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Safety and efficacy of Calsporin® (*Bacillus subtilis* DSM 15544) as a feed additive for laying hens and avian species for laying

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

Abstract

The additive Calsporin® is a preparation containing viable spores of a single strain of *Bacillus subtilis*. The product was previously assessed by the European Food Safety Authority and is currently authorised for use in chickens for fattening, weaned piglets, chickens reared for laying, turkeys, minor avian species and other ornamental and game birds. The present application is for an extension of use in feed for laying hens and minor avian species for laying and for game, sporting and ornamental birds for laying. No evidence of toxigenic potential or resistance to antibiotics of human and veterinary importance was found, as judged by the current guidelines. Thus the conclusion reached in previous opinions that this strain of *B. subtilis* is presumed safe for target animals, consumers and the environment is still considered valid. This conclusion automatically covers the use of the additive in feed for laying hens and for all avian species for laying. Use of the additive in feed for laying hens and for all avian species for laying will not introduce hazards for users not already considered. In the three trials conducted with laying hens the amount of feed needed to produce a unit of egg mass was significantly reduced when Calsporin® was included at the minimum recommended dose of 3×10^8 colony-forming units per kilogram of feed. While the Panel notes that one of the studies involved layers in the second half of production rather than from the onset of laying, the positive outcome is taken to indicate a potential for efficacy over the entire laying period. This conclusion on efficacy for laying hens can be extended to all avian species for laying when the additive is used at the same minimum dose.

Keywords: Calsporin®, *Bacillus subtilis,* safety, efficacy, laying hens, avian species for laying, eggs

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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.

The European Commission received a request from Calpis Co., Ltd[2] for authorisation of the product Calsporin®, *Bacillus subtilis* DSM 15544, when used as a feed additive for laying hens and minor avian species for laying (game birds, ducks, geese, pigeons, sporting & ornamental birds) (category: zootechnical additives; functional group: gut flora stabilisers).

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). 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 16 February 2015.

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 safety for the target animals, consumer, user and the environment and the efficacy of the product Calsporin® (*Bacillus subtilis* DSM 15544), when used under the proposed conditions of use (see Section 3.1.3).

1.2. Additional information

The additive Calsporin® is a preparation containing viable spores of a single strain of *Bacillus subtilis* (DSM 15544). EFSA has issued several opinions on the safety and efficacy of this product when used in chickens for fattening (EFSA, 2006, 2007a), with weaned piglets (EFSA FEEDAP Panel, 2010a) and in turkeys for fattening, ducks, geese, pigeons and other game birds for meat production, ducks, geese, pigeons, game birds, ornamental and sporting birds for rearing to point of lay, turkeys reared for breeding and chickens reared for laying (EFSA FEEDAP Panel 2010b). Subsequent to these opinions the additive was authorised for use in chickens for fattening,[3] weaned piglets,[4] chickens reared for laying, turkeys, minor avian species and other ornamental and game birds.[5]

The species *B. subtilis* is considered by EFSA to be suitable for the qualified presumption of safety (QPS) approach to establishing safety for the target species, consumers and the environment (EFSA, 2007b; EFSA BIOHAZ Panel, 2013). This approach requires the identity of the active agent to be established and the absence of toxigenic potential and susceptibility to a selected range of antibiotics to be demonstrated. EFSA considered these issues in its opinion on the safety and efficacy of Calsporin® as a feed additive for weaned piglets (EFSA FEEDAP Panel, 2010a), following the provisions of the guidance applicable at the time, and concluded that the strain could be presumed safe for the target species, consumers and the environment. Subsequently, EFSA has introduced new guidance on the determination of antibiotic susceptibility (EFSA FEEDAP Panel, 2012) and assessing

[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] Calpis Co. Ltd, represented in the EU by Calpis Co. Ltd Europe Representative Office, 46 rue Paul Valery, 75516 Paris, France.
[3] Commission Regulation (EC) No 1444/2006 of 29 September 2006 concerning the authorisation of *Bacillus subtilis* C-3102 (Calsporin) as a feed additive. OJ L 271, 30.9.2006, p. 19 plus amendments.
[4] Commission Regulation (EU) No 333/2010 of 22 April 2010 concerning the authorisation of a new use of *Bacillus subtilis* C-3102 (DSM 15544) as a feed additive for weaned piglets (holder of authorisation Calpis Co. Ltd. Japan, represented in the European Union by Calpis Co. Ltd. Europe Representative Office). OJ L 102, 23.4.2010, p. 19
[5] Commission Regulation (EU) No 184/2011 of 25 February 2011 concerning the authorisation of *Bacillus subtilis* C-3102 (DSM 15544) as a feed additive for chickens reared for laying, turkeys, minor avian species and other ornamental and game birds (holder of authorisation Calpis Co. Ltd Japan, represented by Calpis Co. Ltd Europe Representative Office). OJ L 53, 26.2.2011, p. 33.

the toxigenic potential of *Bacillus* species (EFSA FEEDAP Panel, 2014). Consequently, these elements of the previous assessment are reconsidered in this opinion.

The use of the additive with laying hens and minor avian species for laying is considered unlikely to introduce hazards for users of the product not already considered as part of the first assessment (EFSA, 2006). Consequently, in the present opinion the FEEDAP Panel has focused only on the verification of the compliance of the active agent with the qualifications currently applied using the QPS approach and the data specific to the use of the additive in the new target species.

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[6] in support of the authorisation request for the use of Calsporin® as a feed additive. The technical dossier was prepared following the provisions of Article 7 of Regulation (EC) No 1831/2003 and the applicable EFSA guidance documents.

The EURL considered that the conclusions and recommendations reached in the previous assessment are valid and applicable for the current application.[7]

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of Calsporin® is in line with the principles laid down in Regulation (EC) No 429/2008[8] and the relevant guidance documents: Guidance on zootechnical additives (EFSA, 2012); Technical guidance on tolerance and efficacy studies in target animals (EFSA FEEDAP Panel, 2011); Guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition (EFSA FEEDAP Panel, 2014); Technical guidance on the extrapolation of data from major species to minor species regarding the assessment of additives for use in animal nutrition (EFSA, 2008, revised in 2009); and Technical guidance on the update of the criteria used in the assessment of bacterial resistance to antibiotics of human or veterinary importance (EFSA, 2008, revised in 2012).

3. Assessment

Calsporin® is a preparation of viable spores of a single strain of *Bacillus subtilis* intended for use as a zootechical additive (gut flora stabiliser) in feed for laying hens and all avian species for laying (ducks, geese, pigeons) and for game, sporting and ornamental birds for laying.

3.1. Characterisation

3.1.1. Characterisation of the active agent

The active agent in the additive consists of viable endospores of a single strain of *B. subtilis* originally isolated from soil in Japan and deposited in the German Collection of Microorganisms and Cell Cultures (DSMZ) with the accession number DSM 15544.[9]

The battery of antibiotics currently recommended for the testing of antibiotic resistance among species of *Bacillus* were all included in the recommended list applicable at the time of the 2008 submission for the use of the additive in piglets.[10] The results presented in this application are still valid and, as the cut-off values remain the same, the conclusion on the susceptibility of the strain to all tested antibiotics is unchanged.

[6] FEED dossier reference: FAD-2014-0042.
[7] The full report is available on the EURL website: https://ec.europa.eu/jrc/sites/default/files/FinRep-FAD-2009-0013.pdf
[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/Annex II.2.1.2.1.
[10] Technical dossier/Section II/Annexes II.2.2.2.4 and II.2.2.2.5.

The technical guidance on the assessment of the toxigenic potential of *Bacillus* species introduced in 2014 contains a substantial revision applicable to *B. subtilis*. The emphasis of the assessment changed from the detection of *B. cereus*-like toxins in the former guidance to the detection of cyclic lipopeptides able to cause measurable cell cytotoxicity. It recommends the use of a single cytotoxicity assay preferably done with Vero cells and using an established method, such as inhibition of radio-labelled leucine uptake, as evidence of toxicity. The data set generated in 2003 and used for subsequent applications included an assay with Vero cells done with a 10-fold concentrated culture supernatant of the Calsporin® strain.[11] The presence of the concentrated supernatant did not inhibit leucine uptake, unlike the supernatant from the positive control, which fully inhibited uptake. Consequently, these data are considered to fulfil the requirements of the present guidance confirming the absence of toxigenic potential.

3.1.2. Characterisation of the additive

The additive that is the subject of the present application has the same formulation and method of manufacture as that considered in previous applications. Thus, the data pertaining to impurities and physical properties still apply. Since the first application, the applicant has continued to monitor the shelf life of the product and now has evidence, based on three batches, that losses are virtually non-existent after 10 years' storage in commercial packaging under conditions designed to mimic ambient (25 ºC/60 % relative humidity).[12] Although no specific data on stability in feed for laying hens or mixing in such feed were provided, the data on mixing and stability in premixes, mash and pelleted feeds variously produced for chickens for fattening, turkeys and piglets are considered sufficient given the similarity in feed formulation.

3.1.3. Conditions of use

The product is intended for use in feed for laying hens and all avian species for laying (ducks, geese, pigeons) and for game, sporting and ornamental birds for laying at a minimum dose of 3×10^8 colony-forming units (CFU)/kg complete feedingstuff.

3.2. Safety

No evidence of toxigenic potential or resistance to antibiotics of human and veterinary importance was found, as judged by the current requirements. Thus, the conclusion reached in the previous opinion on weaned piglets (EFSA FEEDAP Panel, 2010a) that the strain *B. subtilis* DSM 15544 is presumed safe for target animals, consumers and the environment, is still considered to apply. This conclusion automatically covers the use of the additive in feed for laying hens and all avian species for laying.

The use of the additive in laying hens and all avian species for laying is considered unlikely to introduce hazards for users of the product not already considered as part of the first assessment (EFSA, 2006).

3.3. Efficacy

3.3.1. Efficacy for laying hens

Three trials conducted in two Member States are described. In each case trials were of 168 days' duration and consisted of a comparison of a control group of layers with a group given the additive at the minimum recommended dose of 3×10^8 CFU/kg feed (confirmed by analysis). Birds were fed mash diets based on wheat/soybean (trial 1[13]) or wheat/maize/soybean (trials 2[14] and 3[15]), and the breeds used were Hy-Line layers (trial 1) and Tetra SL (trials 2 and 3). The total numbers of layers used and number of replicates per treatment for each trial are shown in Table 1. In trial 1, birds were purchased at 36 weeks of age and randomly allocated to cages. They were allowed four weeks

[11] Technical dossier/Section II/Annex II.2.2.2.1.
[12] Technical dossier/Section II/Annex II.4.1.2.
[13] Technical dossier/Section IV and Supplementary information June 2015/Annex IV.3.1 and Addendum.
[14] Technical dossier/Section IV/Annex IV.3.2.
[15] Technical dossier/Section IV/Annex IV.3.3.

acclimatisation, during which time they were fed the control feed, with the start of the trial at 40 weeks of age. In trials 2 and 3, observations were made from the start of laying at 19 weeks of age.

Hen performance, egg production, feed consumption and feed conversion ratio were measured or calculated in all trials. Trials 2 and 3 also included the egg quality parameters (e.g. egg length, egg width, shell breaking strength, shell thickness, shell weight, shell density, shell colour, yolk weight, yolk colour, albumen height, egg cholesterol content). A more limited assessment of egg quality was made in trial 1 (cracked/dirty eggs, shell thickness and yolk colour). Faecal numbers of *Clostridium perfringens* and *Campylobacter* spp. were also monitored in trial 1.

Overall production data were analysed as a randomised two-factorial design by analysis of variance. The model included the block as fixed effect and the dietary treatment and period as main effects. In trial 1, numbers of faecal microorganisms were analysed by Student's *t*-test for *Campylobacter* spp. and Fisher's exact test for detection rates of *C. perfringens.* The overall performance results for the three trials are summarised in Table 1.

Table 1: Summary of the overall performance results of the three trials made with laying hens

Trial no	Total number of animals No of replicates per treatment × no of birds per replicate	Treatment (CFU/kg feed)	Laying rate (%)	Average egg weight (g)	Daily egg mass (g/cage)	Feed intake (g/bird)	Egg to feed ratio
1	384	0	90.3	64.3	232.8	123.1	0.47
	48 × 4	3×10^8	91.2	64.3	234.8*	122.4	0.48*
2	120	0	91.0	60.9	110.8	122.5	0.50
	30 × 2	3×10^8	90.9	62.0*	112.7	121.9	0.51*
3	120	0	89.6	60.8	109.0	123.9	0.49
	30 × 2	3×10^8	91.5	61.3	112.1	123.6	0.50*

*Significantly different from the control value at at least $P < 0.05$.

No hens died or were culled in any of the three trials and no health problems were recorded. Daily egg mass increased with treatment in all three trials but reached significance in only one. This increase was accompanied in all trials by a non-significant reduction in feed intake in the Calsporin® group compared with the control group, leading to a significant improvement in egg to feed ratio. The Panel notes that the first study was done with hens in the second half of production, whereas the second and third studies were done at the start of laying. Although hens used in the first trial were older than the age recommended in the EFSA Guidance (EFSA FEEDAP Panel, 2011), evidence was provided confirming that there was no significant difference in laying performance (average egg weight, laying intensity and number of eggs per bird) at the start of the study, which might have confounded the outcome. The positive results in this trial would appear to indicate a potential benefit of Calsporin® addition throughout laying.

Inclusion of Calsporin® in diets had no significant effects on egg quality parameters, colour or cholesterol content in any of the studies, other than an effect on shell colour in trial 2. However, there was a significant shift in egg size classification in the two trials in which this was recorded, with more eggs classified as large/extra-large. This was most marked in trial 2, in which the average egg weight was significantly increased, but a similar pattern was also seen in trial 3, where the increase in egg weight did not reach significance.

Analysis of faecal data after eight weeks showed a small but significant reduction in faecal *Campylobacter* numbers in birds fed Calsporin® compared with control birds in trial 1 (6.9 vs. 6.2 $\log_{10}$/g faeces, $P = 0.04$).

Conclusions on efficacy for laying hens

In all three studies the amount of feed needed to produce a unit of egg mass was significantly reduced when Calsporin® was included at the minimum recommended dose of 3×10^8 CFU/kg feed. While the Panel notes that one of the studies involved layers in the second half of production, the positive outcome is taken to indicate a potential for efficacy over the entire laying period.

3.3.2. Efficacy for minor avian species for laying and for game, sporting and ornamental birds for laying

The dose proposed for use in egg-laying avian species is the same as that demonstrated as effective in a physiologically similar major species (laying hens), and it can be reasonably assumed that the mode of action is the same. Consequently, the conclusion on efficacy for laying hens can be extended to all avian species for laying without the need for specific studies.

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[16] and Good Manufacturing Practice.

4. Conclusions

No evidence of toxigenic potential or resistance to antibiotics of human and veterinary importance is found, as judged by the current guidelines. The conclusion reached in previous opinions, that the strain *B. subtilis* DSM 15544 is presumed safe for target animals, consumers and the environment, is still considered valid. This conclusion automatically covers the use of the additive in feed for laying hens and all avian species for laying.

Use of the additive in feed for laying hens and all avian species for laying will not introduce hazards for users not already considered.

In all three studies the amount of feed needed to produce a unit of egg mass was significantly reduced when Calsporin® was included at the minimum recommended dose of 3×10^8 CFU/kg feed. While the Panel notes that one of the studies involved layers in the second half of production, the positive outcome is taken to indicate the potential for efficacy over the entire laying period. The conclusion on efficacy for laying hens can be extended to all avian species for laying when the additive is used at the same minimum dose.

[16] 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.

Documentation provided to EFSA

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2. Calsporin® *Bacillus subtilis* C-3102, DSM 15544 Zootechnical feed additive for laying hen & minor avians in lay. Supplementary information. June 2015. Submitted by Calpis Co. Ltd.

3. Comments from Member States.

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Abbreviations

CFU	colony-forming unit
EURL	European Union Reference Laboratory
FEEDAP	EFSA Panel on Additives and Products or Substances used in Animal Feed
QPS	qualified presumption of safety