SCIENTIFIC OPINION

Scientific Opinion on the safety and efficacy of *Bacillus subtilis* PB6 (*Bacillus subtilis*) as a feed additive for chickens for fattening[1]

EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP)[2]

European Food Safety Authority (EFSA), Parma, Italy

SUMMARY

Following a request from the European Commission, the European Food Safety Authority (EFSA) was asked to deliver a scientific opinion on the safety and efficacy of the product *Bacillus subtilis* PB6 as feed additive for chickens for fattening.

Bacillus subtilis PB6 is the trade name for a feed additive based on viable spores of a strain of *Bacillus subtilis*. It is intended to be used in feed for chickens for fattening at a minimum content of 1×10^7 and a maximum content of 5×10^7 CFU/kg of complete feedingstuff.

The active agent was identified as *Bacillus subtilis*, a species that is considered by EFSA to be suitable for QPS assessment. The sensitivity to antibiotics and the absence of toxigenic potential qualified the strain for QPS status. Consequently the additive is presumed safe for chickens for fattening, the consumer and the environment. The QPS status of the additive is further supported by the results of a tolerance study and several toxicological tests.

Evidence was provided that the additive is not a skin/eye irritant and does not induce skin sensitisation. Data on the dusting potential of the additive do not give concerns of sensitisation via respiratory route. The FEEDAP Panel concludes that the additive is safe for users.

Three studies made in two European countries were provided to support the efficacy of *Bacillus subtilis* PB6. A significant improvement in one or more zootechnical parameters (final body weight, feed to gain ratio) was observed in all three studies. Therefore, the FEEDAP Panel considers that evidence of efficacy of *Bacillus subtilis* PB6 in chickens for fattening has been provided at the minimum recommended dose.

The compatibility of *Bacillus subtilis* PB6 with the coccidiostats decoquinate, diclazuril, salinomycin sodium, narasin/nicarbazin and lasalocid A sodium has been demonstrated.

[1] On request from the European Commission, Question No EFSA-Q-2008-473, adopted on 15 September 2009.

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KEY WORDS

zootechnical additive, gut flora stabiliser, *Bacillus subtilis*, chickens for fattening, efficacy, safety, coccidiostats

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BACKGROUND

Regulation (EC) No 1831/2003[3] 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 the company Kemin Europa N.V.[4] for authorisation of the product *Bacillus subtilis* PB6, to be used as a feed additive for chickens for fattening (category: zootechnical additive; functional group: gut flora stabiliser) under the conditions mentioned in Table 1.

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). EFSA received directly from the applicant the technical dossier in support of this application.[5] According to Article 8 of that Regulation, 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. The particulars and documents in support of the application were considered valid by EFSA as of 15 January 2009.

The additive *Bacillus subtilis* PB6 has not been previously authorised in the Community.

TERMS OF REFERENCE

According to Article 8 of Regulation (EC) No 1831/2003, EFSA shall determine whether the feed additive complies with the conditions laid down in Article 5. EFSA shall deliver an opinion on the efficacy and the safety for the target animal, user and consumer and the environment of the product *Bacillus subtilis* PB6 when used under the conditions described in Table 1.

ACKNOWLEDGEMENTS

The European Food Safety Authority wishes to thank the members of the Working Group on microorganisms, including Guido Rychen and Atte von Wright for the preparation of this opinion.

[3] OJ L 268, 18.10.2003, p.29
[4] Kemin Europa N.V., Toekomstlaan 42, 2200 Herentals, Belgium
[5] Dossier reference: FAD-2008-0039

Table 1: Description and conditions of use of the additive as proposed by the applicant

Additive	*Bacillus subtilis*
Registration number/EC No/No	To be assigned
Category of additive	Zootechnical additive
Functional group of additive	Gut flora stabilisers

Description			
Composition, description	**Chemical formula**	**Purity criteria**	**Method of analysis**
Preparation of *Bacillus subtilis* spores containing minimum 1 x 10^{10} CFU per gram.	N/A	Not appropriate	Enumeration spread plate method using tryptone soya agar with preheat treatment of feed samples.

Trade name	*Bacillus subtilis* PB6
Name of the holder of authorisation	Kemin Europa N.V.

Conditions of use				
Species or category of animal	**Maximum Age**	**Minimum content**	**Maximum content**	**Withdrawal period**
		CFU/kg of complete feedingstuffs		
Chickens for fattening	-	1 x 10^7	5 x 10^7	Not appropriate

Other provisions and additional requirements for the labelling	
Specific conditions or restrictions for use	Stable for pelleting up to 90°C
Specific conditions or restrictions for handling	Store in a dry place
Post market monitoring	Not appropriate
Specific conditions for use in complementary feedingstuffs	Not appropriate

Maximum Residue Limit (MRL)			
Marker residue	**Species or category of animal**	**Target tissue(s) or food products**	**Maximum content in tissues**
Not appropriate	Not appropriate	Not appropriate	Not appropriate

ASSESSMENT

1. Introduction

Bacillus subtilis PB6 is the trade name for a feed additive based on viable spores of a strain of *Bacillus subtilis*, a species which EFSA recognises as having qualified presumption of safety (QPS) status (EFSA, 2008a). The product is intended for use in chickens for fattening to improve growth and feed conversion. The applicant has requested the authorisation of this product as a zootechnical additive (functional group: gut flora stabiliser). The product and its active component have not been previously assessed or authorised for use as feed additive in the European Community.

2. Identity, characterisation and conditions of use

2.1. Characterisation of the additive

The final product is a dry, free flowing powder standardised to a minimum concentration of 1.0×10^{10} CFU of *B. subtilis* spores per gram. The variation between different batches was determined in four batches of the additive. The mean concentration was found 1.6×10^{10} CFU/g.[6]

The product is the result of the aerobic fermentation of *Bacillus subtilis* (ATCC PTA-6737) in a liquid medium containing food grade raw materials. Following fermentation the cells are recovered by centrifugation or filtration, spray dried and mixed with maltodextrin (used as carrier material).

The particle size distribution of *Bacillus subtilis* PB6 was measured by laser diffraction analysis.[7] The mean particle size was 44 µm (range of 0.1 to 250 µm) with approximately 2% by weight of product having a particle size <10 µm and potentially respirable. However, the dusting potential as determined by the Stauber-Heubach method appeared to be essentially zero.[8] Confirmatory tests (Stauber Heubach test and particle size distribution test) were performed on a different batch. The results of the Stauber Heubach test showed that the additive is nearly dust free (0.0025 g/m^3). This apparent anomaly was probably due to the hygroscopic nature of the product and the tendency to form agglomerates which prevented the release of airborne fines.[9]

Chemical impurities (heavy metals, i.e. lead, mercury and cadmium; arsenic, mycotoxins i.e. ochratoxin A, aflatoxins) were tested in three different batches (two for mycotoxins). However each batch was analysed for a different set of compounds/elements. Overall, the data provided showed that the observed results fall below concentrations that could give cause of concern.

Microbial contamination was tested on three different batches (i.e. *E. coli*, total coliforms, *Salmonella* sp., *Staphylococcus aureus*, filamentous fungi and yeasts) according to standardised methods. Results showed no microbial contamination of concern.[10]

Three batches of the additive and the carrier alone were tested for the presence of *Bacillus cereus* using a selective *B. cereus* medium (standard method ISO 7932:2004). No *B. cereus* was detected in the maltodextrin carrier (LOD, 100 CFU/g), or when aliquots of the additive were plated. This confirmed that any *B. cereus* presence was consistently below the infective dose for humans (EFSA, 2005).[11]

[6] Technical dossier/Section II
[7] Technical dossier/Section II/Annex II.4
[8] Technical dossier/Section II/Annex II.5
[9] Technical dossier/Supplementary information July 09/Annex I.8
[10] Technical dossier/Supplementary information July 09/Annexes I.4, I.5 and I.6
[11] Technical dossier/Supplementary information July 09/Annex I.7

2.2. Characterisation of the active agent

Bacillus subtilis PB6 was isolated from the intestinal tract of a chicken and has not been subjected to any genetic modification. The strain is deposited at the American Type Culture Collection with the accession number ATCC PTA-6737.[12]

Biochemical tests identified strain PB6 as *B. subtilis*. The 16S rRNA gene sequence confirmed the taxonomical identification of strain PB6 as *B. subtilis*. This strain does not harbour any plasmids.[13]

The FEEDAP Panel concludes that identity has been established and that QPS can be applied.

2.2.1. Toxigenic potential

Bacillus subtilis qualifies for QPS status provided that the absence of enterotoxic activity and emetic food poisoning toxins with surfactant activity is demonstrated.

In a first study, filtrates or cells of *Bacillus subtilis* PB6 were evaluated for the presence of toxins using immunological assays, cytotoxicity tests and PCR-based methods as suggested by SCAN (EC, 2000). The negative results with the PCR-based methods show that *Bacillus subtilis* PB6 lacks the capacity to produce haemolytic, non-haemolytic enterotoxins and cytotoxin K under the same conditions that allowed detection for a known toxigenic strain of *B. cereus*. ELISA tests for components of the haemolytic and non-haemolytic enterotoxins using commercially available assays were also negative.[14]

Culture filtrates were further subjected to cytotoxicity tests with Vero and HEp-2 cell lines. No cytotoxicity was observed. In order to test for the presence of the emetic toxin a boar semen motility test was performed. No change in sperm mobility was seen in the presence of *Bacillus subtilis* PB6, this result indicates the absence of toxins with surfactant activity.[15]

2.2.2. Antibiotic resistance

Bacillus subtilis PB6 was assessed for its susceptibility towards antibiotics currently used in human or animal medicinal practices. An *in vitro* quantitative MIC assay was conducted for the assessment of susceptibility of *Bacillus subtilis* PB6 to the battery of antibiotics included in the Technical Guidance on the update of the criteria used in the assessment of bacterial resistance to antibiotics of human and veterinary importance (EFSA, 2008b).[16]

The test was performed according to the standard method for dilution antimicrobial susceptibility for bacteria. No MIC values of any of the antimicrobials tested against *Bacillus subtilis* PB6 were identified above the breakpoints, therefore, the strain is susceptible to the tested antibiotics.

2.2.3. Genetic stability

A comparison of Pulsed Field Gel Electrophoresis DNA profiles of the master seed culture with two production batches of the additive showed no detectable differences. Profiles from *Bacillus subtilis* PB6 could, however, be differentiated from corresponding profiles from other strains of *B. subtilis* and from the closely related strains of *Bacillus amyloliquefaciens*.[17]

[12] Technical dossier/Section II/Annex II.9
[13] Technical dossier/Section II/Annex II.8
[14] Technical dossier/Annex II.12
[15] Technical dossier/Annex II.2
[16] Technical dossier/Annex II.13
[17] Technical dossier/Supplementary information July 09/Annex I.9

2.3. Stability and homogeneity

Stability of *Bacillus subtilis* PB6 (1.74 x 10^{10} CFU/g) was studied when stored at 5°C and at 25°C.[18] No statistical differences in cell counts were found over one year of storage.

Stability to pelleting was examined at different pelleting temperatures (ranging from 70 to 95 ºC).[19] Viability of *Bacillus subtilis* PB6 was not affected by the highest pelleting temperatures (90 – 95°C). Moreover, bacilli counts remained stable for three months of storage after pelleting.

Stability of *Bacillus subtilis* PB6 in premixtures was studied for a period of six months when stored at room temperature.[20] No significant decrease in bacterial counts was observed during the storage period.

The homogeneity of mixing of *Bacillus subtilis* PB6 in premixtures (containing trace elements) and mash feedingstuffs was established during the stability studies. Ten premixtures samples and ten feed samples were analysed for homogeneous distribution of *Bacillus subtilis* PB6. The coefficient of variation of samples from the premixture was on average 19%, and on average 28% in finished mash feed. This variation may be due to the hygroscopic nature of the product and/or the limitation of microbial enumeration techniques.[20]

2.4. Proposed conditions of use

The additive is intended for use in feed for chickens for fattening at a minimum content of 1 x 10^7 and a maximum content of 5 x 10^7 CFU/kg of complete feedingstuffs.

2.5. Compatibility with coccidiostats

The compatibility of *Bacillus subtilis* PB6 with various coccidiostats was studied using the stepwise approach proposed by the FEEDAP Panel (EFSA, 2008c). Three chemical coccidiostats (decoquinate, robenidine and diclazuril) and six ionophores (lasalocid A sodium, narasin, salinomycin sodium, maduramycin ammonium, monensin sodium and semduramycin sodium) and one combined product (narasin/nicarbazin) were evaluated for their minimum inhibitory concentrations (MIC) against *Bacillus subtilis* PB6.[21]

The MIC values of decoquinate and diclazuril were above four times the maximum authorised concentration in feed, therefore compatibility is assumed with these two coccidiostats. Incompatibility could not be excluded for the remaining coccidiostats.

An *in vivo* study was performed to investigate the compatibility of *Bacillus subtilis* PB6 with salinomycin sodium, narasin/nicarbazin and lasalocid A sodium.[22]

A total of 959 one-day-old male Ross 308 chickens for fattening were distributed in 30 cages (31 or 32 birds per cage) until day 14. At day 14 they were distributed into 60 cages with 15 broilers per cage, until day 42. The treatments comprised a negative control, and the same diet supplemented with *Bacillus subtilis* PB6 alone (5 x 10^7 CFU/kg) or in conjunction with either salinomycin sodium, narasin/nicarbazin or lasalocid A sodium at the maximum authorised use level (doses confirmed by analyses).

On day 31, six birds per pen (treatment) were randomly selected and killed (total 30 animals). Ileum and caeca contents of those birds were collected for *Bacillus* analysis (Table 2). The samples were

[18] Technical dossier/Annex II.15
[19] Technical dossier/Annex II.17
[20] Technical dossier/Annexes II.16
[21] Technical dossier/Supplementary information July 09/Annex I.11
[22] Technical dossier/Supplementary information April 09/Annex I.4

subjected to heat treatment to differentiate spores (heat treatment) from vegetative cells + spores (without heat treatment).

Table 2: **Recoveries of *Bacillus subtilis* PB6 from intestinal samples (log_{10} CFU/g intestinal content)**

	Ileum		Caeca
	Without heat treatment	With heat treatment	With heat treatment
Negative control	2.08[a]	2.27[b]	2.13[a]
B. subtilis PB6	3.67[b]	3.76[b]	4.03[b]
B. subtilis PB6 + salinomycin sodium (70 mg/kg)	3.75[b]	3.80[b]	3.79[b]
B. subtilis PB6 + narasin/nicarbazin (100 mg/kg)	3.79[b]	4.09[b]	3.75[b]
B. subtilis PB6 + lasalocid A sodium (125 mg/kg)	3.91[b]	3.99[b]	3.66[b]

[a, b] : Means in a column with different superscripts are statistically different (P<0.0001)

Since the differences between the counts of *Bacillus subtilis* are lower than one log, it is considered that compatibility of *Bacillus subtilis* PB6 with salinomycin, narasin/nicarbazin and lasalocid A sodium is demonstrated.

2.6. Evaluation of the analytical methods by the Community Reference Laboratory (CRL)

EFSA has verified the CRL report as it relates to the methods used for the control of the *Bacillus subtilis* PB6 in animal feed. The Executive Summary of the CRL report can be found in the Appendix A.

3. Safety

The species *Bacillus subtilis* is considered by EFSA to have QPS status. In the view of the FEEDAP Panel, the identity of the production strain and the lack of toxigenic potential are established and therefore no further assessment of safety, except for user safety, is required for the active agent. However, the dossier contains experimental data relevant to the safety assessment of the product which have been reviewed by the FEEDAP Panel and summarised below.

3.1. Safety for the target species

3.1.1. Tolerance study

A combined efficacy-tolerance trial was performed with a total of 2784 one-day-old chicks (Ross 308) distributed into four treatments (12 replicates of 58 birds per treatment).[23] A basal diet based on wheat, maize and soybean meal was supplemented with *Bacillus subtilis* PB6 at 0, 1 x 10^7 (minimum recommended dose), 5 x 10^7 (maximum recommended dose), and 5 x 10^9 (100-fold the maximum recommended dose) CFU/kg feed (confirmed by analyses). Mortality and culling were recorded throughout the experimental period. Performance of the animals was monitored on day 21, 35 and day 42. Birds were individually weighed and total feed intake was controlled by pen basis, feed to gain ratio was calculated. At the end of the rearing period the litter condition was checked visually.

On day 42, seven chickens from each treatment were slaughtered and a macroscopic evaluation was carried out. Also, fragments of ileum were collected to determine the number of coccidia oocysts.

[23] Technical Dossier/Section III/ Annexes III.1, III.2 and III.3

No adverse effects from a 100-fold overdose with the additive were observed on the performance, health or the results of the macroscopic evaluation of chickens for fattening.

3.2. Safety for the consumer

The applicant provided an *in vivo* micronucleus study in mice, an acute toxicity test in rats and a 28-day sub-chronic oral toxicity study also in rats. No adverse effects were seen in these studies.

3.3. Safety for the user

An eye irritation and dermal irritation test in rabbits was provided by the applicant in compliance with OECD guidelines 405 and 404.[24] No signs of dermal irritation were observed when 1 g of *Bacillus subtilis* PB6 (1 x 10^{10} CFU/g) was applied as a paste. No signs of eye irritation were observed when 100 µL of at 10% suspension of *Bacillus subtilis* PB6 (1 x 10^{10} CFU/g) was instilled in the eye.

No inhalation study has been provided by the applicant. However, data on dusting potential do not give concerns of sensitisation via respiratory route.

4. Efficacy

The applicant has provided three studies carried out in two European countries.

Trial 1

A total of 960 one-day-old male Ross 308 chickens were allocated to three dietary treatments (eight replicates of 40 birds per treatment) for 42 days (feeding *ad libitum*). The treatments resulted from the supplementation of a wheat-soy based diet with *Bacillus subtilis* PB6 at 0, 1 x 10^7 and 5 x 10^7 CFU /kg feed (confirmed by analyses).[25] Feed intake and body weight were determined at days 21 and 42. Health status was monitored daily. Data were analysed by ANOVA.

Birds receiving *Bacillus subtilis* PB6 showed a significant higher weight gain and improved feed to gain ratio compared to those in the control group (Table 3). Mortality was low (3.8%) and not treatment related.

Table 3: **Performance data of chickens for fattening (42 days) treated with *Bacillus subtilis* PB6**

***Bacillus subtilis* PB6 (CFU/kg)**	**Weight gain (kg/bird)**	**Feed intake (kg/bird)**	**Feed/gain (kg/kg)**
0	2.61[a]	4.70	1.80[a]
1 x 10^7	2.71[b]	4.64	1.72[b]
5 x 10^7	2.73[b]	4.61	1.69[b]

[a, b] : Means in a column with different superscripts are statistically different (P<0.05)

Trial 2

The second study was made in conjunction with the tolerance study.[26] The description of the trial can be found under the tolerance section.

[24] Technical Dossier/Section III/Annex III.10
[25] Technical Dossier/Section IV/Annex IV.1
[26] Technical Dossier/Section IV/Annex IV.4

The overall results of the trial can be found in Table 4. Mean mortality (3.1%) was not affected by treatments. Results showed that birds fed the product irrespective of the dose had a significant higher body weight compared to control birds.

Table 4: **Performance data of chickens for fattening (42 days) treated with *Bacillus subtilis* PB6**

***Bacillus subtilis* PB6 (CFU/kg)**	**Final body weight (kg)**	**Feed intake (kg/bird)**	**Feed/gain (kg/kg)**
0	2.44[a]	4.70	1.93
$1x10^7$	2.52[b]	4.77	1.91
$5x10^7$	2.49[b]	4.77	1.93
$5x10^9$	2.49[b]	4.80	1.93

[a,b] : Means in a column with different superscripts are statistically different (P<0.05)

Trial 3

A total of 960 one-day-old male Ross 308 chickens were distributed into three treatments (eight replicates of 40 chickens per pen).[27] The treatments resulted from the supplementation of a wheat-soy based diet with *Bacillus subtilis* PB6 at 0, 1 x 10^7 and 5 x 10^7 CFU/kg (confirmed by analysis). The trial lasted for 42 days. Body weight and feed intake were determined at days 21, 35 and 42. Health status was monitored daily.

Chickens receiving the additive showed a significant higher weight gain at both dosages. Feed to gain ratio was also significantly improved at 5 x 10^7 CFU/kg (Table 5). Mean mortality (3.4%) was not affected by treatments.

Table 5: **Performance data of chickens for fattening (42 days) treated with *Bacillus subtilis* PB6**

***Bacillus subtilis* PB6 (CFU/kg)**	**Weight gain (kg/bird)**	**Feed intake (kg/bird)**	**Feed/gain (kg/kg)**
0	2.69[a]	4.71	1.75[a]
1 x 10^7	2.80[b]	4.77	1.70[ab]
5 x 10^7	2.81[b]	4.74	1.69[b]

[a, b] : Means in a column with different superscripts are statistically different (P<0.05)

4.1. Conclusions on the efficacy for chickens for fattening

Efficacy of the additive has been demonstrated in three studies on zootechnical performance parameters of chickens for fattening at the lowest recommended dose.

5. Post-market monitoring

No risks associated with the use of the product are foreseen. It is considered that there is no need for specific requirements for a post-market monitoring plan other than those established in the Feed Hygiene Regulation (Regulation (EC) No 183/2005) and Good Manufacturing Practice.

[27] Technical Dossier/Section IV/Annex IV.7

CONCLUSIONS

The active agent was identified as *Bacillus subtilis*, a species that is considered by EFSA to be suitable for QPS assessment. The sensitivity to antibiotics and the absence of toxigenic potential qualified the strain for QPS status. Consequently the additive is presumed safe for chickens for fattening, the consumer and the environment. The QPS status of the additive is further supported by the results of a tolerance study and several toxicological tests.

Evidence was provided that the additive is also safe for users.

A significant improvement of zootechnical parameters was observed in three studies when chickens for fattening were fed *Bacillus subtilis* PB6 at 1 x 10^7 CFU/kg feedingstuffs. Therefore, the FEEDAP Panel considers that evidence of efficacy of *Bacillus subtilis* PB6 has been demonstrated at the minimum recommended dose.

The compatibility of *Bacillus subtilis* PB6 with the coccidiostats decoquinate, diclazuril, salinomycin sodium, narasin/nicarbazin and lasalocid A sodium has been demonstrated.

DOCUMENTATION PROVIDED TO EFSA

1. *Bacillus subtilis* PB6. July 2008. Submitted by Kemin Europa N.V., Belgium.
2. Additional information on *Bacillus subtilis* PB6 for chickens for fattening, April 2009. Submitted by Kemin Europa N.V., Belgium.
3. Additional information on *Bacillus subtilis* PB6 for chickens for fattening, July 2009. Submitted by Kemin Europa N.V., Belgium.
4. Evaluation report of the Community Reference Laboratory for Feed Additives on the methods(s) of analysis for *Bacillus subtilis*.
5. Comments from Member States received through the ScienceNet.

REFERENCES

EC (European Commission), 2000. Opinion of the Scientific Committee on animal nutrition on the safety of use of *Bacillus* species in animal nutrition http://ec.europa.eu/food/fs/sc/scan/out41_en.pdf

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APPENDIX

Executive Summary of the Evaluation Report of the Community Reference Laboratory for Feed Additives on the Method(s) of Analysis for *Bacillus subtilis* PB6

In the current application authorisation is sought for the microbial feed additive *Bacillus subtilis* ATCC PTA-6737 (*Bacillus subtilis* PB6) under the category 'zootechnical additives', functional group 'gut flora stabilisers' according to Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought for the use of *Bacillus subtilis* PB6 as a gut flora stabiliser for chickens for fattening. The feed additive has a minimum of $1x10^{10}$ colony-forming units (CFU) per gram of viable spores of *Bacillus subtilis* ATCC PTA-6737 as active agent in maltodextrin carrier. The feed additive is intended to be mixed into complete feedingstuffs at a final concentration ranging from $1x10^{7}$ to $5x10^{7}$ CFU/kg of feedingstuffs.

For the enumeration of *Bacillus subtilis* ATCC PTA-6737 in the feed additive, premixtures and feedingstuffs, the applicant proposes the draft CEN method - prEN 15784:2008 E – an internationally recognised spread plate method. This method was ring-trial validated using the premixtures and feedingstuffs samples containing *Bacillus subtilis* spores. The performance characteristics of the draft CEN method reported after logarithmic transformation of measured values (CFU) are:

- For the premixtures: (1) a standard deviation for repeatability (sr) of 0.09 $\log_{10}$ and (2) a standard deviation for between-laboratory reproducibility (sR) of 0.32 $\log_{10}$.

- For the feedingstuffs: (1) a sr of 0.07 $\log_{10}$ and (2) a sR of 0.35 $\log_{10}$.

The applicant used the above mentioned spread plate method to analyse the various matrices containing *Bacillus subtilis* ATCC PTA-6737 spores and reported the following results: (a) $1x10^{9}$ to $1.5x10^{11}$ CFU/g of feed additive; (b) $1x10^{7}$ to $1.5x10^{9}$ CFU/kg for premixtures and (c) $1x10^{7}$ to $1.5x10^{8}$ CFU/kg for feedingstuffs. The results obtained for feed additive and premixtures are considered acceptable; this method is therefore recommended for official controls for the feed additives and premixtures in the frame of the authorisation.

As regards feedingstuffs, the CRL notes that the limit of quantification reported by the applicant upon request (LOQ = $1x10^{7}$ CFU/kg feedingstuffs) is identical to the minimum dose proposed and is below the LOQ reported in the draft CEN method ($2x10^{7}$ CFU/kg). On the basis of the available information, the draft CEN method is recommended for official control of the feedingstuffs containing *Bacillus subtilis* PB6 at the dosages above the LOQ reported by CEN. Below $2x10^{7}$ CFU/kg the CRL is not able to conclude on the suitability of this method for official control purposes.

Molecular methods were used by the applicant for identification of the active agent. For official controls pulsed field gel electrophoresis (PFGE), a generally recognised standard methodology for microbial identification, is recommended.

Further testing or validation is not considered necessary.