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Safety and efficacy of BioWorma® (*Duddingtonia flagrans* NCIMB 30336) as a feed additive for all grazing animals

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Abstract

Following a request from the European Commission, the EFSA Panel on Additives and products or Substances used in Animal Feed (FEEDAP) was asked to deliver a scientific opinion on the safety and efficacy of BioWorma® (*Duddingtonia flagrans* NCIMB 30336) when used as a zootechnical feed additive for all grazing animals. *Duddingtonia flagrans* belongs to a group of nematophagous fungi that physically entrap nematodes through an adhesive hyphal net. The additive contains the fungus in the form of chlamydospores and is intended to control pathogenic nematodes on pasture, with subsequent benefits for grazing animals. No conclusions could be drawn on the safety for the target species due to lack of data. [redacted] As it is not possible to exclude the presence of secondary metabolites (other than flagranones) produced during fermentation and their potential carry-over into animal products, safety for the consumer could not be established. The Panel concluded that the additive is not irritant to skin and eyes but is irritant to the respiratory tract and a respiratory sensitiser. No conclusion could be drawn on its skin sensitisation potential. Since *D. flagrans* is a naturally inhabiting soil organism of world-wide distribution, the Panel considered that use of an additive based on this organism does not pose a risk for the environment under the intended conditions of use. The strain under application reduced the number of parasitic nematodes on pasture to the benefit of grazing animals when used at the recommended application rate of 3×10^4 chlamydospores/kg bodyweight and day.

Keywords: *Duddingtonia flagrans*, nematodes, eelworm, grazing animals, safety, efficacy

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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.

The European Commission received a request from International Animal Health Product Pty Ltd,[2] for authorisation of the product BioWorma® (*Duddingtonia flagrans* NCIMB 30336[3]) when used as a feed additive for all grazing animals (category: zootechnical additives; functional group: other zootechnical additives).

According to Article 7(1) of Regulation (EC) No 1831/2003, the Commission forwarded the application to the European Food Safety Authority (EFSA) as an application under Article 4(1) (authorisation of a feed additive or new use of a feed additive). The particulars and documents in support of the application were considered valid by EFSA as of 10 April 2017.

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 BioWorma® (*Duddingtonia flagrans* NCIMB 30336), when used under the proposed conditions of use (see Section 3.1.4).

1.2. Additional information

BioWorma® (*Duddingtonia flagrans* NCIMB 30336) is not currently authorised as a feed additive in the EU.

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[4] in support of the authorisation request for the use of BioWorma® (*Duddingtonia flagrans* NCIMB 30336) as a feed additive.

EFSA has verified the European Union Reference Laboratory (EURL) report as it relates to the methods used for the control of the active agent in animal feed. The Executive Summary of the EURL report can be found in Annex A.[5]

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of Bioworma® 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, 2011), Guidance for establishing the safety of additives for the consumer (EFSA FEEDAP Panel, 2012b) and Guidance on studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012c).

3. Assessment

BioWorma® (*D. flagrans* NCIMB 30336) is intended for use as a feed additive (category: zootechnical additive; functional group: other zootechnical additives) to control pathogenic nematodes (eelworm) in the soil, with subsequent benefits for ruminants, horses and other grazing animals.

[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] GAB Consulting GmbH on behalf of International Animal Health Product Pty Ltd 18 Healey Circuit Huntingwood NSW 2148, Australia.
[3] Identified by the applicant as: *Duddingtonia flagrans* IAH 1297.
[4] FEED dossier reference: FAD-2016-0067.
[5] The full report is available on the EURL website: https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2016-0067-duddingtonia-flagrans.pdf

3.1. Characterisation

Duddingtonia flagrans (Dudd) Cooke belongs to a group of nematophagous fungi that physically entrap nematodes by means of a specialised adhesive hyphal net. The species is widely distributed and has been isolated from pasture soils in many countries throughout the world (Ahren et al., 2004). Reproduction is by vegetative means and conidiospores. Under adverse conditions and in the absence of suitable prey, the fungus survives by the production of chlamydospores, thick-walled resting spores.

3.1.1. Characterisation of the active agent

The strain under application was isolated from Australian pasture and formed part of a collection of *D. flagrans* strains held by the Commonwealth Scientific and Industrial Research Organisation (CSIRO) Australia. It is deposited in the National Collection of Industrial, Food and Marine Bacteria with the accession number NCIMB 30336[6] and has not been genetically modified.[7] Identification was made on the basis of DNA sequencing and comparison of the internal transcribed spacers (ITS) region of the ribosomal RNA genes.[8] It was also shown that polymorphisms in the ITS sequence could be used to distinguish this isolate from all other isolates of *D. flagrans* whose sequences are lodged in GenBank.

Chlamydospores were shown *in vitro* not to germinate under anaerobic conditions at 37°C.

Little is known about secondary metabolites production by *Duddingtonia* other than the presence of flagranones, compounds structurally related to the farnesylated cyclohexenoxides isolated from another nematode-trapping fungus *Arthrobotrys oligospora* (Anderson et al., 1999).[9]

3.1.2. Manufacture and characterisation of the additive

The active agent is produced [redacted] according to specifications set by the applicant. [redacted]

Each batch of the spore concentrate is specified to contain a minimum of 1×10^6 chlamydospores/g and analysis of 11 batches gave a mean count of 0.94×10^6 chlamydospores/g (range 0.41–2.0×10^6 chlamydospores/g).[10] The minimum content of spores in the additive is specified as 5×10^5 chlamydospores/g BioWorma®.[11] Three out of six batches of the additive tested showed compliance with the minimum specifications, while the remaining three showed values below the specifications (mean count of 7.5×10^5 chlamydospores/g, range 2.2–17×10^5 chlamydospores/g).[12]

A raw material specification for the spore concentrate is set which specifies maximum acceptable values for some microbiological parameters (total bacteria $< 5 \times 10^4$ CFU/g, total fungi other than *Duddingtonia* sp. $< 1 \times 10^2$ CFU/g, bile-tolerant bacteria $<10^2$ CFU/g, *Staphylococcus aureus* and *Escherichia coli* each absent in 1 g and *Salmonella* spp., absent in 10 g).[13] Analysis of three batches of

[6] Technical dossier/Section II.
[7] Technical dossier/Section II_2.2.1.2.
[8] Technical dossier/Section II/Annex 2.2.1.2_03.
[9] Technical dossier/Section II/Annex 2.2.2.2_01.
[10] Technical dossier/Section II/2.1.3.
[11] Technical dossier/Section II/Annex 2.4.1.1_01.
[12] Technical dossier/Supplementary information July 2017/Annex SupInfo_FAD_2016_0067_Req_1_1_Bioworma Batch Assay.
[13] Technical dossier/Section II/Annex 2.2.1.2_04.

the additive and three batches of a vitamin/mineral premixture containing the additive among other ingredients showed compliance with the limits for total bacteria and total fungi. Compliance with limits with the other microbial contaminants specifications was shown in the only batch of the additive and premixture tested.[14] Although shown in only single batches of the spore concentrate and the additive, only trace amounts of heavy metals and arsenic contents were detected and are of no concern (As: 0.08/0.02, Cd: 0.01/0.03, Pb: 0.1/0.09 mg/kg spore concentrate/additive).[15] Mercury was below the limit of detection (< 0.01 mg/kg). A single batch of the spore concentrate and of the additive and two batches of the premixture mentioned above were tested for the presence of a range of mycotoxins.[16] None were detected, except for zearalenone in the only batch of the additive tested (61 μg/kg).[17]

Results for the analysis of flagranone A in nine batches of *D. flagrans* spore concentrate showed a range of 4.4–73.9 μg/g, with a mean of 34.6 μg/g.[6] Since the spore concentrate represents approximately one-third of the additive, concentrations of flagranone A in BioWorma® could be in the region of 11 mg/kg additive. Flagranones B and C could not be detected.

The additive is described as a free-flowing brown powder. Particle size analysis of a single batch made by dry sieving showed that 90% of particles were retained by a 125-μ diameter mesh and none passed a 50-μ mesh.[18] An estimate of the dustiness was made using the same additive batch following the protocol developed by the Collaborative International Pesticide Analytical Council (CIPAC) test MT 171 (Dustiness of granular products).[19] In this test, a weighed amount of a granular product is allowed to fall under standard conditions in a test chamber, releasing dust. The generated dust is then removed by an airflow, collected on a filter and weighed. Following the criteria established by CIPAC MT 171 the results obtained (1.3 mg of dust) indicated that the addictive should be considered as 'nearly dust free'.

3.1.3. Stability and homogeneity

The product is based on chlamydospores able to withstand adverse conditions and so no loss of viability would be expected under the recommended storage conditions for the additive (< 30°C). This was confirmed by analysis of three batches of the additive stored for at least 24 months (36 months in one case) at temperatures of < 8°C, 25°C, and 30°C.[20] Although viable counts made during storage fluctuated, they remained within 0.5 log of the initial value showing that viability was retained over the storage period independent of temperature.

The viability of the chlamydospores when mixed into a vitamin/mineral premixture (three batches of 2.1, 3.0 and 7.9 $\times$ 10^7 chlamydospores/kg) was monitored for a period of 24 months with storage temperatures of < 8°C, 25°C, and 30°C.[21] Results showed that the viability of spores in the product was retained independent of temperature and duration.

Although no data on the stability of the additive in feed or its capacity for homogeneous mixing in feed have been provided, these are not considered necessary. The additive is intended for use with grazing animals which normally requires its supply to animals in the form of a premix/complementary feed. In addition, the target animal simply provides a passive conduit to distribution.

3.1.4. Conditions of use

The additive is intended for use with grazing animals at a daily dose of 1.5 g additive per 25 kg body weight to be incorporated directly in feed or via a premixture. This approximates to 3 $\times$ 10^4 chlamydospores/kg body weight (bw) and day. No withdrawal period is proposed.

[14] Technical dossier/Supplementary information July 2017/Annex FAD_2016_0067_Req_2_1_Microbial contaminant testing CofAs.
[15] Technical dossier/Supplementary information July 2017/Annex SupInfo_FAD_2016_0067_Req_2_2_Heavy metals testing CofAs.
[16] Technical dossier/Supplementary information July 2017/Annex SupInfo_FAD_2016_0067_Req_2_3_Mycotoxin testing CofAs.
[17] Limits of detection: aflatoxin A1, B2, G1 and G2: 1.0 μg/kg, deoxynivalenol: 250 μg/kg, fumonisin B1 and B2: 200 μg/kg, HT2: 100 μg/kg, nivalenol: 250 μg/kg, ochratoxin A: 1.0 μg/kg, T2: 100 μg/kg, total aflatoxin: 4 μg/kg and zearalenone: 25 μg/kg.
[18] Technical dossier/Section II/Annex 2.1.5_04.
[19] Technical dossier/Section II/Annex 2.1.5_05.
[20] Technical dossier/Section II/Annexes 2.4.1.1_02-04.
[21] Technical dossier/Section II/Annexes 2.4.1.2_02-04.

3.2. Safety

3.2.1. Toxicological studies

An acute oral toxicity study (OECD 423) was made with three female CRL:(WI) rats given a single oral (gavage) dose of *D. flagrans* NCIMB 30336 spore concentrate (3.8×10^6 spores/g) formulated in 1% methyl cellulose at the limit dose of 5,000 mg/kg bw.[22] No mortality was observed during the study and no adverse effects were observed over the study period. Accordingly, the additive is considered of low acute toxicity.

An *in silico* assessment of the potential toxicity of flagranone A made with Derek Nexus and Leadscope software allocated this compound to Cramer Class III but indicated that it would be unlikely to be mutagenic, carcinogenic or toxic to reproduction.[23] In particular, it was predicted that steric hindrance would prevent the opening of the epoxide ring required for alkylation.

3.2.2. Safety for the target species

Studies made with cattle,[24] sheep[25] and horses[26] were submitted in which animals were given an overdose of the additive compared to the recommended level. However, none could be further considered due to flaws in the experimental design (e.g. no use level tested, lack of experimental replication, wrong experimental unit).

In the absence of data, no conclusions can be drawn on the safety of BioWorma® for the target species.

3.2.3. Safety for the consumer

Although the fungus in form of chlamydospores is very unlikely to be metabolically active in the digestive tract of the target species, the presence of fungal metabolites produced during cultivation of the fungus and capable of absorption cannot be excluded. [redacted]

The applicant addressed this potential safety issue by demonstrating that the group of secondary metabolites already identified in this species (flagranones) were represented only by flagranone A at low concentration (approximately 11 mg/kg additive).[27] An *in silico* assessment of the potential toxicity of flagranone A indicated that it would be unlikely to be mutagenic, carcinogenic or toxic to reproduction. However, no studies intended to survey the species or strain for the presence of other metabolites was made available. These were presumed absent by examination of chromatograms optimised for the isolation of flagranones.

Although an acute oral toxicity study in rats (Section 3.2.1) showed no evidence of toxicity at the limit dose, such acute studies are not sufficient to exclude toxic effects following chronic exposure or the possible presence of genotoxic agents.

3.2.3.1. Conclusions on safety for the consumer

No issues of concern were raised by the available studies. However, it is not possible to exclude the presence of secondary metabolites, other than flagranone A, produced during the growth of the fungus and their potential carry-over into animal products. Consequently, safety for the consumer cannot be established.

[22] Technical dossier/Section III/Annex 3.2.2.1_01.
[23] Technical dossier/Section III/Annex 3.2.2.2.1_01.
[24] Technical dossier/Section III/Annex 3.1.1_01.
[25] Technical dossier/Section III/Annex 3.1.1_03.
[26] Technical dossier/Section III/Annex 3.1.1_02.
[27] Technical dossier/Section II/Annex 3.2.1.2/02 .

3.2.4. Safety for the user

3.2.4.1. Effects on skin and eyes

Skin[28] and eye[29] irritation studies were performed with New Zealand white rabbits following OECD 405 and 406 guidelines. However, the test item was the premixture described under Section 3.1.3, and not the additive itself. No local dermal signs were seen throughout the 72 h of skin irritation study. Instillation of the test item into the eye produced an initial conjunctivitis which disappeared within 72 h. Fluorescent staining of the cornea remained negative throughout. Consequently, the test item is considered non-irritant to skin and eyes.

An additional *in vitro* test was conducted with isolated chicken eyes following OECD 438 protocol.[30] The test item was similar to that used in the two previous studies. No significant corneal swelling was observed and the test item could not be considered a severe irritant. However, staining showed particles adhering to the cornea which could not be removed by washing. Since these might cause mechanical abrasions, the results of this *in vitro* study were considered equivocal.

In the absence of studies, no conclusions can be drawn on the skin sensitisation potential of the additive.

3.2.4.2. Respiratory tract

The feed additive BioWorma® has been shown to be nearly dust-free and the sieve analysis indicated that there were no particles of less than 50 μm diameter. Thus, inhalation is considered not to be a likely route of exposure. Nonetheless, the applicant made an acute pulmonary toxicity and infectivity study in the rat following US EPA Health Effect Test Guideline OPTTS 855 3150.[31]

Test animals were treated with a single dose of *D. flagrans* NCIMB 30336, in the form of the dry spore powder or the inactivated equivalent by instillation of the spore powder to the lung. The dose was calculated to be approximately 5.8×10^4 spores/animal or 2.0×10^5 spores/kg body weight. Five rats/sex and treatment were used for the data collected on days 21 and 42 and three rats/sex and treatment were used for the other test periods. Animals were observed for signs of toxicity and pathogenicity with animals killed on days 0, 3, 21 and 42. A concurrent untreated control group was included in the study. A microbiological analysis was made to detect the presence of viable spores in the organs of the experimental animals.

Necropsy confirmed that spore deposits spread within the lungs following instillation. The only significant clinical finding was initial difficulties in respiration in all animals which disappeared after 3–4 days. After this period, there were no treatment related effects on body weight/weight gain. *D. flagrans* was not detected in any organs (kidneys, brain, liver, spleen, blood, mesenteric lymph nodes and caecum) except the lung, during the first 21 days. Thereafter there was some evidence of clearing from the lung and no viable counts were obtained after 42 days. In all animals necropsied on day 3, 21 or 42, the lungs were enlarged, multifocal, dark red discoloration could be observed and the peribronchial lymph nodes were enlarged. However, there were no differences between animals treated with living or attenuated spores. All other organs appeared normal.

It was concluded from this study that *D. flagrans* spore preparation administered to rats as a single intratracheal dose does not induce signs of toxicity, infectivity, or pathogenicity, but is irritant to the respiratory tract.

3.2.4.3. Conclusions on safety for the user

Although in tests on skin and eyes the test item used was a commercial mix of the additive with a premix, this is probably the most likely form in which the additive will be used. Results with this test item indicated that BioWorma® in this form is not irritant to skin and eyes. No conclusions can be drawn on the skin sensitisation potential of the additive. The physical properties of the additive make exposure by a respiratory route unlikely. In addition, it was confirmed that *D. flagrans* spore preparations, do not induce signs of toxicity, infectivity, or pathogenicity but are irritant to the respiratory tract. Owing to the proteinaceous nature of the active agent, the additive is considered a respiratory sensitiser.

[28] Technical dossier/Section III/Annex 3.3.1.2.1_01.
[29] Technical dossier/Section III/Annex 3.3.1.2.2_02.
[30] Technical dossier/Section III/Annex 3.3.1.2.2_01.
[31] Technical dossier/Section III/Annex 3.3.1.1_01.

3.2.5. Safety for the environment

D. flagrans is a naturally inhabiting soil organism of widespread distribution. Use of the product is very unlikely to measurably increase numbers in soils where the fungus is already prevalent. Consequently, the Panel considers that use of an additive based on chlamydospores of *D. flagrans* will not pose a risk for the environment.

3.3. Efficacy

The deliberate introduction of nematophagous fungi into pasture as a means of controlling eelworm was first proposed in 1957 by Duddington (Duddington, 1957). Since this date the practical use of *D. flagrans* (and other predaceous fungi) for the control of gastro-intestinal nematodes in sheep, goats, cattle and horses has been extensively studied. Chlamydospores are extracted from cultures of the fungus and are fed to the target species usually by incorporation into the feed and occasionally by addition to mineral licks (Sagüés et al., 2011). Ingested spores pass through the digestive tract apparently without germination and are deposited on pasture with the faeces. Here the environmental conditions favour germination of the chlamydospores and the production of mycelium with its hyphal traps. In principle, trapping reduces the number of nematodes able to migrate to herbage and infect/re-infect the grazing animals.

The results of eight short-term and four long-term studies made with BioWorma® in New South Wales or Queensland, Australia with cattle, goats, sheep and horses are described in support of the efficacy of the additive. In addition, reference is made to 41 studies concerned with the control of nematode parasites made *in vitro* or after passage of *D. flagrans* strains through the digestive tract. A further 40 published studies made with grazing animals throughout the world (including the EU) are also cited involving strains of *D. flagrans* other than the one under application.

3.3.1. Studies on the control of nematodes *in vitro* or after passage through the digestive tract

The published *in vitro* studies submitted were made with a variety of *D. flagrans* isolates. Strains are obligate aerobes and as such grow and are effective only at the surface of dung pats where nematode numbers are generally highest. Growth is optimal between 20 and 30°C and the presence of nematodes is essential for the induction of trap production by the fungus. On agar, the fungus loses trap inducibility after 2–3 weeks with a concomitant production of chlamydospores (Grønvold et al., 1996, 1999). Agar plate assays in which nematode larvae are incubated in the presence or absence of *D. flagrans* conidia have demonstrated the ability of the fungus to significantly reduce larval counts or larval emergence of a wide range of nematode parasites of ruminants and horses (e.g. Casillas–Aguila et al., 2008; Braga et al., 2010).

In other studies, animals were given *D. flagrans* chlamydospores and then faeces collected directly from the rectum or from pasture. Faecal material was incubated for varying periods and the emerging larval populations counted and compared with counts from faeces of control animals (e.g. Flores-Crespo et al., 2003; Paraud and Chartier, 2003; Waghorn et al., 2003; Paraud et al., 2005).These coproculture experiments confirmed than the various isolates of *D. flagrans* were effective in substantially reducing numbers of infective larvae of a wide range of nematode parasites.

3.3.2. Short-term studies with BioWorma®

A series of eight short-term trials made in Australia are described in which the effects of BioWorma® supplementation of feed for cattle, goats or horses on pasture nematode populations were measured in different seasons (Table 2). In each case, animals were given a premixture containing BioWorma® to reach an intended daily application rate of 3.4×10^4 chlamydospores/kg body weight for periods of 5–9 weeks. The inclusion level was confirmed by the analysis of the *D. flagrans* spores in the premixture. The trial animals harboured a variety of parasitic nematodes, which in the case of the goat studies were augmented via artificial infection. The animals were fed the premix alone for about 1 week prior to collection of faecal material over a 24-h period (cattle and horses) or 48-h period (goats). Four sample pats per animal (of ~ 1 kg horses, ~ 500 g cattle, ~ 100 g goats) were manually placed on pasture at each of two trial sites in seven studies and at a single site in one study. The pastures chosen were considered representative of the region and had not been grazed for at least two months previously. The same animals were then treated with BioWorma® for about 1 week and faeces again collected as previously and placed on pasture at the same sites as the control samples. At two-weekly intervals thereafter for 8 weeks pasture samples from around (40 cm radius) and under

the selected deposited faecal samples were collected and examined for nematode larvae. The larvae found were then identified and the total parasitic larval numbers for each sample was calculated.

In the cattle trials, the nematode types encountered were *Cooperia* spp., *Trichostrongylus* spp., *Oesophagostomum* spp., *Ostertagia* spp. and *Haemonchus* spp. In goats, the nematodes were *Teladorsagia* spp., *Trichostrongylus* spp., *Nematodirus* spp., *Haemonchus* spp. and *Cooperia* spp. Goats were also challenged with a mixture of gastrointestinal strongyle larvae. In horses the dominant species were cyathostomes; however, some *Strongylus* spp. and *Trichostrongylus axei* were also present.

Individual animal data were used to calculate group means. Treatment efficacies were determined by comparison of treated and untreated group mean data using Abbott's formula. The results of the eight trials are summarised in Table 2.

Table 2: Effects of *D. flagrans* NCIMB 30336 on mean numbers of parasitic nematodes found in and around faecal pats of approximately 0.5 m^2. Results were pooled over a 2- to 8-week period

Trial (Season)	Species (No of animals)	Trial site 1			Trial site 2		
		Control samples	Test samples	Reduction (%)	Control samples	Test samples	Reduction (%)
1[32] (Spring)	Cattle (6)	113,086	25,967	75	669	116	75
2[33] (Autumn)	Cattle (6)	96,377	17,246	82	46,484	5,470	88
3[34] (Spring)	Goat (6)	126,107	17,247	85	4,698	4,306	8[1]
4[35] (Autumn)	Goat (12)	860	165	81	52,578	635	99
5[36] (Spring/ summer)	Goat (12)	110,683	21,998	80	13,856	307	98
6[37] (Autumn)	Horse (5)[2]	6,081	307	94	–	–	–
7[38] (Spring)	Horse (6)	16,996	479	97	1,512	705	53
8[39] (Autumn)	Horse (6)	18,562	6,431	65	4,719	302	94

[1]Unexpected freezing conditions after deposition of faeces.
[2]Single paddock used.

The results of the eight trials showed that supplementation of feed for cattle, goats and horses with *D. flagrans* chlamydospores in the form of BioWorma® can reduce the number of parasitic nematode larvae present on pasture. However, in the absence of a proper statistical analysis, these results are taken as supportive evidence only.

3.3.3. Long-term studies with BioWorma®

The short-term studies made with BioWorma® were designed only to test the effects of *D. flagrans* when added to feed on the population of parasitic nematodes on pasture and not the subsequent consequences for the grazing animal. To address this issue, four long-term trials were made to assess the impact of reducing pasture numbers of nematode larvae on the parasitic nematode burden of grazing sheep.

[32] Technical dossier/Section IV/Annex 4.2_01.
[33] Technical dossier/Section IV/Annex 4.2_02.
[34] Technical dossier/Section IV/Annex 4.2_03.
[35] Technical dossier/Section IV/Annex 4.2_04.
[36] Technical dossier/Section IV/Annex 4.2_05.
[37] Technical dossier/Section IV/Annex 4.2_06.
[38] Technical dossier/Section IV/Annex 4.2_07.
[39] Technical dossier/Section IV/Annex 4.2_08.

The four trials followed a common design and location (Australia) but differed in duration and number of animals involved (Table 3). In each case two groups of sheep were used with a different purpose:

- Seeder sheep, which harboured natural infections of a range of parasitic worms, used to contaminate pasture by dropping faeces bearing worm eggs.
- Tracer sheep, young animals free of any worm burden, used to assess the degree of worm contamination of the pasture on which they grazed.

In each trial, a pair of matched paddocks was used; one paddock was grazed with a group of seeder sheep that received a daily supplement of the premix (control group) and the other by a matching group that received a supplement of the same premix including BioWorma,® intended to provide a daily application rate of 3×10^4 chlamydospores/kg body weight (Table 3). The seeder sheep were allowed to graze the pasture for periods of 57–125 days. To consider seasonality, the trials were conducted in summer/autumn (1 and 4) or spring/summer (2 and 3). Faecal egg counts (FECs) in individual seeder sheep and group bulk coprocultures were monitored fortnightly for the level of infection and the potential for egg deposition on pasture. In addition, at the start of the trial ten faecal samples from each group were analysed for the presence of *D. flagrans*.

In the first trial at the end of the first phase, seeder sheep were removed from the paddocks and replaced with two groups of tracer sheep. Prior to their introduction, all tracer sheep were treated once orally with the clean-out formulation and were confirmed to be free of worm infestation using FECs post treatment. One group of tracers grazed the control paddock and a matching group grazed the BioWorma® paddock for a period of 3 weeks after which the tracer animals were transferred to raised pens to allow their worm burdens to 'mature'. The degree of infection was determined by total worm counts (TWC) made after killing and gut washing of ten animals per group. In the remaining three trials, groups of tracer sheep were introduced at two different time points in the presence of the seeder sheep and allowed to graze for 21 days followed by removal, killing and the determination of TWCs.

Group mean (arithmetic and geometric) FEC and TWC results were analysed for significant differences by a one-way analysis of variance. When data from groups were not normally distributed as shown by a Barletts test, a non-parametric Kruskal–Wallis test was applied. Differences in pairwise comparison of group means were considered significant at $P < 0.05$. A summary of the results of the four trials is shown in Table 3. Only group data expressed as arithmetic means are shown.

Table 3: Effects of supplementation of the diets of grazing sheep with *D. flagrans* NCIMB 30336 on parasitic nematode larval development, migration from faeces on pasture and potential infection of other sheep grazing the same pasture

Trial No	Seeder sheep				Tracer sheep			
	No of animals	Duration of grazing (days)	Final faecal egg count Control	Final faecal egg count BioWorma®	No of animals	Total worm count[1] Control group	Total worm count[1] BioWorma® group	Reduction (%)
1[40]	20/20	57	1,292[a]	424[b]	23/23	10,024[a]	4,296[b]	57
2[41]	30/30	119	393[a]	261[b]	2 × 10/10	10,295[a]	4,480[b]	57
3[42]	30/30	122	683[a]	616[b]	2 × 10/10	14,487[a]	2,357[b]	84
4[43]	30/30	125	76	259	2 × 10/10	3,037[a]	770[b]	75

[1]Data from second introduction only for trials 2–4 (from day 98 in trial 2, day 101 in trial 3 and day 104 in trial 4).
[a,b]: Means within the same row and means type with different superscripts are significantly different at $p < 0.05$.

[40] Technical dossier/Section IV/Annex 4.3_01.
[41] Technical dossier/Section IV/Annex 4.3_02.
[42] Technical dossier/Section IV/Annex 4.3_03.
[43] Technical dossier/Section IV/Annex 4.3_04.

The results showed that supplementation of sheep with *D. flagrans* NCIMB 30336 in the form of BioWorma® resulted in a lower level of infectivity of pasture with a concomitant reduction in total worm counts in tracer animals grazing on the same pasture. However, in the three trials where tracer sheep were introduced at two time-points after the introduction of the seeder sheep, significant benefits were seen only with the later introduction (shown in Table 3).

3.3.4. Published efficacy studies made with other strains of *D. flagrans*

Although the concept of using nematophagous fungi, and in particular *Duddingtonia*, was developed in Australia and much of the proof of concept was done there, evidence of the capacity of *D. flagrans* to biologically control nematodes on pasture has been shown in many countries including EU Member States. This work has been extensively reviewed by Knox (2003), Soder and Holden (2005), Ketzis et al. (2006), Jagla et al. (2013) and Terry (2013).

Studies in EU Member States with strains of *D. flagrans* have been made with cattle in Sweden (Dimander et al., 2003a,b), in Denmark (Grønvold et al., 1993; Wolstrup et al., 1994; Larsen et al., 1995; Nansen et al., 1995; Fernández et al., 1999a,b,c,d,e) and in Switzerland (Hertzberg et al., 2007). Corresponding studies have been made with goats in Spain (Gomez-Rincon et al., 2007) and France (Chartier and Pors, 2003; Paraud et al., 2007) and with sheep in the Netherlands (Githiga et al., 1997; Faedo et al., 2000; Eysker et al., 2006a,b), Germany (Epe et al., 2008, 2009), Spain (Gomez-Rincon et al., 2006), Sweden (Waller et al., 2001) and Switzerland (Faeesler et al., 2007). Other studies made in Denmark have included horses (Larsen et al., 1996; Fernández et al., 1997) and pigs (Nansen et al., 1996).

The European studies were similar in design to the short- and long-term studies made in Australia and described above. Some investigated only the degree of pasture infection when faeces from worm-infected animals that had been fed with *D. flagrans* were collected and manually placed on pasture. The pasture surrounding these faecal pats was then periodically tested for parasitic nematode larvae and results were compared with those for matching faecal pats from control animals not given *D. flagrans* chlamydospores. Other studies, following the design of the long-term studies, involved the use of tracer animals to provide a direct (FEC, coproculture, TWC) or indirect (improved weight gain) measure of the degree of pasture infectivity.

Overall the results of studies made in Europe with a range of *D. flagrans* isolates mirror those made with *D. flagrans* NCIMB 30336 in Australia. Although the strain under application has not been tested under European conditions, it is unlikely that geographical location would affect its nematophagous properties.

3.3.5. Conclusions on efficacy

Based on the data provided, BioWorma® can reduce number of parasitic nematodes on pasture to the benefit of grazing animals when used at the recommended application rate.

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[44] and Good Manufacturing Practice.

4. Conclusions

In the absence of data, no conclusions can be drawn on the safety of BioWorma® for the target species

As it is not possible to exclude the presence of secondary metabolites produced during the growth of the fungus and their potential carry-over into animal products, safety for the consumer cannot be established.

The Panel concludes that the additive is not irritant to skin and eyes but is irritant to the respiratory tract and a respiratory sensitiser. No conclusion can be drawn on its skin sensitisation potential.

D. flagrans is a naturally inhabiting soil organism of widespread distribution. Use of the product is very unlikely to measurably increase numbers in soils where the fungus is already prevalent.

[44] 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.

Consequently, the Panel considers that use of an additive based on chlamydospores of *D. flagrans* will not pose a risk for the environment.

The additive can reduce the number of parasitic nematodes on pasture to the benefit of grazing animals when used at the recommended application rate of 3×10^4 chlamydospores/kg bw and day.

5. Documentation as provided to EFSA/Chronology

Date	Event
12/10/2016	Dossier received by EFSA. BioWorma® (*Duddingtonia flagrans* NCIMB 30336) for grazinbg animals. Submitted by International Animal Health Product Pty Ltd represented in the EU by GAB Consulting GmbH
20/12/2016	Reception mandate from the European Commission
10/04/2017	Application validated by EFSA – Start of the scientific assessment
23/05/2017	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. *Issues: characterisation and safety and*
08/06/2017	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. *Issues: methods of analysis*
10/07/2017	Comments received from Member States
27/07/2017	Reception of supplementary information from the applicant - Scientific assessment still on hold
14/01/2020	Reception of supplementary information from the applicant - Scientific assessment re-started
22/01/2020	Reception of the Evaluation report of the European Union Reference Laboratory for Feed Additives
02/07/2020	Opinion adopted by the FEEDAP Panel. End of the Scientific assessment

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EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2012b. Guidance for establishing the safety of additives for the consumer. EFSA Journal 2012;10(1):2537, 12 pp. https://doi.org/10.2903/j.efsa.2012.2537

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Abbreviations

bw	body weight
CFU	colony forming unit
CIPAC	Collaborative International Pesticide Analytical Council
CSIRO	Commonwealth Scientific and Industrial Research Organisation
EURL	European Union Reference Laboratory
FEC	Faecal egg counts
FEEDAP	EFSA Panel on Additives and products or Substances used in Animal Feed
ITS	internal transcribed spacers
MPN	most probable number
NCIMB	National Collection of Industrial, Food and Marine Bacteria
PCR	polymerase chain reaction
TWC	total worm counts
YMA	yeast mannitol agar

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

In the current application authorisation is sought under Article 4(1) for *Duddingtonia flagransIAH 1297*[45] under the category / functional group 4(d) 'zootechnical additives' /'other zootechnical additives', according to Annex I of Regulation (EC) No 1831/2003. Specifically, the authorisation is sought for the use of *Duddingtonia flagrans IAH 1297* as feed additive for all grazing animals.

According to the Applicant, the *feed additive* contains the viable spores of non-genetically modified *Duddingtonia flagrans IAH 1297* as active agent. The feed additive is intended to be marketed as a preparation under the trade name of BioWorma®, containing a minimum of 5×10^5 spores of *Duddingtonia flagrans IAH 1297* /g *feed additive*.

The *feed additive* is to be used in *premixtures* and *feedingstuffs*. The Applicant proposed the minimum dose of the feed additive expressed in terms of 3×10^4 spores of *Duddingtonia flagrans IAH 1297* per kg of body weight of animal per day.

For the identification/characterisation of the feed additive the EURL recommends for official control a polymerase chain reaction (PCR) method as specified by the Applicant for the genetic identification of *Duddingtonia flagrans IAH 1297*.

For the enumeration of the active agent (viable spores) of *Duddingtonia flagrans IAH 1297* in the *feed additive, premixtures* and *feedingstuffs* the Applicant submitted a single-laboratory validated and further verified method based on yeast mannitol agar (YMA) containing the antibiotics streptomycin and chloramphenicol by using a most probable number (MPN) procedure described in European Pharmacopeia monographs (01/2008:201612 and 20613).

Based on the overall experimental evidence available the EURL recommends for official control the above mentioned single-laboratory validated and further verified YMA-MPN method for the enumeration of the active agent (viable spores) of *Duddingtonia flagrans IAH 1297* in the *feed additive* and *premixtures.*

As the dose of the active agent (viable spores of *Duddingtonia flagrans IAH 1297*) presented by the Applicant was not expressed as number of the spores per mass unit of *feedingstuffs*, the EURL cannot recommend for official control the YMA-MPN method for the enumeration of the active agent (viable spores) of *Duddingtonia flagrans IAH 1297* in *feedingstuffs*. However, the method is fit-for-purpose for the enumeration of the active agent (viable spores) of *Duddingtonia flagrans IAH 1297 in feedingstuffs* at the validated and verified range of content of *Duddingtonia flagrans IAH 1297*.

[45] Now renamed as *Duddingtonia flagrans* NCIMB 30336