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Safety and efficacy of Probion Forte[®] (*Bacillus subtilis* KCCM 10941P and *Bacillus coagulans* KCCM 11093P) as a feed additive for chickens for fattening

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Abstract

Following a request from the European Commission, the EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) was asked to deliver a scientific opinion on the safety and efficacy of Probion Forte[®] when used in feed for chickens for fattening. Probion Forte[®] is a preparation containing viable spores of a strain of *Bacillus subtilis* and *Bacillus coagulans* intended for use in feed for chickens for fattening at the minimum dose of 1×10^8 and a maximum of 2×10^8 colony-forming units (CFU)/kg feedingstuffs. In the view of the FEEDAP Panel, the identity of the bacterial strains is established as *B. subtilis* and *B. coagulans* and their susceptibility to relevant antibiotics is demonstrated. However, both bacterial strains belong to species capable of producing toxins. The absence of a toxigenic potential has not been demonstrated and, therefore, these strains do not qualify for a qualified presumption of safety (QPS) approach to safety assessment and separate studies on target animals and consumer safety are required. The data provided did not allow concluding on the safety for chickens for fattening or the consumers. Probion Forte[®] is not a skin or eye irritant but should be considered a potential respiratory sensitiser. No conclusion can be drawn on the skin sensitisation potential of the additive. In the absence of evidence that the two active agents are not toxigenic, Probion Forte[®] is considered as a hazard for those handling the additive. Probion Forte[®] is considered safe for the environment. The FEEDAP Panel is not in the position to conclude on the efficacy of Probion Forte[®] for chickens for fattening from the data provided.

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Keywords: zootechnical additive, Probion Forte[®], *Bacillus subtilis*, *Bacillus coagulans*, safety, efficacy, chickens for fattening

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Table of contents

Abstract.....	1
1. Introduction.....	4
1.1. Background and Terms of Reference.....	4
1.2. Additional information.....	4
2. Data and methodologies.....	4
2.1. Data.....	4
2.2. Methodologies.....	4
3. Assessment.....	5
3.1. Characterisation.....	5
3.1.1. Characterisation of the active agents.....	5
3.1.2. Production process and characterisation of the additive.....	5
3.1.3. Stability and homogeneity.....	6
3.1.4. Conditions of use.....	6
3.2. Safety.....	6
3.2.1. Safety for chickens for fattening.....	6
3.2.2. Safety for the consumer.....	6
3.2.3. Safety for the user.....	7
3.2.4. Safety for the environment.....	7
3.3. Efficacy for chickens for fattening.....	7
3.4. Post-market monitoring.....	8
4. Conclusions.....	8
Documentation provided to EFSA.....	8
References.....	8
Abbreviations.....	9
Annex A – Executive Summary of the Evaluation Report of the European Union Reference Laboratory for Feed Additives on the Method(s) of Analysis for Probion Forte®.....	10

1. Introduction

1.1. Background and Terms of Reference

Regulation (EC) No 1831/2003¹ establishes the rules governing the Community authorisation of additives for use in animal nutrition. In particular, Article 4(1) of that Regulation lays down that any person seeking authorisation for a feed additive or for a new use of a feed additive shall submit an application in accordance with Article 7.

The European Commission received a request from WooGene B&G Co., Ltd.² for authorisation of the product Probion Forte®, *Bacillus subtilis* KCCM 10941P and *Bacillus coagulans* KCCM 11093P, when used as a feed additive for chickens for fattening, chickens reared for laying and laying hens (category: zootechnical additive; functional group: gut flora stabiliser). During the assessment, the applicant requested a change in the species by withdrawing the request for authorisation for chickens reared for laying and laying hens.³

According to Article 7(1) of Regulation (EC) No 1831/2003, the Commission forwarded the application to the European Food Safety Authority (EFSA) as an application under Article 4(1) (authorisation of a feed additive or new use of a feed additive). The particulars and documents in support of the application were considered valid by EFSA as of 4 May 2015.

According to Article 8 of Regulation (EC) No 1831/2003, EFSA, after verifying the particulars and documents submitted by the applicant, shall undertake an assessment in order to determine whether the feed additive complies with the conditions laid down in Article 5. EFSA shall deliver an opinion on the safety for the target animals, consumer, user and the environment and on the efficacy of the product Probion Forte® (*Bacillus subtilis* KCCM 10941P and *Bacillus coagulans* KCCM 11093P), when used under the proposed conditions of use (see Section 3.1.4).

1.2. Additional information

The additive Probion Forte® is a preparation of spores of one strain of *Bacillus subtilis* and one of *Bacillus coagulans*. This product has not been previously authorised in the EU.

The species *B. subtilis* and *B. coagulans* are considered by EFSA to be suitable for the qualified presumption of safety (QPS) approach to establishing safety for the target species, consumers and the environment (EFSA, 2007; EFSA BIOHAZ Panel, 2013). This approach requires the identity of the strains to be conclusively established and evidence that the strains lack of toxigenic potential and do not show resistance to antibiotics of human and veterinary importance.

2. Data and methodologies

2.1. Data

The present assessment is based on data submitted by the applicant in the form of a technical dossier⁴ in support of the authorisation request for the use of Probion Forte® (*Bacillus subtilis* KCCM 10941P and *Bacillus coagulans* KCCM 11093P) as a feed additive. The technical dossier was prepared following the provisions of Article 7 of Regulation (EC) No 1831/2003, Regulation (EC) No 429/2008⁵ and the applicable EFSA guidance documents.

EFSA has verified the European Union Reference Laboratory (EURL) report as it relates to the methods used for the control of the active agent in animal feed. The Executive Summary of the EURL report can be found in Annex A.⁶

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of Probion Forte® is in line with the principles laid down in Regulation (EC) No 429/2008 and the relevant guidance

¹ Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. OJ L 268, 18.10.2003, p. 29.

² WooGene B&G Co. Ltd., represented in the EU by Agencja Inwestycyjno, Wojska Polskiego 65, 82 – 825, Bydgoszcz, Poland.

³ Technical dossier/Supplementary information February 2016/Administrative documents/CoIdentity.

⁴ FEED dossier reference: FAD-2014-0038.

⁵ Commission Regulation (EC) No 429/2008 of 25 April 2008 on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the preparation and the presentation of applications and the assessment and the authorisation of feed additives. OJ L 133, 22.5.2008, p. 1.

⁶ The full report is available on the EURL website: <https://ec.europa.eu/jrc/sites/jrcsh/files/finirep-fad-2014-0038-probion-forte.pdf>

documents: Guidance on zootechnical additives (EFSA FEEDAP Panel, 2012a), Technical guidance: Tolerance and efficacy studies in target animals (EFSA FEEDAP Panel, 2011), Guidance on studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012b), Guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition (EFSA FEEDAP Panel, 2014) and Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance (EFSA FEEDAP Panel 2012c).

3. Assessment

Probion Forte® is a preparation of spores of *B. subtilis* and *B. coagulans* intended for use as a feed additive to improve the performance of chickens for fattening (category: zootechnical additive; functional group: gut flora stabiliser).

3.1. Characterisation

3.1.1. Characterisation of the active agents

The *B. subtilis* strain was isolated from the Korean traditional fermented food Chung-gook-jang (ground fermented soybean paste) and is deposited in the Korean Culture Center of Microorganisms with the accession number KCCM 10941P. The *B. coagulans* strain (of non-specified origin) is deposited in the same culture collection with the accession number KCCM 11093P.⁷

The taxonomical identification of the product strains as *B. subtilis* and *B. coagulans* was established by analysis of the full sequence of the 16S rRNA gene.⁸ No methods for strain specific identification were provided.

Both strains were tested for antibiotic susceptibility using twofold broth dilutions. The battery of antibiotics tested was that recommended by EFSA (EFSA FEEDAP Panel 2012c).⁹ All minimum inhibitory concentration (MIC) values for the two strains were equal or fell below the corresponding cut-off values defined by the FEEDAP Panel. Consequently, no further investigation is necessary and the strains are considered to be susceptible to all relevant antibiotics.

A cytotoxicity study following ISO 10993-5:2009 (Biological Evaluation of Medical Devices, Part 5: Tests for *in vitro* Cytotoxicity) was provided using fibroblast cells.¹⁰ However, the Panel considered that this study is unacceptable as a means of excluding the toxigenic potential due to a series of deficiencies (e.g. unknown test item, inappropriate negative and positive controls, inappropriate cell line).

3.1.2. Production process and characterisation of the additive

The two organisms are produced separately using typical industrial media containing sucrose and starch as sugar sources, yeast extract and salts. After sporulation, the fermentation broth is concentrated and the resulting slurry is spray-dried. After drying, the spores are mixed in the appropriate proportion and blended with carriers (zeolite (25%) and diatomite (51%)).

Probion Forte® is a 1:1 mixture of the two active agents, *B. subtilis* (KCCM 10941P) and *B. coagulans* (KCCM 11093P) with a minimum content of spores of each strain of 1×10^8 colony-forming units (CFU)/g additive.

Analysis of five batches of the additive showed compliance with specifications (mean: 1.6×10^8 CFU *B. subtilis*/g and 1.3×10^8 CFU *B. coagulans*/g; range: $1.2\text{--}2.0 \times 10^8$ CFU *B. subtilis*/g and $1.2\text{--}1.4 \times 10^8$ CFU *B. coagulans*/g).¹¹

The applicant set specifications for some microbial and chemical contaminants (absence of *Salmonella*, *Shigella* and *Staphylococcus*, absence of mycotoxins, lead < 20 mg/kg, mercury < 1 mg/kg, arsenic < 5 mg/kg and chromium < 100 mg/kg).¹² Analysis of three batches showed values compliant with these specifications and that do not give rise for concerns (lead ≤ 2.33 mg/kg, arsenic ≤ 4.31 mg/kg,

⁷ Technical dossier/Supplementary information July 2016/Section II/C1/KCCM11093P.

⁸ Technical dossier/Supplementary information July 2016/Section II/C1/1,2,3_taxonomic_identifi_morphological_susceptibility_and_COA_Molecular_analysis.

⁹ Technical dossier/Supplementary information July 2016/Section II/C2/Test on susceptibility to antimicrobials.

¹⁰ Technical dossier/Supplementary information July 2016/Section II/C2/Study on Safety of Probiotic combination of *Bacillus subtilis* and Test report cytotoxicity test.

¹¹ Technical dossier/Supplementary information February 2016/Section II/Annex II_9.

¹² Technical dossier/Supplementary information February 2016/Section II/Annex II_10.

chromium < 5 mg/kg, mercury, mycotoxins, *Salmonella*, *Shigella* and *Staphylococcus* were not detected).^{13,14} The action limit set for chromium is high; however, the Panel notes that analysis of the additive the batches tested provided values that do not give rise to concerns.

The additive is described as a light red or brown powder. One batch was examined for particle size distribution by laser diffraction.¹⁵ Results showed that approximately 66% by volume of the additive consists of particles with diameters below 50 µm and 14% below 10 µm. The total aerosol mass using the National Institute of Occupational Safety and Health Test Method 0500 was determined in one batch and found to be 0.45 mg/m³. The test was performed in the mixing and packing area of the plant during one rotation which is equivalent to 8 h shift.

3.1.3. Stability and homogeneity

To study the shelf-life of Probion Forte®, three batches were stored in its commercial packaging at 25°C/60% relative humidity (RH) and 40°C/75% RH and *B. subtilis* and *B. coagulans* counts measured at 24 and 6 months, respectively.¹⁶ Individual counts of the two species remained constant (losses < 0.5 log) under both tested conditions.

Stability of three batches of Probion Forte® when mixed at the recommended dose with a minerals/vitamins premixture, mash feed and pelleted feed for chickens was tested at 25°C/60% RH for 6 months.¹⁶ No significant differences were observed in the enumeration of the two species in any of the tested materials (losses < 0.5 log). No information on the stability of the additive to pelleting was provided.

The ability of the additive (one batch) to be uniformly distributed in a minerals/vitamins premixture and a mash feed for chickens was examined.¹⁶ Analyses of individual counts of 10 subsamples showed a coefficient of variation of 2% (for both bacterial species) in premixtures and of 3% (*B. subtilis*) and 2% (*B. coagulans*) in feed.

3.1.4. Conditions of use

The additive is intended for use in feed for chickens for fattening at the minimum recommended dose 1×10^8 CFU/kg feedingstuffs (equivalent to 500 mg/kg feedingstuffs) and a maximum dose of 2×10^8 CFU/kg feedingstuffs (equivalent to 1,000 mg/kg feedingstuffs).

3.2. Safety

In the view of the FEEDAP Panel, the identity of the bacterial strains is established as *B. subtilis* and *B. coagulans* and their susceptibility to relevant antibiotics demonstrated. However, both bacterial strains belong to species capable of producing toxins. The absence of a toxigenic potential has not been demonstrated and, therefore, these strains do not qualify for a QPS approach to safety assessment and separate studies on target animals and consumer safety are required.

3.2.1. Safety for chickens for fattening

Two studies were presented to support the safety of Probion Forte® for chickens for fattening. Neither could be further considered due to inadequate reporting and flaws in the study design (i.e. in one case, no information on housing conditions of chickens was provided and the study lacked replication,¹⁷ and in the second case, no overdose was included¹⁸). Therefore, in the lack of data, the FEEDAP Panel cannot conclude on the safety of the additive for the target species.

3.2.2. Safety for the consumer

Both bacterial strains belong to species of *Bacillus* capable of producing toxins. The spores will survive passage through the gastrointestinal tract of animals and be a potential source of contamination of the food of animal origin. In the absence of evidence that the two strains are not

¹³ Technical dossier/Supplementary information February 2016/Section II/Additional information/Reply to SIn May 22.

¹⁴ Limits of detection not specified.

¹⁵ Technical dossier/Supplementary information July 2016/Section II/C4/Particle size and dusting potential.

¹⁶ Technical dossier/Supplementary information July 2016/Section II/C5/Stability and homogeneity.

¹⁷ Technical dossier/Section III and Section IV/3.1.2 and 4.1.3/Annex_IV_24_4.1.3.

¹⁸ Technical dossier/Section IV/Annex_IV_23_4.1.3.

toxigenic, and given the potential for carcass contamination, safety for the consumers cannot be established.

3.2.3. Safety for the user

A study of acute dermal irritation was performed with two batches of Probion Forte®, using three female New Zealand White rabbits and following a protocol that conformed to OECD Guideline 404.¹⁹ No reactions were seen in any of the animals after 72 h; thus, it is concluded that the additive is not a skin irritant.

A study of acute eye irritation was performed with two batches of Probion Forte®, using three male New Zealand White rabbits and following a protocol that conformed to OECD Guideline 405.²⁰ The test item did not produce any effects on the cornea, iris or conjunctiva. Therefore, the additive is not an eye irritant.

Skin sensitisation potential was not studied. Therefore, in the absence of data, no conclusion can be drawn on the skin sensitisation potential.

A fraction (66%) of the particles of the product has the potential to reach the respiratory surface of the lungs when inhaled. In the absence of evidence that the two active agents are not toxigenic and given their proteinaceous nature, the additive should be considered a hazard for those handling it in the feed production site.

Conclusions on safety for the user

Probion Forte® is not a skin or eye irritant but should be considered a potential respiratory sensitiser. No conclusion can be drawn on the skin sensitisation potential. In the absence of evidence that the two active agents are not toxigenic, Probion Forte® is considered as a hazard for those handling the additive.

3.2.4. Safety for the environment

The strains composing the additive belong to species which are ubiquitous soil saprophytes. Consequently, use of the strains in animal nutrition is not expected to measurably increase their numbers in the environment. Therefore, its use as a feed additive is considered safe for the environment.

3.3. Efficacy for chickens for fattening

Six studies were provided to support the efficacy of Probion Forte® when administered to chickens for fattening. Of them, two were not further considered since they lacked replication.^{21,22} The reporting of the other four studies did not allow the Panel to properly assess their suitability to demonstrate efficacy. For instance, relevant information on the statistical analysis performed and the raw data were missing in all cases.^{23,24} Furthermore, in two of these, there was no clear indication of replication.^{25,26} In the course of the assessment, the applicant was requested to complete the information necessary to carry out a full assessment of the studies. However, the replies provided did not address the concerns expressed. As a consequence, the FEEDAP Panel is not in the position to conclude on the efficacy of Probion Forte® for chickens for fattening.

¹⁹ Technical dossier/Supplementary information July 2016/Section III/C6/Acute dermal irritation study.

²⁰ Technical dossier/Supplementary information July 2016/Section III/C6/Acute eye irritation study.

²¹ Technical dossier/Section III and Section IV/3.1.2 or 4.1.3 Test on Tolerance for Probion Forte®/Annex_IV_24_4.1.3.

²² Technical dossier/Sections III and IV/Annexes III_3.1.2 and IV_23 and Supplementary information July 2016/C7/Study on the effectiveness of probiotic Probion Forte® and Supplementary information November 2016/Final supplementary information.

²³ Technical dossier/Section IV/Annex IV_24 and Supplementary information July 2016/Files/Technical dossier/Section IV/C8/Report Probion and Supplementary information November 2016/Final supplementary information.

²⁴ Technical dossier/Section IV/4.1.5. Effects of Probion Forte® Supplementation on Growth Performance in chickens for fattening -Growth Performance in Broiler Chickens, Annex IV_25 and Supplementary information July 2016/Files/Technical dossier/Section IV/C7/Effect on supplementation and Supplementary information November 2016/Question 4.

²⁵ Technical dossier/Section IV/4.1.1. Tests for possible use the Probion Forte® probiotic in chickens for fattening feeding, Annex_IV_22_4.1.2 and Supplementary information July 2016/Files/Technical dossier/Section IV/C7/Report 1 and Supplementary information November 2016/Annexes.

²⁶ Technical dossier/Section IV/4.1.2. Tests for possible use the Probion Forte® probiotic in chickens for fattening feeding and Annex_IV_21_4.1.1 and Supplementary information July 2016/Files/Technical dossier/Section IV/C7/Report 2 and Supplementary information November 2016/Annexes.

3.4. Post-market monitoring

The FEEDAP Panel considers that there is no need for specific requirements for a post-market monitoring plan other than those established in the Feed Hygiene Regulation²⁷ and Good Manufacturing Practice.

4. Conclusions

In the view of the FEEDAP Panel, the identity of the bacterial strains is established as *B. subtilis* and *B. coagulans* and their susceptibility to relevant antibiotics demonstrated. However, both bacterial strains belong to species capable of producing toxins. The absence of a toxigenic potential has not been demonstrated and, therefore, these strains do not qualify for a QPS approach to safety assessment and separate studies on target animals and consumer safety are required.

The data provided did not allow concluding on the safety for chickens for fattening or the consumers.

Probion Forte® is not a skin or eye irritant but should be considered a potential respiratory sensitiser. No conclusion can be drawn on the skin sensitisation potential of the additive. In the absence of evidence that the two active agents are not toxigenic, Probion Forte® is considered as a hazard for those handling the additive.

Probion Forte® is considered safe for the environment. The FEEDAP Panel is not in the position to conclude on the efficacy of Probion Forte® for chickens for fattening from the data provided.

Documentation provided to EFSA

- 1) Probion Forte® (*Bacillus subtilis* KCCM 10941P and *Bacillus coagulans* KCCM 11093P). October 2014. Submitted by WooGene B&G Co. Ltd., represented in the EU by Agencja Inwestycyjno.
- 2) Probion Forte® (*Bacillus subtilis* KCCM 10941P and *Bacillus coagulans* KCCM 11093P). Supplementary information. February 2016. Submitted by WooGene B&G Co. Ltd., represented in the EU by Agencja Inwestycyjno.
- 3) Probion Forte® (*Bacillus subtilis* KCCM 10941P and *Bacillus coagulans* KCCM 11093P). Supplementary information. July 2016. Submitted by WooGene B&G Co. Ltd., represented in the EU by Agencja Inwestycyjno.
- 4) Probion Forte® (*Bacillus subtilis* KCCM 10941P and *Bacillus coagulans* KCCM 11093P). Supplementary information. November 2016. Submitted by WooGene B&G Co. Ltd., represented in the EU by Agencja Inwestycyjno.
- 5) Evaluation report of the European Union Reference Laboratory for Feed Additives on the Methods(s) of Analysis for Probion Forte®.
- 6) Comments from Member States.

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- EFSA BIOHAZ Panel (EFSA Panel on Biological Hazards), 2013. Scientific Opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed (2013 update). EFSA Journal 2013;11(11):3449, 108 pp. doi:10.2903/j.efsa.2013.3449
- EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2011. Technical guidance: tolerance and efficacy studies in target animals. EFSA Journal 2011;9(5):2175, 15 pp. doi:10.2903/j.efsa.2011.2175
- EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2012a. Guidance for the preparation of dossiers for zootechnical additives. EFSA Journal 2012;10(1):2536, 19 pp. doi:10.2903/j.efsa.2012.2536
- EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2012b. Guidance on studies concerning the safety of use of the additive for users/workers. EFSA Journal 2012;10(1):2539, 5 pp. doi:10.2903/j.efsa.2012.2539

²⁷ Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 laying down requirements for feed hygiene. OJ L 35, 8.2.2005, p. 1.

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2012c. Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. EFSA Journal 2012;10(6):2740, 10 pp. doi:10.2903/j.efsa.2012.2740

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2014. Guidance on the assessment of the toxigenic potential of Bacillus species used in animal nutrition. EFSA Journal 2014;12(5):3665, 10 pp. doi:10.2903/j.efsa.2014.3665

Abbreviations

CFU	colony-forming unit
EURL	European Union Reference Laboratory
FEEDAP	EFSA Panel on Additives and Products or Substances used in Animal Feed
LOD	limit of detection
KCCM	Korean Culture Center of Microorganisms
MIC	minimum inhibitory concentration
OECD	Organisation for Economic Co-operation and Development
QPS	qualified presumption of safety
RH	relative humidity

Annex A – Executive Summary of the Evaluation Report of the European Union Reference Laboratory for Feed Additives on the Method(s) of Analysis for Probion Forte®

In the current application authorisation is sought under Article 4(1) for *Probion Forte*®, under the “category”/“functional group” 4(b), “zootechnical additives”/“gut flora stabilisers”, according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought for the use of the feed additive for laying hens, and chickens for fattening and reared for laying.

According to the Applicant, the product is intended to be marketed as a light red or brown powder containing at least 1×10^8 Colony Forming Units (CFU)/g of *Bacillus subtilis* (KCCM 10941P), and at least 1×10^8 CFU/g of *Bacillus coagulans* (KCCM 11093P), on a zeolite-diatomite carrier. The feed additive is intended to be used directly in feedingstuffs at a minimum/maximum content of 0.5/1.0 g/kg of Probion Forte® in feedingstuffs. Taking into account (i) the content of the microorganisms in the product and (ii) the conditions of use suggested for the product in the feed, the content of the sum of both microorganisms in feedingstuffs would range from 1×10^8 to 2×10^8 CFU/kg.

For the identification of *Bacillus subtilis* (KCCM 10941P) and *Bacillus coagulans* (KCCM 11093P), the EURL recommends for official control Pulsed Field Gel Electrophoresis (PFGE), a generally recognised standard methodology for genetic identification.

For the enumeration of the sum of *Bacillus subtilis* (KCCM 10941P) and *Bacillus coagulans* (KCCM 11093P) in the feed additive and feedingstuffs the Applicant applied a spread plate procedure based on the ISO 6887-1. The EURL identified instead the ring-trial validated spread plate method EN 15784, already evaluated by EURL in the frame of previous *Bacillus* spp. dossiers. Based on the performance characteristics available, the EURL recommends for official control this CEN method (EN 15784) for the enumeration of the sum of the two microorganisms in the feed additive and feedingstuffs.

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.