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Safety and efficacy of Calsporin® (*Bacillus subtilis* DSM 15544) as a feed additive for ornamental fish

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

Abstract

Following a request from the European Commission, the European Food Safety Authority (EFSA) Panel on Additives and Products or Substances used in Animal Feed was asked to deliver a scientific opinion on the safety and efficacy of Calsporin® when used in feed for ornamental fish. Calsporin® is currently approved as a zootechnical feed additive for use with chickens for fattening and rearing, turkeys, piglets, and minor avian species to point of lay. The additive contains viable spores of a single strain of *Bacillus subtilis*. This species is considered by the EFSA to be suitable for the qualified presumption of safety approach to establishing safety for target species, consumers and the environment. The additive was found to meet the criteria for this approach in the context of previous opinions and, as a result, is presumed safe for all target species, including ornamental fish. The use of the additive with feed for ornamental fish is considered unlikely to introduce hazards for users of the product not already considered in previous assessments. The minimum dose proposed for use in feed for ornamental fish is 1×10^{10} colony-forming units (CFU)/kg of complete feedingstuff. This application makes reference to a published study describing the effects of adding Calsporin® to the diet of juvenile koi carp (*Cyprinus carpio*). A significant increase in final body weight and improvement in feed to gain ratio was observed in fish given the additive compared with controls. Since the effect of Calsporin® addition in all of the major species previously examined was an improvement in one or more performance-related parameters, and since the same outcome was observed in koi carp, it can be presumed that Calsporin® has a potential to improve the growth and feed utilisation of developing ornamental fish.

Keywords: *Bacillus subtilis* DSM 15544, Calsporin®, ornamental fish, QPS, safety, efficacy

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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* C-3102[3]), when used as a feed additive for Koi carp and other ornamental fish (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 28/05/2015.

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 on the efficacy of the product Calsporin® (*Bacillus subtilis* C-3102), when used under the proposed conditions of use (see Section 3.2).

1.2. Additional information

The additive Calsporin® is a preparation containing viable spores of a single strain of *Bacillus subtilis*. EFSA has issued several opinions on the safety and efficacy of this product when used with poultry (chickens for fattening and rearing, turkeys for fattening and rearing, ducks, geese, pigeons and other game birds for meat production and rearing and hens and minor poultry species for laying) (EFSA, 2006, 2007a; EFSA FEEDAP Panel, 2010a, 2015) and weaned piglets (EFSA FEEDAP Panel, 2010b). Subsequently, the additive was authorised for use with chickens for fattening,[4] weaned piglets,[5] chickens reared for laying, turkeys, minor avian species and other ornamental and game birds.[6]

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 a toxigenic potential and susceptibility to antibiotics of human clinical and veterinary importance 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, 2010b) 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. Since then, EFSA has introduced new guidance on the determination of antibiotic susceptibility (EFSA FEEDAP Panel, 2012) and assessing the toxigenic potential of *Bacillus* species (EFSA FEEDAP Panel, 2014). In a more recent opinion (EFSA FEEDAP Panel, 2015), EFSA reviewed the data provided in previous opinions 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] Calpis Co. Ltd. Europe Representative Office, 46 rue Paul Valery, 75116, Paris, France.
[3] C-3102 is an in-house identifier; the active agent is *Bacillus subtilis* DSM 15544.
[4] 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.
[5] 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.
[6] 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.

concluded that Calsporin® meets the current requirements and that *Bacillus subtilis* DSM 15544 can be presumed safe for target animals, consumers and the environment.

The use of the additive with ornamental fish 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 data specific to the use of the additive with the new target species.

2. Data and Methodologies

2.1. Data

The present assessment is based on data submitted by the applicant in support of the request for authorisation of the use of Calsporin® as a feed additive. The data were received as a technical dossier[7] containing detailed information on the identification and the conditions of use of the product, with the relevant supporting studies. Other published studies were also taken into consideration.

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 FEEDAP Panel, 2012), Technical guidance on tolerance and efficacy studies in target animals (FEEDAP Panel, 2011a), Guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition (EFSA FEEDAP Panel, 2014), Guidance on the assessment of additives intended to be used in pets and other non food-producing animals, (FEEDAP Panel, 2011b), Technical Guidance on 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).

The European Union Reference Library (EURL) considered that the conclusions and recommendations reached in the previous assessment are valid and applicable for the current application.[9]

3. Assessment

Calsporin® is a preparation of viable spores of a single strain of *Bacillus subtilis* intended for use as a zootechnical additive (gut flora stabiliser) in feed for koi carp and other ornamental fish.

3.1. Characterisation of the additive

The additive which is the subject of the present application has the same formulation and method of manufacture as that considered in previous applications. The data pertaining to impurities, physical properties and shelf life still apply.

The stability of the active agent in feed and premixes for pigs and poultry was established in previous opinions. Given the commonality of feed ingredients in diets for ornamental fish and terrestrial livestock and the inherent resistance of bacterial endospores, the FEEDAP Panel is of the opinion that the existing data are sufficient to establish the stability of the additive in fish feed.

Most feed for ornamental fish is subject to some form of physical processing, usually involving heat (pelleting/extrusion cooking) with or without subsequent grinding/flaking. The stability of the additive to pelleting has been tested in feeds for turkeys[10] (at 65–70 °C) and chickens (at 70, 80 and 90 °C) without loss.[11] A similar result is to be expected when the additive is used in pelleted fish feed.

[7] FEED dossier reference: FAD-2015-0010.
[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] The full report is available on the EURL website: https://ec.europa.eu/jrc/sites/default/files/FinRep-FAD-2009-0013.pdf
[10] Technical dossier/Section II/Annex II.4.1.4.
[11] Technical dossier/Section II/Annexes II.4.1.5-6.

3.2. Conditions of use

The additive is proposed to be used in feed for ornamental fish, including koi carp, at a minimum concentration of 1×10^{10} CFU/kg of complete feedingstuff.

3.3. Efficacy

It may be considered sufficient that a demonstration of the efficacy of a zootechnical additive in pets and non-food-producing animals is based on a single *in vivo* study. However, this is accompanied by a proviso that the additive has been previously authorised/assessed for use in a major animal species/category and that the mode of action/effect claimed can be considered the same in both cases (EFSA FEEDAP Panel, 2011b).

The use of microbial feed additives with ornamental fish may serve different purposes. For fish producers an improvement in performance, whether measured in terms of time to reach a marketable size or in feed to gain ratio, will have economic benefits. However, this is not the case for many fish owners, for whom increased growth rate is a secondary consideration. For most owners, maintenance of health and coloration are of greater significance.

This application makes reference to a published study by He et al. (2011) describing the effects of adding Calsporin® to the diet of koi carp (*Cyprinus carpio*). A total of 384 juveniles (starting weight of approximately 4.5 g) were randomly assigned to one of 16 100 L tanks with a stocking density of 24 fish per tank. Fish in eight tanks were provided with a basal diet in pelleted form (fish meal/soybean meal/wheat flour) and those in the remaining eight tanks were provided with the basal diet supplemented with Calsporin® at the recommended dose of 1×10^{10} CFU/kg feed (confirmed by analysis of feed). Water temperature was 23–26 °C and 10 % of the water was exchanged daily. At the start and end of the 35-day feeding trial, fish were batch weighed and counted. Fish were fed twice daily at 1–2 % body weight per day. Uneaten feed was removed from each tank and weighed to allow the calculation of feed intake and feed to gain ratio.

In the original publication, performance data were analysed using a paired t-test with significant differences accepted at $P < 0.05$. However, for the purposes of this application, the raw data were reanalysed by analysis of variance (ANOVA). There was no significant difference in starting weight between the two groups (4.49 g vs. 4.47 g), but a significant increase was seen in final body weight (11.20 g vs. 11.73 g; $P = 0.006$) and improvement in feed to gain ratio (1.38 vs. 1.31; $P = 0.009$). Uncertainty on the interpretation of results arises from the removal of fish during the study. No deaths were recorded over the 35-day feeding period.

Since the effect of Calsporin® addition in all of the major species examined was an improvement in one or more performance-related parameters (EFSA, 2006, 2007a; EFSA FEEDAP Panel, 2010a, b), and since the same outcome was observed in koi carp, it can be presumed that Calsporin® has the potential to improve the growth and feed utilisation of developing ornamental fish.

A number of other parameters were included in the study. Three fish per tank were sampled at days 0, 10, 20 and 35 for gut microbial community analysis and for selected gene expression in the liver, kidney and intestinal tract. In addition, at the end of the feeding study, on day 35, all remaining fish were challenged with an intraperitoneal injection of a known pathogenic strain of *Aeromonas hydrophilia* and monitored for survival for a further seven days.

For the microbiological analysis, intestines were aseptically removed and washed and genomic DNA extracted. The V3 region of *rrs* was amplified for denaturing gradient gel electrophoresis (DGGE) and a comparison of banding patterns made with known bacteria. Although changes with time in the relative abundance of some bacterial groups could be seen, this generally occurred in both control and treatment groups. Changes which might be ascribed to the presence of Calsporin® were not evident.

Organ samples from each tank taken for gene expression were pooled and total RNA extracted. The reverse transcription polymerase chain reaction (RT-PCR) was performed using primers selected for interleukin 1β (IL-1β), tumour necrosis factor α (TNF-α), heatshock protein 70 (HSP70), transforming growth factor β (TGF-β) and IL-10. A trend towards elevated cytokine expression was seen in the intestine and liver, but not in the kidney. However both pro- and anti-inflammatory cytokines were similarly affected, and no consistent pattern of change was evident over time. The single attempt to link such changes to the maintenance of health within the test population by challenging with a known

pathogen did not reveal any significant differences in survivability between the control and test groups.

The data provided in the paper by He et al. (2011) do not provide evidence of changes induced by the addition of Calsporin® which might indicate a potential to increase resistance to infection and aid the maintenance of health.

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[12] and Good Manufacturing Practice.

4. Conclusions

The active agent fulfils the requirements of the QPS approach to the assessment of safety and can be presumed safe when used in feed for ornamental fish.

The use of the additive with feed for ornamental fish is considered unlikely to introduce hazards for users of the product not already considered in previous assessments of Calsporin®.

Since the effect of Calsporin® addition in all of the major species examined was an improvement in one or more performance-related parameters, and since the same outcome was observed in koi carp, it can be presumed that Calsporin® has the potential to improve the growth and feed utilisation of developing ornamental fish at the dose of 1×10^{10} CFU/kg of complete feedingstuffs.

The data provided do not provide evidence of changes induced by the addition of Calsporin® which might indicate a potential to increase resistance to infection and aid the maintenance of health.

[12] 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.

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2. Comments from Member States.

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Glossary

ANOVA	analysis of variance
BW	body weight
CFU	colony-forming unit
DGGE	denaturing gradient gel electrophoresis
EC	European Commission
EFSA	European Food Safety Authority
EURL	European Union Reference Laboratory
FEEDAP	Panel on Additives and Products or Substances used in Animal Feed
HSP70	heatshock protein 70
IL	interleukin
PCR	polymerase chain reaction
QPS	qualified presumption of safety
RT	reverse transcription
TGF	transforming growth factor
TNF	tumour necrosis factor