above.

#### 4. CONCLUSIONS AND RECOMMENDATIONS

The applicant provided methods for the enumeration of the active agents *Lactobacillus acidophilus* NBIMCC 8242, *Lactobacillus helveticus* NBIMCC 8269, *Lactobacillus bulgaricus* NBIMCC 8244, *Lactobacillus lactis* NBIMCC 8250, *Streptococcus thermophilus*

NBIMCC 8253, *Enterococcus faecium* NBIMCC 8270 that allow a determination of the concentration of lactic acid bacteria and some differentiation in Probiotic LACTINA<sup>®</sup>. The proposed methods are referenced in the dossier for Probiotic LACTINA<sup>®</sup> and are considered appropriate. For official controls ring-trial validated methods are recommended for the enumeration and identification of *Lactobacillus acidophilus* NBIMCC 8242, *Lactobacillus helveticus* NBIMCC 8269, *Lactobacillus bulgaricus* NBIMCC 8244, *Lactobacillus lactis* NBIMCC 8250. MRS is as well suitable to enumerate *Streptococcus thermophilus* NBIMCC 8253 and *Enterococcus faecium* NBIMCC 8270 in the feed additive, premixtures and feedingstuffs in the context of official controls.

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

Enumeration: Spread plate method using MRS agar and 37 °C as incubation temperature

## **5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL**

In accordance with the requirements of Regulation (EC) No 1831/2003, samples of the additive Probiotic LACTINA<sup>®</sup> for chickens for fattening, piglets and pigs 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] Proposal of Register entry Annex III
- [2] Technical dossier. Section II, Biological origin
- [3] IDF 146:(1997)2003. ISO 9232:2003. Yoghurt – Identification of characteristic microorganisms (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*)
- [4] IDF 149:1997. Dairy starter cultures of lactic acid bacteria – Standard of identity
- [5] Leuschner R.G.K., Bew J., Coeuret V., Vernoux J.-P., Gueguen, M. 2003. A collaborative study of a method for the enumeration of probiotic lactobacilli in animal feed. Food Microbiol., 20, 57-66

- [6] Van de Castele S., Vanheuverzwijn T., Ruysen T., Van Asche P., Swings J., Huys G. 2006. Evaluation of culture media for selective enumeration of probiotic strains of lactobacilli and bifidobacteria in combination with yoghurt or cheese starters. *Int. Dairy J.* 16, 1470-476
- [7] Leuschner R.G.K., Bew J., Domig K.J., Kneifel W. 2004. A collaborative study of a method for enumeration of probiotic enterococci in animal feed. *J. Appl. Microbiol.* 93, 781-786
- [8] Technical dossier. Section II.6 Appendix to Section II. Identity cards
- [9] EFSA Public Summary, II.5 Control methods
- [10] ISO 4833 (2003) 'Microbiology of food and animal feeding stuffs -- Horizontal method for the enumeration of microorganisms -- Colony-count technique at 30 degrees C'.

## **7. RAPPORTEUR LABORATORY**

The Rapporteur Laboratory for this evaluation was the Community Reference Laboratory for Feed Additive (CRL-FA), Geel, Belgium

## **8. ACKNOWLEDGEMENTS**

The following National Reference Laboratories contributed to this report:

- NRL – RO Praha, Motol, Czech Republik
- Thüringer Landesanstalt für Landwirtschaft, Jena, Germany
- Laboratoire de Rennes, Rennes, France
- National Veterinary Research Institute, Pulawy, Poland
- National Veterinary Institute, Ljubljana, Slovenia