SCIENTIFIC OPINION

EFSA Journal

ADOPTED: 12 July 2016

doi: 10.2903/j.efsa.2016.4558

Safety and efficacy of BioPlus 2B® (*Bacillus subtilis* DSM 5750 and *Bacillus licheniformis* DSM 5749) as a feed additive for sows, piglets, pigs for fattening, turkeys for fattening and calves

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

Abstract

BioPlus 2B® is a preparation of *Bacillus subtilis* and *Bacillus licheniformis* authorised for use with piglets, pigs and turkeys for fattening, sows and calves. The EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) was asked to re-evaluate the additive when used in feeds for these species and to assess a new use in water for drinking. The additive subject of the evaluation is BioPlus 2B® 10, a formulation 10-fold more concentrated than that currently authorised. The active agents have been identified as strains of *B. licheniformis* and *B. subtilis*; they are susceptible to relevant antibiotics and do not show toxigenic potential. Consequently, they meet the qualifications required by the qualified presumption of safety (QPS) approach and are presumed safe for the target species, consumers and the environment. In the absence of data, the FEEDAP Panel cannot conclude on the irritancy of the additive to skin and eyes or its dermal sensitisation. The dustiness of BioPlus 2B® 10 indicated a potential for users to be exposed via inhalation. Given the proteinaceous nature of the active agents, the additive should be considered as a potential respiratory sensitiser. BioPlus 2B® and BioPlus 2B® 10 are considered equivalent when used to deliver the same dose. BioPlus 2B® has the potential to improve performance of piglets (weaned and suckling plus weaned), pigs for fattening, sows and calves for rearing at a minimum dose of 1.3×10^9 colony forming units (CFU)/kg complete feed or when used in water for drinking at an equivalent dose of 6.5×10^8 CFU/L water. The additive has the potential to reduce mortality of piglets when used over a complete reproductive cycle of sows. No conclusion could be drawn on the efficacy of BioPlus 2B® for turkeys for fattening.

Keywords: zootechnical additive, *Bacillus subtilis* and *Bacillus licheniformis*, piglets, pigs for fattening, sows, turkeys for fattening, calves for rearing

Requestor: European Commission

Question number: EFSA-Q-2009-00680

Correspondence: feedap@efsa.europa.eu

Panel members: Gabriele Aquilina, Giovanna Azimonti, Vasileios Bampidis, Maria de Lourdes Bastos, Georges Bories, Andrew Chesson, Pier Sandro Cocconcelli, Gerhard Flachowsky, Jürgen Gropp, Boris Kolar, Maryline Kouba, Secundino López Puente, Marta López-Alonso, Alberto Mantovani, Baltasar Mayo, Fernando Ramos, Guido Rychen, Maria Saarela, Roberto Edoardo Villa, Robert John Wallace and Pieter Wester

Acknowledgements: The Panel wishes to thank the members of the Working Group on Microorganisms for the preparatory work on this scientific opinion. The Panel also wishes to thank the members of the previous WG on Microorganisms 2012–2015, including Ingrid Halle.

Suggested citation: EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2016. Scientific opinion on the safety and efficacy of BioPlus 2B® (*Bacillus subtilis* DSM 5750 and *Bacillus licheniformis* DSM 5749) as a feed additive for sows, piglets, pigs for fattening, turkeys for fattening and calves. EFSA Journal 2016;14(9):4558, 19 pp. doi:10.2903/j.efsa.2016.4558

ISSN: 1831-4732

The EFSA Journal is a publication of the European Food Safety Authority, an agency of the European Union.

Table of contents

1. Introduction

1.1. Background and Terms of Reference

Regulation (EC) No 1831/2003[1] 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. In particular, Article 10(2) of that Regulation also specifies that for existing products within the meaning of Article 10(1), an application shall be submitted in accordance with Article 7, at the latest 1 year before the expiry date of the authorisation given pursuant to Directive 70/524/EEC for additives with a limited authorisation period, and within a maximum of 7 years after the entry into force of this Regulation for additives authorised without a time limit or pursuant to Directive 82/471/EEC.

The European Commission received a request from Chr. Hansen A/S[2] for authorisation and re-evaluation of the product BioPlus 2B® (*Bacillus subtilis* DSM 5750 and *Bacillus licheniformis* DSM 5749), when used as a feed additive for piglets, pigs for fattening, sows, turkeys for fattening and calves (category: zootechnical additives; functional group: gut flora stabilisers). During the assessment, the applicant requested to extend the application period for sows from the one currently authorised ("from 2 weeks before farrowing and during the lactation") to the whole reproductive cycle.

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) and under Article 10(2) (re-evaluation of an authorised feed additive). EFSA received directly from the applicant the technical dossier in support of this application. The particulars and documents in support of the application were considered valid by EFSA as of 7 July 2010.

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 BioPlus 2B® (*Bacillus subtilis* DSM 5750 and *Bacillus licheniformis* DSM 5749), when used under the proposed conditions of use (see Section 3.1.3).

1.2. Additional information

The additive BioPlus 2B®[3] is a preparation of *B. subtilis* (DSM 5750) and *B. licheniformis* (DSM 5749). The Scientific Committee on Animal Nutrition (SCAN) issued two opinions on the use of BioPlus® 2B as a feed additive (European Commission, 1997, updated 2003, and European Commission, 2000). EFSA has issued several opinions on the compatibility of BioPlus 2B with coccidiostats (EFSA, 2003, 2005, 2006, 2007, 2008 and EFSA FEEDAP Panel, 2011a) and one opinion on the use of the additive with sows (EFSA FEEDAP Panel, 2011b).

The additive is currently authorised for use in feed for pigs for fattening and piglets,[4] sows,[5] turkeys for fattening and calves.[6] With the current application, the applicant is requesting the re-evaluation of the additive administered through feed and the new authorisation for use in water for drinking. The additive subject of the authorisation is BioPlus 2B® 10 in a formulation which is 10-fold more concentrated than the formulation currently authorised.

The bacterial species *B. licheniformis* and *B. subtilis* are considered by EFSA to be suitable for the qualified presumption of safety (QPS) approach to safety assessment (EFSA, 2007, EFSA BIOHAZ Panel 2013). This approach requires the identity of the strains to be conclusively established and

[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] Chr. Hansen A/S, 10-12 Boege Allé, 2970 Hoersholm, Denmark.
[3] The Applicant intends to market the product also under the tradename Nemix.
[4] Commission Regulation (EC) No 2148/2004 of 16 December 2004 concerning the permanent and provisional authorisations of certain additives and the authorisation of new uses of an additive already authorised in feedingstuffs. OJ L 370, 17.12.2004, p. 24.
[5] Commission Regulation (EC) No 1453/2004 of 16 August 2004 concerning the permanent authorisation of certain additives in feedingstuffs. OJ L 269, 17.8.2004, p. 3.
[6] Commission Regulation (EC) No 600/2005 of 18 April 2005 concerning a new authorisation for 10 years of a coccidiostat as an additive in feedingstuffs, the provisional authorisation of an additive and the permanent authorisation of certain additives in feedingstuffs. OJ L 99, 19.4.2005, p. 5.

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 dossier[7] in support of the authorisation request for the use of BioPlus 2B® (*B. subtilis* DSM 5750 and *B. licheniformis* DSM 5749) as a feed additive. The technical dossier was prepared following the provisions of Article 7 of Regulation (EC) No 1831/2003, Regulation (EC) No 429/2008[8] 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 agents in animal feed. The Executive Summary of the EURL report can be found in Annex A.

2.2. Methodologies

The approach followed by the EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) to assess the safety and the efficacy of BioPlus 2B® is in line with the principles laid down in Regulation (EC) No 429/2008 and the relevant guidance documents: Guidance on zootechnical additives (EFSA FEEDAP Panel, 2012a), Technical guidance: Tolerance and efficacy studies in target animals (EFSA FEEDAP Panel, 2011c), 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), Technical Guidance: microbial Studies (EFSA, 2008), and Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance (EFSA FEEDAP Panel, 2012c).

3. Assessment

The additive BioPlus 2B® is a preparation of *B. subtilis* (DSM 5750) and *B. licheniformis* (DSM 5749) intended for use with piglets (suckling and weaned), pigs for fattening, sows to have benefits in piglets, turkeys for fattening and calves for rearing (category: zootechnical additives; functional group: gut flora stabilisers).

3.1. Characterisation

3.1.1. Characterisation of the active agents

The *B. licheniformis* strain was originally isolated from soil and the *B. subtilis* strain from soy bean mash.[9] They are deposited in the Deutsche Sammlung von Mikro-organismen und Zellkulturen, under the accession numbers DSM 5749 (*B. licheniformis*) and DSM 5750 (*B. subtilis*).[10]

The identification of *B. licheniformis* DSM 5749 was achieved by analysing the partial *gyrA* and *rpoB* sequences. *B. subtilis* DSM 5750 was identified using multilocus sequence analysis comparing the partial sequences of *groEL, gyrA, polC, purH* and *rpoB*.[11] The strains have not been genetically modified. Strain-specific identification and genetic stability analysis are based on the use of pulsed field gel electrophoresis (PFGE) after cleavage with restriction enzymes used individually.[12] Using this method, the master culture is compared with the working cultures used to inoculate fermentation batches. No differences in the resultant patterns have been observed to date.

Cytotoxicity of the two strains was assessed on Vero cells using the culture supernatants in accordance to the FEEDAP Panel guidance document (EFSA FEEDAP Panel, 2014). Neither strain was

[7] FEED dossier reference: FAD-2009-0023.
[8] 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.
[9] Technical dossier/Section II.
[10] Technical dossier/Section II/Annex II.2.1.2a.
[11] Technical dossier/Section II/Supplementary information September 2012/Annex 5.
[12] Technical dossier/Section II/Supplementary information September 2012/Annex 6.

shown to be toxigenic.[13] A sperm mobility test with a water/methanol extracts did not significantly reduce the motility of spermatozoa.[14,15] Both strains are non-haemolytic when tested on blood agar plates for 72 h.[16]

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).[17] All minimum inhibitory concentration (MIC) values for the *B. subtilis* strain fell below the corresponding cut-off values defined by the FEEDAP Panel. The MIC values for *B. licheniformis* DSM 5749 were equal or fell below the corresponding cut-off values defined by the FEEDAP Panel, with the exception of streptomycin (16 vs 8 mg/L). This is within the normal variation around the mean, and thus, does not raise concerns for safety.

3.1.2. Characterisation of the additive

BioPlus 2B® 10 is a 1:1 mixture of the two active agents, *B. licheniformis* DSM 5749 and *B. subtilis* DSM 5750, with a minimum content of spores in the additive of 3.2×10^{10} colony forming units (CFU)/g. The carrier materials include calcium carbonate (92%) and 1% of kieselgur[18] as anticaking agent.[19]

Data on five batches of BioPlus 2B® 10 showed that the minimum specification was exceeded in all samples (mean value 4.2×10^{10} CFU/g, range $3.8–4.8 \times 10^{10}$ CFU/g) and that the 1:1 ratio between *B. licheniformis* and *B. subtilis* was complied with in all the analysed batches.[20]

The additive is routinely monitored for microbial and chemical contamination at various points in the manufacturing process and in the final product. Limits are set for total coliforms (< 100 CFU/g), *Escherichia coli* (< 10 CFU/g), yeasts and filamentous fungi (< 100 CFU/g), and *Salmonella* (absent in 25 g). Analysis of five batches of the additive demonstrated compliance with these limits.[21] Apparently, no specifications are set for undesirable substances but the applicant declares that levels are in accordance with legal limits, when these exist. The analysed values on three batches (mean values: aflatoxin B1 < 0.64 μg/kg, mercury 0.01 mg/kg, lead 0.44 mg/kg, cadmium 0.11 mg/kg, arsenic 0.87 mg/kg, and dioxins and polychlorinated biphenyls (PCBs): octachlorodibenzo-*p*-dioxin (OCDD) < 0.198 ng/kg, octachlorodibenzofuran (OCDF) < 0.208 ng/kg, WHO-polychlorinated dibenzodioxins/dibenzofurans-toxic equivalent (WHO-PCDD/F-TEQ) < 0.241 ng/kg and WHO-PCDD/F-PCB-TEQ < 0.255 ng/kg) do not raise concerns.[22]

One batch of BioPlus 2B® 10 was examined for particle size distribution by laser diffraction and dusting potential with a Heubach dustometer.[23] Results showed that 8.7% by volume of the additive consists of particles with diameters below 50 μm and 4.5% below 10 μm. The value for dusting potential was 6.3 g/m^3, which is considered high.

3.1.3. Manufacturing process[24]

The manufacturing process is detailed in the dossier.[25]

3.1.4. Stability and homogeneity

Most of the studies presented were performed with BioPlus 2B®, the currently authorised form. As the bacterial counts are independent of the form of the additive, these results are considered to equally apply to BioPlus 2B® 10.

[13] Technical dossier/Section II/Annex II.2.2.2c.
[14] Technical dossier/Section II/Annex II.2.2.2a.
[15] Technical dossier/Section II/Supplementary information September 2012/Toxigenic potential/Annex 3.
[16] Technical dossier/Section II/Supplementary information September 2012/Annexes 1 and 5.
[17] Technical dossier/Section II and Supplementary information September 2012/Annexes II.2-8 and 8.
[18] Currently under re-evaluation according to Article 10(2) of 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.
[19] Technical dossier/Section II and Supplementary information September 2012/Overview of Q and answers.
[20] Technical dossier/Section II/Annex II.1.3b.
[21] Technical dossier/Supplementary information April 2014/Annexes II.1.3b, II.1.4.1a and b.
[22] Technical dossier/Section II/Annex II.1.4.1.
[23] Technical dossier/Supplementary information September 2012/Annexes 3 and 4.
[24] This section has been amended following the provisions of Article 8(6) and Article 18 of Regulation (EC) No 1831/2003.
[25] Technical dossier/Section II.

3.1.4.1. Shelf life

Three studies were provided with a total of six batches of BioPlus 2B® to support the shelf life at 5, 20–25 and 37°C.[26] Total counts of bacilli and differential enumeration of the two species remained unvaried (< 0.5 log) over 36 months at temperature equal or lower than 20–25°C. The shelf life of the more concentrated form (BioPlus 2B® 10) is not expected to differ. This was confirmed by stability studies made with the concentrated cell mass (intermediate product containing 2.5×10^{11} CFU/g) which showed similar viability over time.

3.1.4.2. Stability in premixtures and feed

Stability of three batches of BioPlus 2B® when mixed with a minerals/vitamins premixture (containing choline chloride) for sows was tested at 25°C for 3 months.[27] No significant differences in the total counts of bacilli and differential enumeration of the two species were observed. An additional study with premixtures for calves at 25°C for 4 months, performed on five subsamples, confirmed the stability of the product over this period. However, none of the studies extended to 6 months.

The stability of BioPlus 2B® to pelleting conditions (75, 85 and 95°C) was tested when mixed with piglets' feed in two studies.[28] The enumeration of total counts of bacilli showed a recovery close to 80% after the thermal treatment, regardless of the process temperature. In a different study, the total counts and the differential enumeration of the two species showed a recovery higher than 87% after a pelleting at 84°C for 10 minutes.

The stability of several batches of BioPlus 2B®, when mixed with pelleted feeds for piglets, pigs for fattening, sows and poultry at the recommended dose was tested. No significant differences in the total counts of bacilli and differential enumeration of the two species were observed up to 16 months at 20–25°C.[29]

The stability of BioPlus 2B® suspended in water for drinking at 25°C was tested after 1, 2 and 7 days. Total recovery after 7 days was over 90% with both strains showing equal stability.[30]

3.1.4.3. Homogeneity

The capacity of the additive to homogeneously distribute in feed was studied using the current authorised product BioPlus 2B®. One batch of BioPlus 2B® was incorporated into a mash feed and a second batch into a feed prior to pelleting (type not specified) at the recommended dose.[31] In both cases, eight subsamples were taken from the product stream during the packaging process. Analyses of total counts showed a coefficient of variation (CV) of 9% for the mash feed and 11% for the pelleted feed.

3.1.5. Conditions of use

BioPlus 2B® 10 is intended for use in feedingstuffs and water for drinking for suckling and weaned piglets until 35 kg, pigs for fattening, sows during the whole reproductive cycle, turkeys for fattening and calves until 3 months of age. The minimum recommended dose for use in feed is 1.3×10^9 CFU/kg feed. The minimum dose proposed by the applicant for use in water for drinking is 6.4×10^8 CFU/L water for pigs, 6.3×10^8 CFU/L water for turkeys and 6.6×10^8 CFU/L water for calves.[32]

3.2. Safety

3.2.1. Safety for the target species, consumer and the environment

In the view of the FEEDAP Panel, the identity of the production strains (DSM 5750 and DSM 5749) is established as *B. licheniformis* and *B. subtilis*, respectively. Moreover, the toxigenic potential and the antibiotic resistance qualifications have been met. Therefore, they are presumed safe for the target species, consumer of products fed with the additive and the environment.

[26] Technical dossier/Section II/Annexes II_4_1a, Annexes II_4_1b and II_4_1g.
[27] Technical dossier/Section II/Annexes II_4_1c and II_4_1e.
[28] Technical dossier/Section II/ Annex II_4_1d.
[29] Technical dossier/Section II/Annexes II_4_1d and II_4_1e.
[30] Technical dossier/Supplementary information September 2012/Annex 9.
[31] Technical dossier/Section II/Annex II_4_2a.
[32] Technical dossier/Supplementary information September 2012/Annex 11.

3.2.2. Safety for the user

No data are available on skin/eye irritation or skin sensitisation for the additive BioPlus 2B® 10. A small fraction (9%) of the particles of the product has the potential to reach the respiratory surface of the lungs when inhaled and the dusting potential measured is high. Given the proteinaceous nature of the active agents, the additive should be considered to be a potential respiratory sensitiser.

3.3. Efficacy

The studies presented were performed with BioPlus 2B®, the currently authorised form. The FEEDAP Panel considers that results of the efficacy studies apply to all forms of the additive when delivering the same dose in terms of CFU/kg feed.

3.3.1. Efficacy for sows

The current authorisation of BioPlus 2B® for sows covers the period from 2 weeks before farrowing until weaning. The applicant has now requested the authorisation of BioPlus 2B® 10 in sows covering the whole reproduction cycle.

In 2011, the FEEDAP Panel already evaluated the extension of the use of the additive from 2 weeks before farrowing until weaning to the whole reproductive cycle of sows (EFSA FEEDAP Panel, 2011b). In that instance, the applicant provided five efficacy trials, one covering two reproductive cycles, two covering one reproductive cycle and two covering the period '2 weeks before farrowing and lactation'. The latter two studies were not considered because they did not cover the full period under evaluation. The aim of the three remaining studies, performed in two Member States, was to evaluate the efficacy of BioPlus 2B® on sow reproductive characteristics with special emphasis on the benefits for the piglets (weight gain and mortality). In all trials, the animals were randomly allocated to the experimental groups in order to make the reproductive and physiological status between groups as homogenous as possible. Tests on all feed batches used in the trials confirmed that the correct inclusion rate of spores was applied (1.3×10^9 CFU/kg feed). The animals were fed either a dry sow diet or a lactation diet according to the periods of the reproductive stage. In all trials, zootechnical parameters were recorded for sows (weight, feed intake) and piglets (weight, mortality). Data from each experiment were submitted to analysis of variance (ANOVA). The statistical unit was the sow for all the variables.

The FEEDAP Panel, based on the data provided, concluded the following: 'Three trials were provided covering at least one reproductive cycle. Although, one study showed reduction in piglet mortality, numbers of weaned piglets and litter weight at weaning were unaffected in all studies. Therefore, the FEEDAP Panel finds insufficient evidence of any benefit when sows are treated with BioPlus 2B® over the entire reproductive period' (EFSA FEEDAP Panel, 2011b). The results of the studies previously assessed are described in Table 1 (studies 1–3).

A new study (No 4 in Table 1) was submitted involving 46 sows (PIC 1050/C29) allocated to two treatments at the time of breeding (control and BioPlus 2B® treated animals (1.3×10^8 CFU/kg feed)) based on weight and parity.[33] Sows were individually fed standard gestating and lactating diets in meal form. The study lasted one complete cycle. Litters size was standardised by moving average sized pigs to non-study sows. Piglets did not have access to creep feed or supplemental milk during the course of the assessment. Parameters measured were: sows weight (at breeding, week 8 of gestation, at farrowing and day 18 of lactation), body condition (at breeding, week 8 of gestation, at farrowing and after 18 days) and feed intake, piglets per litter, litter weight at birth and at weaning, mortality and morbidity. Data were analysed by an ANOVA with main effects of treatment and parity. Mortality was analysed using a one-way non-parametric method.

The results of the four studies are summarised in Table 1.

[33] Technical dossier/Supplementary information September 2012/Annex 25.

Table 1: Overview of results of efficacy studies with BioPlus 2B® in sows during a full reproductive cycle

No. sows/ treatment	Dose (CFU/kg feed)	Number of piglets/litter				Litter weight		Piglet mortality(4) (%)
		Born alive	Born dead and mummified	After cross-fostering(1)	Weaned	After cross-fostering(2) (kg)	At weaning(3) (kg)	
24	0	11.9	1.6	11.9	10.2	17.4	78.1	13.8[b]
24	1.3×10^9	10.6	1.0	11.2	10.4	16.9	85.4	7.3[a]
42	0	14.0	2.5	12.3	11.6	25.6	82.0	14.6
43	1.3×10^9	14.2	1.6	12.5	12.0	26.0	81.7	9.6
35	0	12.6	3.0	11.0	10.6	23.2	64.2	15.6
35	1.3×10^9	12.0	2.6	11.0	10.5	22.2	61.5	12.1
23	0	13.2	1.1	12.7	11.0	19.0	75.6	13.5
23	1.3×10^9	12.8	1.1	12.5	11.2	19.0	75.2	10.6

CFU: colony forming units.
[a,b]: Means in a column within a given trial with different superscript letters are significantly different $p < 0.05$.
(1): At day 5 in studies 2 and 3.
(2): At birth in study 1.
(3): At 21 days in studies 3 and 4.
(4): Number of pigs dying during lactation expressed as a percentage of live births (adjusted for fostering).

The only consistent response seen was a reduction in mortality of piglets, however, this reached significance in only one study. The applicant, after having checked for homogeneity, performed a meta-analysis pooling the data of the four studies (number of piglets per litter born, born alive, stillbirth, mummified and weaned, piglet loss during lactation (after cross-fostering), mortality rate and weight of piglets at birth) taking into account the effect of the trial and the BioPlus 2B® inclusion with a non-linear link function (log function) analysis.[34] As mortality rate did not follow a normal distribution, this variable was investigated using the non-parametric Wilcoxon test. The experimental unit was the sow for all parameters. This analysis resulted in a significant decrease of mortality in the BioPlus 2B® group compared with the control (14.4% vs 10.4%, $p = 0.002$). The number of stillborn piglets (1.3 vs 1.0, $p = 0.05$) was also significantly reduced by treatment in the BioPlus 2B® group compared with the control. The other parameters were not influenced by the addition of the additive (number of piglets born: 15.0 vs 14.2; number of piglets born alive: 13.2 vs 12.8; piglet weight at birth: 1.41 vs 1.45 kg).

It can be concluded that the additive has the potential to reduce mortality of piglets when given to sows over the whole reproductive cycle at the minimum dose of 1.3×10^9 CFU/kg feed.

3.3.2. Efficacy for piglets

A total of 19 studies were submitted to demonstrate the efficacy of BioPlus 2B® in piglets. Some involved animals from birth and others from weaning.

3.3.2.1. Efficacy for suckling and weaned piglets

The first four studies were performed in three Member States and involved piglets during the suckling and post-weaning period. A fifth study[35] could not be further considered due to flaws in the statistical approach (i.e., inadequate experimental unit).

All studies shared the same experimental design with sows (Topig 40 dams in trial 1,[36] Large White × Landrace in trial 2,[37] DanBred in trial 3[38] and Large White × Landrace in trial 4[39]) allocated to two treatments to obtain homogeneous groups based on parity and body condition/weight. Sows were fed pelleted lactation feed from farrowing until weaning of piglets which was around 28 days of age in trials 1 and 3, 26 in trial 2 and 27 in trial 4. Cross-fostering was carried out within treatments and within 1 day after farrowing. Suckling piglets had access to feed without (control) or with BioPlus 2B®

[34] Technical dossier/Supplementary information September 2012/Annex 25 Metaanalysis.
[35] Technical dossier/Section IV and Supplementary information September 2012/Annexes IV_3_1: Annexes Piglet trial eff. Ref. 08 and 15-17.
[36] Technical dossier/Supplementary information January 2016/3.1.Piglets suckling + weaned/Annex_IV.3.1.15.
[37] Technical dossier/Supplementary information January 2016/3.1.Piglets suckling + weaned/Annex_IV.3.1.16.
[38] Technical dossier/Supplementary information January 2016/3.1.Piglets suckling + weaned/Annex_IV.3.1.17.
[39] Technical dossier/Supplementary information January 2016/3.1.Piglets suckling + weaned/Annex_IV.3.1.18.

at 1.3 × 10^9 CFU/kg from day 1. They continued receiving the dietary treatments after weaning for 42 days. The dose was confirmed by analysis of feed. Diets were is mash form and based on wheat/rice/fishmeal/whey protein in the suckling period and on barley/maize/wheat/rice/soybean meal in the post-weaning period. At weaning, litters were kept together with each litter in a single pen (except in study 3 where 2 litters were grouped in a single pen). The studies ended 42 days post-weaning, when piglets were approximately 67–70 days of age. Piglets were weighed at day 1 of life, at weaning and at 14, 28 and 42 days post-weaning. Morbidity and mortality were also monitored. Data were analysed using an ANOVA with the farrowing pen of one sow/litter as the experimental unit in the lactation phase and the pen (containing approximately, 13 piglets in the first three studies and 11 in the last) during the post-weaning phase. A summary of the results is presented in Table 2.

Study 1 did not show any significant result either during the suckling period or the post-weaning period. In study 2, the average daily gain (ADG) was significantly improved in BioPlus 2B® treated animals only in the post-weaning period. In study 3, growth and feed to gain ratio during the post-weaning period were significantly improved in treated piglets. In study 4, the mortality of piglets during the suckling period only was significantly reduced in treated animals without any changes in other zootechnical parameters. In the only two studies in which some performance parameters were reported (ADG and mortality in study 1, ADG in study 3) in the overall period (suckling + post-weaning), these were not significantly influenced by treatment (in study 1, ADG: control = 379 g/day vs BioPlus 2B® = 386 g/day; p = 0.498 and mortality: control = 12.4% vs BioPlus 2B® = 11.5%, p = 0.803; study 3, control = 374 g/day vs BioPlus 2B® = 396 g/day, p = 0.084).

Table 2: Overview of results of efficacy studies with BioPlus 2B® in suckling and weaned piglets

Study (duration in days)	Total number of piglets entering the study Replicates/ treatment × piglets/ replicate	BioPlus 2B® (CFU/kg feed)	Suckling period						Post-weaning period				Final weight (kg)
			Initial weight (kg)	Feed intake(3) (g/day)	Final weight (kg)	Average daily gain (g/d)	Feed: gain	Mortality and culls (%)	Feed intake (g/day)	Average daily gain (g/d)	Feed: gain	Mortality (%)	
1	624	0	1.47	82	8.08	235	0.35	6	875	495	1.79	6	27.6
(70)	24 × 13	1.3 × 10^9	1.49	81	8.09	233	0.35	6	863	504	1.72	6	28.1
2	351(1)	0	1.52	51	7.75	240	–	8	520	376[a]	1.39	5	23.6[a]
(68)	16 × 13	1.3 × 10^9	1.55	61	8.09	252		14	553	400[b]	1.38	7	24.9[b]
3	639	0	1.44	6.83	7.18	233	–	12	721	454[a]	1.59[b]	8	26.5[a]
(70)	24 × 13	1.3 × 10^9	1.47	7.50	7.43	240		12	734	484[b]	1.52[a]	4	28.0[b]
4	340(2)	0	1.62	76	7.80	232	–	18[b]	561	400	1.41	4	24.6
(69)	16 × 11	1.3 × 10^9	1.73	71	7.93	234		8[a]	556	390	1.43	4	24.3

CFU: colony forming units.
[a,b]: Means in a column within a given trial with different superscript letters are significantly different $p < 0.05$.
(1): 176 control/175 BioPlus 2B®.
(2): 171 control/169 BioPlus 2B®.
(3): In study 3, total feed intake kg/litter (sum of creep feed fed from day 1–14 and prestarter feed (mix of creep feed and milk replacer) fed from day 15 until weaning).

After having checked for homogeneity, a meta-analysis was applied to the four studies reporting only the data on weight gain at various periods from birth to 42 days post-weaning.[40] Although some benefits were observed in an intermediate period (during suckling + 28 days post-weaning), no significant effects on ADG were seen at the end of the suckling period (235 vs 239 g/day) or at 42 days post-weaning (438 vs 449 g/day) or when both periods were combined (355 vs 364 g/day).

Based on these four studies, there is insufficient evidence to conclude on the efficacy of BioPlus 2B® when supplemented at the recommended dose to piglets during the suckling and post-weaning period.

[40] Technical dossier/Supplementary information January 2016/3.1.Piglets suckling + weaned/Annex_IV.3.1.19.

3.3.2.2. Efficacy for weaned piglets

Fourteen reports of feeding trials performed in several Member States and involving piglets from weaning were submitted. However, 11 studies[41] were not considered due to insufficient reporting and/or flaws in the experimental design and analysis (e.g. insufficient duration, inappropriate production stage of animals, inadequate statistical unit). Results of the three remaining studies fulfilling the minimum requirements are described below.

The detailed design of the studies is presented in Table 3 and the results in Table 4. In all cases (studies 1[42] and 2[43] and 3[44]), piglets (same number of females and males) were distributed in two experimental groups, one receiving the basal diets not supplemented and a second receiving the basal diets supplemented with the additive in order to provide 1.3×10^9 CFU/kg feed. Intended cell counts were confirmed by analysis. In study 1, BioPlus 2B® supplementation started at least 10 days before weaning. The diets were offered to the animals *ad libitum*. Health status was monitored throughout the experimental periods (incidence of diarrhoea was monitored in study 1). Feed intake and body weight of the animals was measured and the feed to gain ratio was calculated. In all studies, an ANOVA was performed on the data obtained using the pen as the experimental unit. In study 2, the initial body weight was introduced as a covariate.

Table 3: Details on the study design for the studies performed in weaned piglets

Study	Breed (Age in days) (Sex)	Total animals Replicates/ treatment × animals/ replicate	Duration of the study (days)	Basal diets (Main ingredients) Form
1	German Landrace (23) ♀,♂	946 10 × 47/48	48	Prestarter and starter (Wheat/soybean meal/maize/fish meal/ skimmed milk powder) Pellet
2	Piétrain × ACMC (28) ♀,♂	144 12 × 6	42	Prestarter and starter (Maize/sweet dried milk whey/soybean/ barley) Mash
3	(Belgian Landrace × Hampshire) × (Large White × Landrace) (28) ♀,♂	540(a) 12 × 15–29	42	Prestarter and starter (Maize/soybean meal/wheat bran/fish meal) Mash

(a): 252 in the control group (1 replicate of 15 animals, 1 of 16, 1 of 20, 4 of 21, 1 of 22, 1 of 23 and 3 of 24) and 288 in the treated group (1 replicate of 17 animals, 1 of 18, 1 of 20, 1 of 21, 2 of 24, 1 of 26, 3 of 27, 1 of 28 and 21 of 29).

Table 4: Overview of results of efficacy studies with BioPlus 2B® in weaned piglets

Study	BioPlus 2B (CFU/kg feed)	Initial weight(1) (kg)	Final weight (kg)	Feed intake (g/d)	Average daily gain (g/d)	Feed:gain	Mortality (%)
1	0 1.3×10^9	6.5 6.4	23.8 25.0	729 731	359^a 389^b	2.03^b 1.88^a	1 2
2	0 1.3×10^9	8.2 8.2	24.1 24.7	588 613	375^a 403^b	1.54 1.52	0 3
3	0 1.3×10^9	6.9 6.9	24.0^a 25.3^b	614 605	406^a 425^b	1.50^b 1.38^a	7^b 4^a

CFU: colony forming units.
[a,b]: Means in a column within a given trial with different superscript letters are significantly different $p \leq 0.05$.
(1): In study 1, BioPlus 2B® supplementation started at least 10 days before weaning.

[41] Technical dossier/Section IV and Supplementary information September 2012/Annexes IV_3_1: Piglet trial eff. Ref. 01, Piglet trial eff. Ref. 03, Piglet trial eff. Ref. 04, Piglet trial eff. Ref. 05, Piglet trial eff. Ref. 06, Piglet trial eff. Ref. 07, Piglet trial eff. Ref. 10, Piglet trial eff. Ref. 11, Piglet trial eff. Ref. 12, Piglet trial eff. Ref. 08 and Annexes 15-17 and Piglet trial eff. Ref. 09 and 18-21.
[42] Technical dossier/Section IV and Supplementary information September 2012/Annexes IV_3_1: Piglet trial eff. Ref. 02 and 12-14.
[43] Technical dossier/Supplementary information September 2012/Annex 22.
[44] Technical dossier/Supplementary information September 2012/Annex 23.

Weight gain was consistently increased and feed to gain ratio improved in animals receiving feed supplemented with BioPlus 2B® in three or two studies, respectively. No other parameter was influenced by treatment, except mortality in study 3 that was significantly reduced in the BioPlus 2B® group compared with the control.

It can be concluded that supplementation of BioPlus 2B® at the recommended dose has the potential to improve performance of weaned piglets.

In addition, taking account of the studies in which BioPlus 2B® was provided during both suckling and weaned periods, and the results of study 1 above in which BioPlus 2B® was provided 10 days prior to weaning, the FEEDAP concludes that the additive has the potential to improve weight gain in piglets from birth to 42 days post-weaning.

3.3.3. Efficacy for pigs for fattening

Reports of 13 feeding trials performed to investigate the efficacy of BioPlus 2B® with pigs for fattening were submitted. Of these, five studies[45] were not considered due to flaws in the experimental design (e.g. inadequate period of application of the additive, insufficient duration). A further study[46] could not be considered because of extensive veterinary interventions throughout the trial, denoting poor health of animals. After having tested for homogeneity, the data from eight studies were pooled together in a meta-analysis.[47] However, this could not be considered as it included the study excluded because of the veterinary interventions.

The designs of the remaining seven studies are presented in Table 5 and the results in Table 6. All studies had two experimental groups, one receiving the basal diets not supplemented and a second receiving the basal diets supplemented with the additive in order to provide 1.3×10^9 CFU/kg feed. Intended cell counts were confirmed by analysis of feed. Animals were distributed in pens so as to have a homogeneous distribution based on body weights and gender. In all cases, there were the same numbers of males and females, with mixed pens in studies 1, 3, 4 and single sex pens in studies 2 (eight pens per sex and treatment), 5 and 6 (six pens per sex and treatment) and 7 (29 pens of males and 15 of females in the control group and 28 pens of males and 18 of females in the treatment group). The diets were offered to the animals *ad libitum*. Health status was monitored throughout the experimental periods. Feed intake and body weight of the animals were measured and the feed to gain ratio was calculated. Morbidity and mortality were also monitored. In study 6, faecal scores were visually measured and days in diarrhoea monitored. In all studies, data were analysed using an ANOVA in a completely randomised block design using the pen as the experimental unit and with initial body weight as a covariate in studies 1, 2, 3 and 5. In study 4, a one way-ANOVA and the Duncan's test were applied to the data using the pen as experimental unit.

Table 5: Details on the study design for the studies performed in pigs for fattening

Study	Breed (sex)	Total animals Replicates/ treatment × animals/replicate	Duration of the study (days)	Basal diets (Main ingredients) Form
1[(a)]	Dan Breed × Duroc ♀,♂	96 24 × 2	81	Starter and finisher (Wheat/barley/maize/soybean meal) Pelleted
2[(b)]	Piétrain × ACMC ♀,♂	384 16 × 12	70	Grower and finisher (Barley/wheat/soy/maize) Mash and pelleted
3[(c)]	(Swedish Yorkshire sires × Swedish Yorkshire dams) × (Swedish Yorkshire dams × Hampshire sires) ♀,♂	294 17 × 7–10	70	Grower and finisher (Wheat/barley/wheat bran/ rapeseed cake/soya bean meal) Pelleted

[45] Technical dossier/Section IV, Supplementary information September 2012 and Supplementary information April 14/Annexes IV_3_2. Pigs for fat. eff. Ref 1, Pigs for fat. trial eff. Ref. 2, Pigs for fat. trial eff. Ref. 3, Annex 24 and IV.3.2.4.
[46] Technical dossier/Supplementary information January 2016/3.2.Pigs for fattening/Annex IV.3.2.09.
[47] Technical dossier/Supplementary information January 2016/3.2.Pigs for fattening/Annex_IV.3.2.15_Meta-analysis.

Study	Breed (sex)	Total animals Replicates/ treatment × animals/replicate	Duration of the study (days)	Basal diets (Main ingredients) Form
4(d)	PIC ♀,♂	308 6 × 26–28	80	Grower and finisher (Triticale/barley/soybean meal) Mash
5(e)	Topigs 20 × Tempo ♀,♂	96 12 × 4	96	Grower and finisher (Wheat/barley/soya/maize/ rapeseed) Mash
6(f)	Large White × Landrace ♀,♂	144 12 × 6	115	Grower and finisher (Wheat/triticale/soy/sunflower/ rapeseed) Mash
7(g)	Topigs Talent × Topigs 40 ♀,♂	1,980 44/46(h) × 22	112	Grower and finisher (Maize/soybean/sunflower/wheat/ fish meal) Pelleted

(a): Technical dossier/Supplementary information April 2014 and January 2016/Annexes Sect.IV Efficacy/Annexes IV.3.2.5 and IV.3.2.13.
(b): Technical dossier/Supplementary information April 14/Annexes Sect.IV Efficacy/Annex IV.3.2.6.
(c): Technical dossier/Supplementary information April 14/Annexes Sect.IV Efficacy/Annex IV.3.2.7.
(d): Technical dossier/Supplementary information April 14/Annexes Sect.IV Efficacy/Annex IV.3.2.8.
(e): Technical dossier/Supplementary information January 2016/3.2.Pigs for fattening/Annex IV.3.2.10.
(f): Technical dossier/Supplementary information January 2016/3.2.Pigs for fattening/Annex IV.3.2.11.
(g): Technical dossier/Supplementary information January 2016/3.2.Pigs for fattening/Annex IV.3.2.12.
(h): 44 in the control and 46 in the treated group.

Table 6: Overview of results of efficacy studies with BioPlus 2B® in pigs for fattening

Study	BioPlus 2B® (CFU/kg feed)	Initial weight (kg)	Final weight (kg)	Total feed intake (kg/day)	Average daily gain (kg/d)	Feed:gain	Mortality and removal (n)
1	0	29.3	111.2	2.54	1.02	2.50	0
	1.3×10^9	28.3	112.0	2.52	1.04	2.44	0
2	0	29.7	76.4	1.54	0.67	2.31	1
	1.3×10^9	29.7	77.4	1.51	0.68	2.22	4
3	0	32.6	107.3	2.63	1.07	2.34	3
	1.3×10^9	32.2	107.5	2.65	1.09	2.33	1
4	0	30.8	100.2^a	2.39	0.87^a	2.79^a	4
	1.3×10^9	30.7	101.6^b	2.37	0.89^b	2.69^b	1
5	0	23.0	111.4^a	2.18^a	0.92^a	2.37	0
	1.3×10^9	23.0	117.0^b	2.32^b	0.98^b	2.37	0
6	0	24.3	114.6^a	2.70	0.79^a	3.45^a	0
	1.3×10^9	24.3	121.1^b	2.73	0.84^b	3.25^b	0
7	0	28.2	110.7^a	2.04	0.73^a	2.78^a	2.4
	1.3×10^9	27.8	113.6^b	2.03	0.76^b	2.66^b	2.1

CFU: colony forming units.
[a,b]: Means in a column within a given trial with different superscript letters are significantly different $p < 0.05$.

The supplementation of the additive at the minimum recommended dose consistently led to an increased weight gain and a better feed to gain ratio in the BioPlus 2B® group compared with the control, reaching a statistical significance in four or three studies, respectively. No other parameter was influenced by treatment.

It can be concluded that supplementation of BioPlus 2B® at the recommended dose has the potential to improve performance of pigs for fattening.

3.3.4. Efficacy for turkeys for fattening

Seven trials were performed in six Member States aiming at investigating the effects of BioPlus 2B® supplementation to turkeys for fattening.

The designs of the studies are presented in Table 7 and the results in Table 8. The studies shared a similar experimental design in which 1-day-old birds were fed standard diets *ad libitum* in 3–5 consecutive phases. In all cases, studies included two experimental groups, one receiving the basal diets not supplemented and a second receiving the basal diets supplemented with the additive in order to provide 1.3×10^9 CFU/kg feed (doses were confirmed by analysis). The parameters monitored were: individual weight, feed intake (per pen), morbidity and mortality. Weight gain and feed to gain ratio (per pen) were calculated thereof. In study 5, some carcass indicators (based on 72 birds, 36 per treatment) were also monitored, and in study 6, faecal analyses (dry matter) were made. Data were analysed using an ANOVA including treatment and replication as factors and considering the pen as experimental unit for all parameters.

In two of the seven studies, there was some evidence of a positive effect of the inclusion of BioPlus 2B® at the minimum recommended dose of 1.3×10^9 CFU/kg feed on growth or feed to gain ratio. No other parameter was influenced by treatment other than some traits in the carcasses quality in study 5 (e.g. thigh weight was increased and abdominal fat reduced in the BioPlus 2B® group).

Table 7: Details on the study design for the studies performed in turkeys for fattening

Study	Breed (Sex)	Total animals Replicates/ treatment × animals/replicate	Duration of the study (days)	Basal diets (Main ingredients) Form
1[(a)]	BIG 6 ♂	600 6 × 50	112	(Wheat/maize/soybean groats) Pelleted
2[(b)]	BUT 6 ♂	1,200 3 × 200	119	(Maize/wheat/soybean/fish meal) Pelleted Contain coccidiostat
3a[(c)] 3b	BUT 9 ♀ ♂	324 6 × 27 240 6 × 20	84 105	(Soybean meal/wheat/maize/field peas) Pelleted Contain coccidiostat
4[(d)]	BUT 9 ♀	720 10 × 36	96	(Wheat/soy beans/maize) Mash and pelleted Contain coccidiostat
5[(e)]	BUT 9 ♀	900 18 × 25	105	(Maize/soybean meal/wheat) Pelleted
6[(f)]	BIG 6 ♂	497[(h)] 10 × 16–33	112	(Wheat/maize/soybean meal) Pelleted Contain coccidiostat
7[(g)]	Not specified ♀	300 10 × 15	84	(Wheat/soybean meal/sunflower meal) Pelleted

(a): Technical dossier/Section IV and Supplementary information January 2016/IV_3_4_Ref. 1 and Annex no. IV.3.4.9.
(b): Technical dossier/Section IV and Supplementary information January 2016/Annex and IV_3_4_Ref. 2 and 1.Report Stat.analysis_T2.
(c): Technical dossier/Section IV and Supplementary information January 2016/IV_3_4_Ref. 3 and Annex no. IV.3.4.10.
(d): Technical dossier/Section IV/IV_3_4_Ref. 4.
(e): Technical dossier/Section IV and Supplementary information January 2016/IV_3_4_Ref. 5 and Annex no. IV.3.4.11.
(f): Technical dossier/Section IV and Supplementary information January 2016/IV_3_4_Ref. 6 and Annex no. IV.3.4.12.
(g): Technical dossier/Supplementary information April 2014/Sect.IV Efficacy/Annex IV.3.4.7 Turkey/1.Report turkeys.
(h): 252 in control and 245 in the treated group. Pens are: 1 of 16 animals, 1 of 20, 2 of 21, 1 of 22, 1 of 29, 2 of 30, 1 of 31 and 1 of 32 in the control and 1 of 19, 1 of 20, 2 of 21, 2 of 22, 2 of 29 and 2 of 31 in the treated group.

Table 8: Overview of results of efficacy studies with BioPlus 2B® in turkeys for fattening

Study	BioPlus 2B® (CFU/kg feed)	Final body weight (kg)	Feed intake (g/day)	Weight gain (g/day)	Feed:gain	Mortality(1) (%)
1	0	14.1[a]	344	125[a]	2.74[b]	4.0
	1.3×10^9	14.9[b]	341	131[b]	2.58[a]	4.3
2	0	15.9	332	133	2.32	3.2
	1.3×10^9	16.1	331	134	2.28	4.5
3a	0	6.8	180	79	2.29	3.7
	1.3×10^9	6.8	179	79	2.26	4.9
3b	0	12.5	268	117	2.29	4.2
	1.3×10^9	12.6	275	120	2.29	5.0
4	0	9.0	191	93	2.05	6.1
	1.3×10^9	9.1	190	94	2.02	4.4
5	0	8.9	286	84	3.48	2.2
	1.3×10^9	9.0	290	85	3.50	1.3
6	0	13.9	320	123	2.60	3.9
	1.3×10^9	14.1	316	124	2.53	3.8
7	0	7.4[a]	173	87[a]	1.94	6.7
	1.3×10^9	7.7[b]	177	91[b]	1.90	8.0

CFU: colony forming units.
[a,b]: Means in a column within a given trial with different superscript letters are significantly different $p < 0.05$.
(1): Including birds culled in studies 1, 4 and 7.

The applicant provided three different meta-analyses: the first was performed on the data of the female turkeys (studies 3a, 4, 5 and 7), the second on the data of the male turkeys (studies 3b, 4, 5 and 7) and the third one on the data of all seven studies.[48] The last one could not be considered as the weight of the animals at 84 days of age (the only parameter tested) had not be measured in two of the studies but determined by calculation, using the daily gain from the final period (ADG days 79–105, study 3b) or an intermediate period (ADG days 63–91, study 2). Data were pooled and analysed statistically; differences were considered significant at a level of at least $p < 0.05$. An ANOVA was performed using the pen as experimental unit. The models included the effect of the treatment, trial location (and pen) and the gender (where relevant). The interaction between treatment and trial location was also investigated and found to be not significant. The model for females considered also the effect of the initial body weight of the birds.

In females, a significantly greater weight gain was found in BioPlus 2B® treated animals. In males, no significant differences were detected between groups.

Overall, there is insufficient evidence to conclude on the efficacy of BioPlus 2B® for turkeys for fattening.

3.3.5. Efficacy for calves for rearing

Three studies were performed in three Member States to investigate the effects of BioPlus 2B® on the performance of calves.

In all cases, calves (Holstein Friesian, 12–25-days-old) were allocated to two treatments (control and BioPlus 2B®). Animals were housed in individual boxes in study 1 (with groups of 5–6 animals sharing a common trough for solid feed), individually in study 2 and in four boxes of 13 calves each in study 3 (Table 9). Study 1 lasted for 9 weeks, and studies 2 and 3 lasted for 8 weeks. BioPlus 2B® was administered via the milk replacer and concentrate (each at 1.3×10^9 CFU/kg feed, doses confirmed by analysis). The parameters monitored were initial and final individual weights, mortality and morbidity (including incidence of diarrhoea). Average daily weight gain was calculated. Milk replacer intake was recorded individually in all the studies. The intake of solid feed (concentrate and forage if fed to the calves) was monitored individually in studies 2 and 3 and per group (sharing a trough) in study 1. Data were analysed using an ANOVA in a randomised block design and considering the calf as experimental unit in studies 2 and 3 and the replicate in study 1. In the last study, the initial weight was used as covariate. Concentrate, hay and maize silage intake data were not statistically analysed in study 1, concentrate intake was not statistically analysed in study 3. The results of the studies are summarized in Table 10.

[48] Technical dossier/Supplementary information January 2016/3.4 Turkeys/New statistical analysis.

Table 9: Details on the study design for the studies performed in calves for rearing

Study	Breed (Sex)	Total animals Replicates/ treatment × animals/ replicate	Duration (days)	Feeding regime	Parameters monitored
1[(a)]	Holstein Friesian ♂	62 6 × 5–6[(d)]	63	Milk replacer (125 g/L, av. 5.2 L/calf per day) and from the second week also maize silage, concentrate and lucerne hay	Individual weight, individual milk replacer and solid feed intake per replicate (silage, hay and concentrate)
2[(b)]	Holstein Friesian ♀	64 32 × 1	56	Milk replacer (100 g/L, 4.5 L/calf per day) twice a day and starter concentrate	Individual weight, individual milk replacer and solid feed intake
3[(c)]	Holstein Friesian ♂	52 26 × 1	56	Milk replacer (126 g/L, av. 5.4 L/calf per day) twice a day and starter concentrate. Grass silage *ad libitum*	Individual weight (at the beginning and end and at two intermediate times), individual feed intake (milk replacer and concentrate only)

(a): Technical dossier/Section IV/IV_3_5_Ref. 1.
(b): Technical dossier/Section IV/IV_3_5_Ref. 2.
(c): Technical dossier/Section IV/IV_3_5_Ref. 3.
(d): Five boxes with five animals and one with six animals.

Table 10: Overview of results of efficacy studies with BioPlus 2B® in calves for rearing

Study	BioPlus 2B® (CFU/kg milk replacer and concentrate)	Initial weight (kg)	Final weight (kg)[(1)]	Daily weight gain (g/d)
1	0	45.4	82.0[a]	581[a]
	1.3×10^9	45.3	87.7[b]	674[b]
2	0	42.3	76.8[a]	617[a]
	1.3×10^9	42.5	79.7[b]	664[b]
3	0	47.3	77.5	538[a]
	1.3×10^9	47.4	84.4	659[b]

CFU: colony forming units.
[a,b]: Means in a column within a given trial with different superscript letters are significantly different $p < 0.05$.
(1): Not statistically analysed in study 3.

Final weight and weight gain for the overall period were improved in the BioPlus 2B® group in all studies; the differences in weight gain reaching significance in two studies. No other parameter was influenced by treatment.

It can be concluded that supplementation of BioPlus 2B® at the recommended dose has the potential to increase the growth rate of calves for rearing.

3.3.6. Efficacy in water

Based on a water to feed ratio of two, the dose of 6.5×10^8 CFU/L water would provide essentially the same exposure as the dose currently authorised for use in feedingstuffs. Consequently, the conclusions on efficacy of the additive when used in feedingstuffs also apply to use in water for drinking for weaned piglets, pigs for fattening, sows and turkeys for fattening and calves.

3.3.7. Conclusions on efficacy

BioPlus 2B® has the potential to improve the performance of piglets from birth to the end of weaning and during the weaning period alone. The additive also has the potential to improve performance of pigs for fattening and calves for rearing when supplemented at a minimum dose of 1.3×10^9 CFU/kg complete feed (approximately 6.5×10^8 CFU/L water for drinking). The data provided in sows show that the additive used over a complete reproductive cycle has the potential to reduce mortality of piglets when supplemented at a minimum dose of 1.3×10^9 CFU/kg feed (approximately 6.5×10^8 CFU/L water for drinking).

The FEEDAP Panel was unable to conclude on the efficacy of BioPlus 2B® for turkeys for fattening.

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 Regulation[49] and Good Manufacturing Practice.

4. Conclusions

The active agents of BioPlus 2B® 10 have been identified as strains of *B. licheniformis* and *B. subtilis*; they are susceptible to clinically relevant antibiotics and do not show toxigenic potential. Consequently, they meet the qualifications required by the QPS approach and can be presumed safe for the target species, consumers of products derived from animals fed the additive and the environment.

In the absence of data, the FEEDAP Panel cannot conclude on the irritancy of the additive to skin and eyes or its dermal sensitisation. The particle size and dustiness of BioPlus 2B® 10 indicated a potential for users to be exposed via inhalation. Given the proteinaceous nature of the active agents, the additive should be considered as a potential respiratory sensitiser.

BioPlus 2B® and BioPlus 2B® 10 are considered equivalent when used to deliver the same dose. BioPlus 2B® has the potential to improve performance of piglets (weaned and suckling plus weaned), pigs for fattening, sows and calves for rearing at a minimum dose of 1.3×10^9 CFU/kg complete feed or when used in water for drinking at an equivalent dose (6.5×10^8 CFU/L water). The data provided in sows show that the additive has the potential to reduce mortality of suckling piglets when used over a complete reproductive cycle. The FEEDAP Panel was unable to conclude on the efficacy of BioPlus 2B® for turkeys for fattening.

Documentation provided to EFSA

1) BioPlus 2B® (*Bacillus licheniformis* DSM5749 and *Bacillus subtilis* DSM5750). Zootechnical feed additive for piglets, pigs for fattening, sows, turkeys for fattening and calves. May 2009. Submitted by Chr. Hansen A/S.
2) BioPlus 2B® (*Bacillus licheniformis* DSM5749 and *Bacillus subtilis* DSM5750). Zootechnical feed additive for piglets, pigs for fattening, sows, turkeys for fattening and calves. Supplementary information. September 2012. Submitted by Chr. Hansen A/S.
3) BioPlus 2B® (*Bacillus licheniformis* DSM5749 and *Bacillus subtilis* DSM5750). Zootechnical feed additive for piglets, pigs for fattening, sows, turkeys for fattening and calves. Supplementary information. March 2014. Submitted by Chr. Hansen A/S.
4) BioPlus 2B® (*Bacillus licheniformis* DSM5749 and *Bacillus subtilis* DSM5750). Zootechnical feed additive for piglets, pigs for fattening, sows, turkeys for fattening and calves. Supplementary information. January 2016. Submitted by Chr. Hansen A/S.
5) Evaluation report of the Community Reference Laboratory for Feed Additives on the methods (s) of analysis for BioPlus 2B.
6) Comments from Member States received through the ScienceNet.

References

EFSA (European Food Safety Authority), 2003. Scientific Opinion on request from the Commission on micro-organism product "BioPlus 2B", authorised as a feed additive in accordance with Council Directive 70/524/EEC. EFSA Journal 2003;1(12):008, 4 pp.

EFSA (European Food Safety Authority), 2005. Scientific Opinion on the modification of terms of authorisation of the micro-organism product *Bacillus licheniformis* (DSM 5749) and *Bacillus subtilis* (DSM 5750) (BioPlus 2B) authorised as a feed additive in accordance with Council Directive 70/524/EEC. EFSA Journal 2005;3(10):272, 8 pp. doi:10.2903/j.efsa.2005.272

EFSA (European Food Safety Authority), 2006. Scientific Opinion on the modification of terms of authorisation of the micro-organism preparation of Bacillus licheniformis and Bacillus subtilis (BioPlus 2B) authorised as a feed additive in accordance with Council Directive 70/524/EEC. Compatibility with the coccidiostat maduramicin ammonium. EFSA Journal 2006;4(7):380, 6 pp. doi:10.2903/j.efsa.2006.380

[49] Regulation (EC) No 183/2005 of the European Parliament and of the Council of 12 January 2005 laying down requirements for feed hygiene. OJ L 35, 8.2.2005, p. 1.

EFSA (European Food Safety Authority), 2007. Scientific Opinion on the compatibility of the microbial preparation *Bacillus licheniformis* and *Bacillus subtilis* (BioPlus 2B) with the coccidiostat lasalocid A sodium in feed for turkeys. EFSA Journal 2007;5(10):573, 5 pp. doi:10.2903/j.efsa.2007.573

EFSA (European Food Safety Authority), 2008. Scientific Opinion on the compatibility of the microbial product BioPlus 2B (*Bacillus licheniformis* and *Bacillus subtilis*) with lasalocid sodium. EFSA Journal 2008;6(11):841, 7 pp. doi:10.2903/j.efsa.2008.841

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), 2011a. Scientific Opinion on the compatibility of BioPlus® 2B (*Bacillus licheniformis* and *Bacillus subtilis*) with semduramicin and formic acid in turkeys for fattening. EFSA Journal 2011;9(1):1953, 7 pp. doi:10.2903/j.efsa.2011.1953

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2011b. Scientific Opinion on the safety and efficacy of BioPlus 2B (*Bacillus licheniformis* DSM 5749 and *Bacillus subtilis* DSM 5750) as a feed additive for sows. EFSA Journal 2011;9(9):2356, 10 pp. doi:10.2903/j.efsa.2011.2356

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2011c. 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

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

European Commission, 1997, updated 2003. Report on the use of certain micro-organisms as additives in feedingstuffs. Available online: http://ec.europa.eu/food/fs/sc/scan/out93_en.pdf

European Commission, 2000. Report of the Scientific Committee on animal nutrition on product BioPlus 2B® for use as feed additive. Available online: http://ec.europa.eu/food/fs/sc/scan/out49_en.pdf

Abbreviations

ANOVA	analysis of variance
ADG	average daily gain
bw	body weight
CEN	European Committee for Standardization
CFU	colony forming unit
CV	coefficient of variation
EURL	European Union Reference Laboratory
FEEDAP	EFSA Panel on Additives and Products or Substances used in Animal Feed
LOD	limit of detection
MIC	minimum inhibitory concentration
OCDD	octachlorodibenzo-*p*-dioxin
OCDF	octachlorodibenzofuran
PCB	polychlorinated biphenyls
PCDD	polychlorinated dibenzo-*p*-dioxins
PFGE	Pulsed Field Gel Electrophoresis
QPS	qualified presumption of safety
s_r	standard deviation for repeatability
s_R	standard deviation for reproducibility
SCAN	Scientific Committee on Animal Nutrition
TEQ	toxic equivalent factor

Annex A – Executive Summary of the Evaluation Report of the Community Reference Laboratory for Feed Additives on the Methods of Analysis for BioPlus 2B[1]

In the current application authorisation is sought for BioPlus 2B under the category 'zootechnical additives', functional group 'gut flora stabiliser' according to Annex I of Regulation (EC) No 1831/2003. The product consists of two active substances, namely *Bacillus subtilis DSM 5750* and *Bacillus licheniformis DSM 5749,* mixed at a 1:1 ratio. Specifically, authorisation is sought for the *feed additive* to be placed on the market in the powder form containing minimum concentration of 1.6×10^{10} CFU/g of each strain. The intended use of the current application is for chickens for fattening, piglets for fattening, sows, turkeys for fattening and calves. A total dosage including both strains of 1.3×10^{9} CFU/kg in complete *feedingstuffs* for all above mentioned species is proposed by applicant. Furthermore, the applicant suggested a minimum content of BioPlus® 2B ranging from 11 to 270 g (depending of the species) per 1,000 animals in drinking *water*. For the enumeration of the sum of the two strains of the active agents in *feed additive*, *premixtures*, *feedingstuffs* and *water* the Applicant proposes the ring trial validated a spread plate method (EN 15784) using tryptone soya agar. The performance characteristics of the CEN method reported after logarithmic transformation of measured CFU values are:

- a standard deviation for *repeatability* (s_r) ranging from 0.07 to 0.09 $\log_{10}$ CFU/g
- a standard deviation for *reproducibility* (s_R) ranging from of 0.32 to 0.35 $\log_{10}$ CFU/g, and
- a limit of detection (LOD) of 10^5 CFU/kg of *feedingstuffs.*

Based on these performance characteristics, the CRL recommends for official control the ring trial validated method EN 15784 for the determination of *Bacillus subtilis DSM 5750* and *Bacillus licheniformis DSM 5749* in *feed additive*, *premixtures*, *feedingstuffs* and *water.*

Molecular methods were used by the Applicant for identification of the active agent. The CRL recommends for official control, Pulsed Field Gel Electrophoresis (PFGE), a generally recognised standard methodology for microbial identification.

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.

[1] The full report is available on the EURL website: https://ec.europa.eu/jrc/sites/default/files/FinRep-FAD-2009-0023.pdf