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Safety and efficacy of a feed additive consisting on the bacteriophages PCM F/00069, PCM F/00070, PCM F/00071 and PCM F/00097 infecting *Salmonella Gallinarum* B/00111 (Bafasal[®]) for all avian species (Proteon Pharmaceuticals S.A.)

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Abstract

Following a request from the European Commission, the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) was asked to deliver a scientific opinion on the feed additive consisting of four bacteriophages infecting *Salmonella Gallinarum* B/00111 (PCM F/00069, PCM F/00070, PCM F/00071 and PCM F/00097, trade name: Bafasal[®]) when used as a zootechnical additive in water for drinking and liquid complementary feed for all avian species. The effects sought are the reduction of the *Salmonella* spp. carriage in chickens for fattening, the improvement of their performance, or both. The host strain harbours an acquired antimicrobial resistance gene. No viable cells or DNA from the host organism were found in the additive. The four phages proved to be strictly lytic and to have a machinery allowing to package a unit-length of the viral genome. The manufacturing process excludes the presence of remnants from the propagation process in the final additive. Consequently, no concerns are expected from the nature and manufacture of the product. Considering this and the results of the tolerance study with chickens for fattening, the Panel concluded that Bafasal[®] is safe for all avian species. Considering the nature and manufacturing process of the additive, Bafasal[®] is not expected to pose a risk for consumers. The results of the subchronic oral toxicity study and genotoxicity studies provided support this conclusion. Exposure of users via inhalation is expected to be low, but Bafasal[®] should be considered a respiratory sensitiser. No conclusions were drawn on the irritancy of Bafasal[®] to skin and eyes or on its dermal sensitisation potential due to lack of data. Considering the nature and manufacturing process of the additive, Bafasal[®] is safe for the environment. The Panel was not in the position to conclude on the efficacy of Bafasal[®] for any avian species due to insufficient data.

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Keywords: Zootechnical additive, bacteriophages, *Salmonella enterica* ser. Gallinarum B/00111, Bafasal[®], safety, efficacy, avian species

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1. Introduction

1.1. Background and Terms of Reference

Regulation (EC) No 1831/2003¹ 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 Proteon Pharmaceuticals S.A.² for authorisation of the feed additive consisting of the bacteriophages PCM F/00069, PCM F/00070, PCM F/00071 and PCM F/00097 infecting *Salmonella enterica* ser. Gallinarum B/00111, when used as a feed additive for all avian species (category: zootechnical additive; functional group: other zootechnicals).

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 22 January 2018.

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 Bafasal® (preparation of the bacteriophages PCM F/00069, PCM F/00070, PCM F/00071 and PCM F/00097 infecting *Salmonella enterica* ser. Gallinarum B/00111), when used under the proposed conditions of use (see Section 3.1.4).

1.2. Additional information

The subject of the assessment is the product consisting of four bacteriophages (PCM F/00069, PCM F/00070, PCM F/00071 and PCM F/00097) infecting *S. enterica* ser. Gallinarum B/00111. It has not been previously authorised as a feed additive in the European Union.

2. Data and methodologies

2.1. Data

The present assessment is based on data submitted by the applicant in the form of a technical dossier³ in support of the authorisation request for the use of the product consisting of four bacteriophages (PCM F/00069, PCM F/00070, PCM F/00071 and PCM F/00097) infecting *S. enterica* ser. Gallinarum B/00111 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 agents in animal feed. The Executive Summary of the EURL report can be found in Annex A.⁴

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of the product consisting of four bacteriophages (PCM F/00069, PCM F/00070, PCM F/00071 and PCM F/00097) infecting *S. enterica* ser. Gallinarum B/00111 is in line with the principles laid down in Regulation (EC) No 429/2008⁵ 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), Technical Guidance for assessing the safety of feed additives for the environment (EFSA, 2008a), Guidance on studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012b), Technical Guidance: Extrapolation of data from major

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 Proteon Pharmaceuticals S.A., ul. Tylina 3a, 90-364, Łódź, Poland.

³ FEED dossier reference: FAD-2017-0039.

⁴ The full report is available on the EURL website: <https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2017-0039-Bafasal.pdf>

⁵ 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.

species to minor species regarding the assessment of additives for use in animal nutrition (EFSA, 2008b), Guidance on the assessment of the safety of feed additives for the consumer (EFSA FEEDAP Panel, 2017) and Guidance on the characterisation of microorganisms used as feed additives or as production organisms (EFSA FEEDAP Panel, 2018).

3. Assessment

The subject of the assessment is a product consisting of four bacteriophages (PCM F/00069, PCM F/00070, PCM F/00071 and PCM F/00097) infecting *S. enterica* ser. Gallinarum B/00111, with the trade name Bafasal®, intended to be used as a zootechnical additive (functional group: other zootechnical additives: animal welfare and food hygiene) in water for drinking and liquid complementary feed for all avian species. It will be hereafter referred to as Bafasal®. The applicant described the effects sought as 'the reduction of the *Salmonella* spp. contamination in broilers or the improvement of performance of treated animals, or both'.⁶

3.1. Characterisation

Bafasal® is a liquid preparation containing four lytic bacteriophages infecting *S. enterica* ser. Gallinarum B/00111 (PCM F/00069, PCM F/00070, PCM F/00071 and PCM F/00097⁷) at a minimum of 5×10^7 total plaque forming units (PFU) per mL of product (1.25×10^7 PFU of each phage/mL additive).

3.1.1. Characterisation of the active agents

The four bacteriophages were isolated from domestic wastewater or broiler faeces and are propagated in a strain of *S. enterica* ser. Gallinarum. This host bacterial strain has not been genetically modified and is deposited at the Polish Collection of Microorganisms (PCM) in the Ludwik Hirsfeld Institute of Immunology and Experimental Therapy of Polish Academy of Sciences, under the deposit number PCM B/00111.⁸ The phages are deposited at the PCM under the following accession numbers: PCM F/00071, PCM F/00070, PCM F/00069 and PCM F/00097.⁹

The host strain used for the phage propagation is *S. Gallinarum* PCM B/00111 and belongs to a serotype known to be pathogenic for poultry. The host strain was typed with polymerase chain reaction-melting profile using *Hind*III as restriction enzyme.¹⁰ The complete whole genome sequence (4.6 MB) has been interrogated for the presence of genes coding for *S. enterica* virulence and toxigenic factors.¹¹

The antimicrobial susceptibility testing of *S. Gallinarum* PCM B/00111 to the antimicrobials recommended by the FEEDAP Panel was determined by broth microdilution following the PN-EN ISO

⁶ Technical dossier/Supplementary information December 2018/Annex 2 FAD-2017-0039_reply-SIn_EFSA_291018.

⁷ In-house identifiers used by the applicant (also in the EURL report): 3sent1 for PCM F/00071, 8sent65 for PCM F/00070, 8sent1748 for PCM F/00069 and 5sent1 for PCM F/00097.

⁸ Technical dossier/Section II/Annex II.2.1.2.1.

⁹ Technical dossier/Supplementary information December 2018/Annex II.2.1.2.1.1.

¹⁰ Technical dossier/Section II/Annex II.2.1.2.2

¹¹ Technical dossier/Section II/Annex II_2_1_2_3.

¹² Technical dossier/Section II.

¹³ Technical dossier/Section II/Annex II.2.1.2.4.Conf.

¹⁴ Technical dossier/Section II/Annex II.2.1.2.3.Conf.

20776-1:2007 standards.¹⁵ With two exceptions, all the minimum inhibitory concentration (MIC) values found were equal or fell below the corresponding cut-off values for Enterobacteriaceae (EFSA FEEDAP Panel, 2018). The exceptions were the MIC values for ciprofloxacin (4 vs 0.06 mg/L) and streptomycin (64 vs 16 mg/L) which were several dilutions higher than the cut-off values.

[REDACTED]

The host strain is resistant to ciprofloxacin and streptomycin and harbours genes coding for the resistance to these critically important antibiotics (WHO, 2016). The resistance to ciprofloxacin is related to a mutation in a housekeeping gene while the aminoglycoside resistance is coded by an acquired antimicrobial resistance gene.

3.1.2. Characterisation of the additive

[REDACTED]

a minimum concentration of 1.25×10^7 PFUs of each phage per millilitre of additive or 5×10^7 total PFU/mL additive.

The batch-to-batch variation of five batches of the additive showed compliance with the minimum specifications based on total PFU counts (mean 1.71×10^8 PFU/mL, range of $1.14\text{--}3.23 \times 10^8$ PFU/mL).¹⁸

No viable cells of the host strain were detected in twelve¹⁹ batches of the additive (25 mL each), confirming absence of the production strain and compliance with the established specifications for *Salmonella* spp. (not detected in 25 mL).²⁰

[REDACTED]

Bafasal® is regularly tested for microbial contaminants and undesirable substances. Limits are set for arsenic (2 mg/kg), lead (10 mg/kg), mercury (0.1 mg/kg), cadmium (2 mg/kg), aflatoxin B1 (0.02 mg/kg), deoxynivalenol (5 mg/kg), fumonisins B1 and B2 (20 mg/kg for the sum of the two), ochratoxin A (0.1 mg/kg), dioxins (1.5 ng WHO-PCDD/F-PCB-TEQ/kg), dioxins and dioxin-like PCBs (1 ng WHO-PCDD/F-PCB-TEQ/kg), sum of non-dioxin-like PCBs (10 µg/kg), β-glucuronidase-positive *Escherichia coli* (absent in 25 mL). Analysis of three batches showed that the product complies with these specifications and that the additional tested contaminants are not a cause of concern (i.e. total aerobic microorganisms count (< 1 CFU/mL), Enterobacteriaceae (< 1 CFU/mL), yeasts and filamentous fungi (< 1 CFU/mL), toxin T-2 (< 0.05 mg/kg), toxin HT-2 (< 0.05 mg/kg) and zearalenone (< 0.01 mg/kg)).¹⁸ The concentration of bacterial endotoxins was tested in nine batches of the additive using two commercial kits based on methods recommended by the European Pharmacopeia

¹⁵ Technical dossier/Supplementary information December 2018/Annex II.2.2.3.

¹⁶ Technical dossier/Supplementary information December 2018/Annex II.2.2.3 and Supplementary information March 2019/Annexes II.2.2.3.1.Conf, II.2.2.3.2.Conf, II.2.2.3.3 and II.2.2.3.4.

¹⁷ Technical dossier/Section II/Annex II.3.1.2.Conf. and Supplementary information December 2018.

¹⁸ Technical dossier/Section II/Annexes II.1.4.2.1-II.1.4.2.4.

¹⁹ Three batches tested using the method PN-EN ISO 6579:2003 and 9 using the method ISO 6579-1:2017.

²⁰ Technical dossier/Section II/Annexes II.1.4.2.1-II.4.2.4 and Supplementary information March 2019/Annexes II.1.4.2.1, II.1.4.2.3. and II.1.4.2.4 and Supplementary information December 2019/Annexes II.1.4.2.5a- II.1.4.2.5f and II.1.4.2.6.

²¹ Technical dossier/Supplementary information December 2018/Annex II.2.2.4 and Supplementary information March 2019/Annex II.2.2.4.Conf.Updated and Supplementary information October 2020/Annex II.2.4.2.8.

(amebocyte lysate and chromogenic techniques). The results showed a high variability (mean: 528 IU/mL, range: 12–1,645 IU/mL).²²

3.1.3. Stability and homogeneity

The applicant proposes a shelf-life of 12 months when stored in its original closed packaging at 2–10°C. Stability was investigated in a test involving three batches stored at 5°C and at 25°C, 60% relative humidity for 12 and 6 months, respectively.²³ Plaque counts at 5°C showed losses < 0.5 log after 12 months whilst those at 25°C decreased by 0.5 and 0.6 log values after 6 months.

A short-term stability test was made with three batches of Bafasal® suspended in three different matrices of poultry liquid complementary feed (the same used in the *in vitro* studies in Section 3.3.1) to reach a concentration of 2×10^9 PFU/L, and stored at 25°C.²⁴ Losses in plaque counts after 2 days were < 0.5 log in all cases except in one in which they reached 0.6 log.

The stability of Bafasal® (one batch) suspended in water for drinking at 25°C was tested after 2 days.²⁵ Losses in plaque counts were < 0.5 log.

The capacity of Bafasal® (one batch) to be homogeneously suspended in the same three matrices of liquid feed (based on 10 subsamples) according to the conditions of use was investigated in one study.²⁶ Analyses of plaque counts showed a coefficient of variation of 0.6%.

3.1.4. Conditions of use

Bafasal® is proposed for use in water for drinking and liquid complementary feed for all avian species at the minimum dose of 2×10^6 PFU/bird per day, equivalent to 0.04 mL additive per bird and day during the whole life of the birds. According to the applicant, this would translate into a variable inclusion level in water for drinking and liquid complementary feed as presented in Table 1.²⁷

Table 1: Proposed inclusion level of Bafasal® in feed and water for avian species

Days of life	PFU/L water	PFU/kg feed
1–7	5.6×10^7	2.9×10^7
8–14	2.2×10^7	5.0×10^6
15–21	1.4×10^7	2.4×10^6
22–28	1.1×10^7	1.3×10^6
29–35	8.5×10^6	8.2×10^6
36–42	7.2×10^6	5.6×10^6
43–49	6.5×10^6	4.2×10^6
After 49	6.1×10^6	3.4×10^6

PFU: plaque forming unit.

3.2. Safety

3.2.1. Safety of the production organism and product

The host strain used for the Bafasal® phages propagation is a known pathogen for poultry, owing to the presence of virulence factors in its genome and was shown to be resistant to ciprofloxacin and streptomycin. The resistance to ciprofloxacin is related to a mutation and therefore considered of no concern. The resistance to aminoglycoside is due to an acquired antimicrobial resistance gene. However, no viable cells or DNA from the host organism were found in the additive.

The Bafasal® phages PCM F/00071, PCM F/00070 and PCM F/00069 showed homology to the lytic phages from genus *Tequintavirus*, while PCM F/00097 showed homology to the lytic phages from

²² Technical dossier/Section II/Annexes II.1.4.2.1-II.4.2.4 and II.1.3 and Supplementary information December 2019/Annex II.1.4.2.6.

²³ Technical dossier/Section II/AnnexesII.4.1.1.1-II.4.1.1.3.

²⁴ Technical dossier/Section II/AnnexesII.4.1.2.1-II.4.1.2.3.

²⁵ Technical dossier/Section II/Annex II.4.1.3.

²⁶ Technical dossier/Section II/Annexes II.4.2.1-II.4.2.3.

²⁷ Technical dossier/Supplementary information January 2021/FAD-2017-0039_AppSIn_Jan21.

genus *Jerseyvirus*. No lysogenic genes were identified in the genome of the four phages tested, denoting that they are strictly lytic.

The manufacturing process of Bafasal® includes several filtration steps which would exclude the presence of remnants from the propagation process. Consequently, no concerns are expected from the nature or manufacturing process of Bafasal®.

3.2.2. Safety for the target species

The applicant has performed a tolerance trial with chickens for fattening that is described below.

A total of 1,294 one-day-old male and female Ross 308 broilers were distributed in groups of 20–23 birds to four dietary treatments (four treatment groups of 16 single sex pens, 8 of females and 8 of males, 20–23 chickens per pen) for a period of 38 days.²⁸ The dietary treatments resulted from the supplementation of Bafasal® via water for drinking in a concentration to reach daily doses of: 0, 2×10^6 PFU/bird ($1 \times$ minimum recommended daily dose), 2×10^7 PFU/bird ($10 \times$) or 2×10^8 PFU/bird ($100 \times$). The specifications of the additive (8.4×10^7 PFU/mL) and intended concentration in water (average concentrations were 8.7×10^6 PFU/L for the $1 \times$ group, 8.9×10^7 PFU/L for the $10 \times$ group and 9.4×10^8 PFU/L for the $100 \times$ group) were confirmed by analysis. Chickens had free access to maize/soybean meal/wheat-based mash diets (free from *Salmonella* spp.) (starter, grower I and grower II) and water was provided *ad libitum*. Pen feed intake was measured daily, body weight (on a pen basis) on days 0, 12, 22 and 35 and from these data, bodyweight gain and feed to gain ratio were calculated. Pen water intake was measured at weekly intervals; however, it was unclear how these measurements were performed. At the end of the trial during 4 consecutive days (35–38), blood samples were collected from 1 bird per pen (one bird at each time point), selected at random, for haematology²⁹ and biochemistry³⁰ analyses. Feed and water intake and performance data were analysed with an analysis of variance and means were compared with Tukey's test. The pen was the experimental unit for all the parameters.

Based on the analysed concentration of the additive in the water and on the average daily water intake of birds ($1 \times$ group: 0.249 L/bird, $10 \times$: 0.249 L/bird and $100 \times$: 0.247 L/bird), Bafasal® daily intake of each experimental group was confirmed (2.2×10^6 , 2.2×10^7 and 2.3×10^8 PFU/bird, for $1 \times$, $10 \times$ and $100 \times$ groups, respectively). The count of bacteriophages in drinking water in the three groups during the first week were lower than intended. Young animals received 0.5–1 log less bacteriophages than the intended dose during this period.

Overall mortality (including culling) was 3.1% in the control group, 4.6% in the $1 \times$ group, 4.0% in the $10 \times$ group and 4.4% in the $100 \times$ group and was not treatment related.

Performance was not affected by any treatment (mean daily feed intake was 100 g (99.2–100.7 g), final body weight was 2.39 kg and feed to gain ratio was 1.51 (1.49–1.53)). Therefore, feeding the birds with the Bafasal® up to 100-fold the recommended inclusion level did not have any negative effects on the performance of the birds receiving it.

No significant differences between treatments were observed in any of the other parameters tested, except for a reduction of the erythroblast proportions in males in the treatment groups receiving the additive at the recommended level and at $10 \times$ overdose, and an increase in blood aspartate aminotransferase and alanine aminotransferase concentrations in females receiving the additive at the recommended level. These differences are considered of no concern.

The bacterial endotoxins concentration measured in nine batches of the additive showed a mean value of 525 IU/mL (ranging from 12 to 1,645 IU/mL). This is not considered of concern for the target species when consumed orally since the values are much lower than those commonly found in feedingstuffs (ca. 1×10^6 IU/g, Cort et al., 1990), and the product, being in liquid form, is not expected to be inhaled.

Considering the results of the tolerance study with chickens for fattening where a margin of safety of up to $100 \times$ was identified, and the nature and manufacturing process of the additive (see Section 3.2.1), the Panel concludes that Bafasal® is safe for all avian species at the proposed conditions of use.

²⁸ Technical dossier/Section III/Annex III.1.1.

²⁹ Erythrocytes, haematocrit, haemoglobin, leucocytes, segmented neutrophils, lymphocytes, monocytes, eosinophils, basophiles, erythroblasts.

³⁰ Total protein, cholesterol, aspartate aminotransferase, alanine aminotransferase, gamma-glutamyltransferase, uric acid, albumin, globulin, bilirubin, calcium, phosphate inorganic.

3.2.3. Safety for the consumer

3.2.3.1. Genotoxicity

The applicant provided two *in vitro* mammalian cell gene mutation tests and three *in vitro* micronucleus assays. One *in vitro* mammalian cell gene mutation test³¹ and one *in vitro* micronucleus assay³² were disregarded since the concentration of the test item used (i.e. 2 µL/mL) was below the level recommended by the relevant OECD TGs. A second *in vitro* micronucleus assay³³ was also disregarded because the frequencies of micronuclei observed in the negative controls after short treatment (i.e. PBS 3 h (-S9): 32‰, 3 h (+S9): 35 ‰; water 3 h (-S9): 28‰, 3 h (+S9): 35‰) were significantly higher than the values reported in the negative control after continuous treatment (i.e. 27h (S-9): 12‰). In addition, the high frequency of micronuclei observed in this study was not consistent with the frequency observed in the study submitted in the original dossier³² and was above literature values for micronuclei frequency in negative controls (OECD, 2014).³⁴ The remaining studies are described below.

3.2.3.1.1. Mammalian cell gene mutation tests

In order to investigate the potential of the bacteriophage preparation to induce gene mutations in mammalian cells, the mouse lymphoma assay (MLA) was performed in L5178Y cell line according to OECD TG 490 (2016) and Good Laboratory Practice (GLP) compliant.³⁵ The phage preparation was tested in two independent experiments applying a short treatment (4 h) in the absence and presence of S9-mix and a continuous treatment (24 h) in the absence of S9-mix. The bacteriophage suspension before the last dilution step with ionised water was tested up to 5 µL/mL in the dose-finding assay and at 2, 1.5, 1 and 0.5 µL/mL in the second experiment. Appropriate positive and negative controls were evaluated concurrently. Positive control chemicals induced statistically significant increases in mutation frequencies, confirming the sensitivity of the assay and the efficacy of the S9-mix. No precipitation and cytotoxicity were induced by treatment with the test item. No biologically relevant increase in mutation frequencies was observed at any concentration tested both in the presence and absence of S9-mix. The Panel concluded that the test item did not induce gene mutations in mammalian cells under the experimental conditions employed in this study.

3.2.3.1.2. In vitro mammalian cell micronucleus tests

The *in vitro* mammalian cell micronucleus assay was performed to evaluate the potential of the test item to induce chromosome damage in human lymphocytes. The bacteriophage suspension before the last dilution step with ionised water³⁶ was tested in two independent experiments conducted in accordance to OECD TG 487 (2016) and GLP compliant. A short treatment (3 + 28 h of recovery) in the presence and absence of metabolic activation and a continuous treatment (28 + 0 h recovery) in the absence of metabolic activation were applied to test four dose levels, i.e. 5, 2, 1 and 0.5 µL/mL. Cytochalasin B was added to the cultures at a final concentration of 5 µg/mL. Appropriate positive control chemicals were used and the results obtained confirmed that the experimental system was sensitive and valid. No significant changes in osmolarity and pH were observed between treated and control cultures. No precipitation and cytotoxicity were induced by treatment in any experimental condition. No increase in the frequency of micronuclei was induced by treatment with the phages preparation compared to concurrent vehicle control in any experimental condition. The Panel concluded that the test item did not induce micronuclei in cultured human peripheral blood lymphocytes under the experimental conditions employed in these studies.

3.2.3.1.3. Subchronic Oral Toxicity Study

The subchronic (90-day) oral toxicity study in rats was conducted following the principles of GLP and in accordance with the OECD TG 408.³⁷ One hundred Wistar rats were divided into six groups: three groups (10 rats/sex per group) receiving different doses of the bacteriophage preparation (2,000

³¹ Technical dossier/Section III/Annex III.2.2.2.1.

³² Technical dossier/Section III/Annex III.2.2.2.

³³ Technical dossier/Supplementary information December 2019/Annex III.2.2.2.3 micronucleus assay.

³⁴ Technical dossier/Supplementary information February 2020/Annexes_III.2.2.2.5 MNA assay comments and III.2.2.2.6 MNA historical data.

³⁵ Technical dossier/Supplementary information December 2019/Annex III.2.2.2.4 mammalian cell gene mutation.

³⁶ Technical dossier/Supplementary information October 2020/Annex II.2.2.2.7 new MNA.

³⁷ Technical dossier/Supplementary information January 2021/Annex_III_2_2_3_90day_rat_study.

mg/kg body weight (bw), 500 mg/kg bw and 125 mg/kg bw), a vehicle control group (10 rats/sex per group), 2 recovery groups (5 rats/sex per group) which received either 2,000 mg/kg bw or the vehicle. The animals were daily given Bafasal by gavage for 90 days, as a water solution.

There were no changes in the health status of the animals, and no statistically significant differences in body weight among the groups. No treatment-related changes in behavioural, neurological, haematological or biochemical (including urinalysis) parameters were found. At necropsy, gross changes and organ weights were not different among the groups. Microscopy performed in control animals, and the group receiving Bafasal® at the dose of 2,000 mg/kg bw did not reveal any histopathological changes that could be related to treatment. A no observed adverse effect level (NOAEL) of 2,000 mg/kg bw per day (the highest dose tested) was derived from this study.

3.2.3.2. Conclusions on safety for the consumer

Considering the nature and manufacturing process of the additive (see Section 3.2.1), Bafasal® is not expected to pose a risk for consumers. The absence of adverse effects in the subchronic oral toxicity study and the negative results in the genotoxicity studies provided, support this conclusion. Consequently, the FEEDAP Panel concludes that the use of Bafasal® in animal nutrition under the proposed conditions of use is safe for the consumers.

3.2.4. Safety for the user

Owing to the proteinaceous nature of the phages, the product should be considered a respiratory sensitiser. The product may contain bacterial endotoxins (mean value of nine determinations 525 IU/mL, ranging from 12 to 1,645 IU/mL) which is considered a hazard for people exposed by inhalation. However, Bafasal® is marketed in liquid form so the risk of exposure of users via inhalation is expected to be low. No data were submitted on skin/eye irritation or skin sensitisation. In the absence of data, the FEEDAP Panel cannot conclude on the irritancy of Bafasal® to skin and eyes or on its dermal sensitisation potential.

3.2.5. Safety for the environment

The host strain used for the phage propagation is a pathogenic component of the gut microbiota of poultry. This strain harbours an acquired antimicrobial resistance gene. However, Bafasal® was shown to be free of viable cells and DNA from the host strain. The active agents of the product are bacteriophages. Bacteriophages are naturally present in all environments where bacteria occur and multiply only when suitable host organisms are present. Consequently, the Panel considers that use of the product in animal nutrition according to the conditions of use does not pose a risk for the environment.

3.3. Efficacy

3.3.1. *In vitro* studies

Four *in vitro* studies were conducted to demonstrate the capacity of the selected phages to lyse the cells of two *S. enterica* serotypes, and to be active in different feed matrices.

The first study³⁸ tested the lytic activity of Bafasal® against *S. enterica* ser. Gallinarum PCM B/00111 and 22 strains of *S. enterica* ser. Enteritidis.³⁹ Cultures of different *S. enterica* strains were prepared by incubation in lysogeny broth until they reached an optical density (600 nm) of about 0.5 (corresponding to approximately 3.0×10^8 CFU/mL), transferred to 96-well plates and the additive was added. Wells not inoculated with bacteriophages were used as negative control (control of bacterial growth). The test included a batch of the additive with a concentration of 1×10^8 PFU/mL. The decrease in optical density (620 nm) of suspensions of *S. enterica* strains and Bafasal® measured every 20 min indicated the capacity of the phages to lyse the cells of all the strains tested.

The remaining three *in vitro* studies tested the lytic activity of Bafasal® in three different liquid feed matrices (i.e. a soy yeast-based liquid complementary feed in study 1,⁴⁰ a sorbitol-based commercially

³⁸ Technical dossier/Section III/Annex IV.1.1.

³⁹ D, WK9, 65, 1021, 1250, 1748, KK3, KK6, KK14, PacK, WK6, 249, 517, 833, 865, 866, 1013, 1014, 1047, 1048, 1067 K3, 1085.

⁴⁰ Technical dossier/Section III/Annex IV.1.2.

available liquid complementary feed in study 2⁴¹ and a glucose-ions based liquid complementary feed in study 3⁴²). The specifications of the batch used in the three studies were confirmed by analysis (9.58×10^7 PFU/mL). The treatments included a control (suspension of the *S. Enteritidis* in the feed matrix) and the same suspension with the addition of Bafasal® (2×10^6 PFU/mL), each with six replicates. The experiments were subject to conditions mimicking the chicken's digestive tract (Chang and Chen, 2000; Martinez-Haro et al., 2009). The system reflected the environment in the crop (0.1 mol/L NaCl and pH 4.5), proventriculus, gizzard (0.1 mol/L NaCl, 0.1% pepsine and pH 4.5) and intestine of birds (3.5% bile salts, 0.35% pancreatin and pH 6.2). To reach the suitable pH in different compartments, NaHCO₃, NaCl and/or HCl was added. Counts of *S. Enteritidis* were analysed at different time points (at the beginning and end, or at the beginning and after 6 and 10 h of incubation) by a miniaturised most probable number (MPN), and the data were subject to Student's *t*-test.

In two studies, Bafasal® was shown to be active against *S. Enteritidis* over the entire experimental period at the tested conditions (i.e. $\Delta \text{Log}_{10}\text{MPN/mL}$ time 10 h – time 0 = -0.46 for control and -5.79 for Bafasal® in one study and -2.06 for control and -5.43 for Bafasal® in the second study, $p < 0.05$). However, the Panel notes that the bacteriophages have been tested against only one strain of *S. Enteritidis*, but not against other serovars with high prevalence in chickens for fattening. In addition, since the effects claimed are to be exerted in the animals, these results can only be used as supportive evidence of efficacy.

3.3.2. *In vivo* studies

A total of five *in vivo* studies with chickens for fattening all sharing the same experimental design were submitted. [REDACTED]

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 - [REDACTED]
 - [REDACTED]
- [REDACTED]
- [REDACTED]
- [REDACTED]

⁴¹ Technical dossier/Section III/Annex IV.1.3.

⁴² Technical dossier/Section III/Annex IV.1.4.

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[REDACTED]

Considering all the above, there are insufficient data to allow the Panel to conclude on potential of Bafasal® to reduce the *S. enterica* carriage in target animals or to improve the zootechnical performance of chickens for fattening.

3.3.3. Conclusions on efficacy for all avian species

The Panel is not in the position to conclude on the efficacy of Bafasal® for any avian species due to insufficient evidence provided.

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 Regulation⁴⁴ and Good Manufacturing Practice.

4. Conclusions

The host strain used for the propagation of Bafasal® phages, *S. enterica* ser. Gallinarum PCM B/00111, is resistant to ciprofloxacin and streptomycin. The resistance to ciprofloxacin is related to a mutation and therefore considered of no concern. The resistance to aminoglycoside is due to an acquired antimicrobial resistance gene. However, no viable cells or DNA from the host organism were found in the additive. No lysogenic genes were identified in the genome of the four phages tested, denoting that they are strictly lytic and they all have a machinery allowing to package a unit-length of the viral genome. The manufacturing process of Bafasal® includes several filtration steps which would exclude the presence of remnants from the propagation process. Consequently, no concerns are expected from the nature or manufacturing process of Bafasal®.

Considering the results of the tolerance study with chickens for fattening where a margin of safety of up to 100× was identified, and the nature and manufacturing process of the additive, the Panel concludes that Bafasal® is safe for all avian species.

Considering the nature and manufacturing process of the additive Bafasal® is not expected to pose a risk for consumers. The results of toxicological studies provided, support this conclusion. Consequently, the FEEDAP Panel concludes that the use of Bafasal® in animal nutrition under the proposed conditions of use is safe for the consumers.

Owing to the proteinaceous nature of the phages, Bafasal® is to be considered a respiratory sensitisation. The product may contain bacterial endotoxins up to a level which is considered a hazard for people exposed by inhalation. However, Bafasal® is in liquid form so the risk of exposure of users via inhalation is expected to be low. No conclusions can be drawn on the irritancy of Bafasal® to skin and eyes or on its dermal sensitisation potential due to lack of data.

The Panel considers that use of the product in animal nutrition according to the conditions of use will not pose a risk for the environment.

⁴³ Technical dossier/Spontaneous supplementary information August 2018/Annex IV.3.6.

⁴⁴ Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 laying down requirements for feed hygiene. OJ L 35, 8.2.2005, p. 1.

The Panel is not in the position to conclude on the efficacy of Bafasal® for any avian species due to insufficient data.

5. Documentation as provided to EFSA/Chronology

Date	Event
05/07/2017	Dossier received by EFSA. Bafasal® for all avian species. Submitted by Proteon Pharmaceuticals S.A.
03/11/2017	Reception mandate from the European Commission
22/01/2018	Application validated by EFSA – Start of the scientific assessment
01/03/2018	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. <i>Issues: method of analysis</i>
22/04/2018	Comments received from Member States
31/08/2018	Reception of spontaneous supplementary information from the applicant
27/09/2018	Reception of supplementary information from the applicant - Scientific assessment re-started
09/10/2018	Reception of the Evaluation report of the European Union Reference Laboratory for Feed Additives
29/10/2018	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. <i>Issues: characterisation of the active additive, conditions of use</i>
21/12/2018	Reception of supplementary information from the applicant - Scientific assessment re-started
06/02/2019	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. <i>Issues: characterisation of the active additive</i>
08/03/2019	Reception of supplementary information from the applicant - Scientific assessment re-started
21/06/2019	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. <i>Issues: characterisation of the active additive, consumer safety</i>
18/07/2019	Teleconference during risk assessment
23/12/2019	Reception of supplementary information from the applicant - Scientific assessment re-started
04/02/2020	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. <i>Issues: consumer safety</i>
12/02/2020	Reception of supplementary information from the applicant - Scientific assessment re-started
21/02/2020	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. <i>Issues: consumer safety</i>
30/10/2020	Reception of supplementary information from the applicant - Scientific assessment re-started
17/12/2020	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. <i>Issues: consumer safety</i>
03/01/2021	Reception of supplementary information from the applicant - Scientific assessment re-started
17/03/2021	Opinion adopted by the FEEDAP Panel. End of the Scientific assessment

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Abbreviations

ADFI	average daily feed intake
ADG	average daily gain
ANOVA	analysis of variance
bw	body weight
CFU	colony forming unit
CV	coefficient of variation
URL	European Union Reference Laboratory
FEEDAP	EFSA Panel on Additives and Products or Substances used in Animal Feed
GLP	Good Laboratory Practice
IU	International unit
LB	Luria–Bertani
LOD	limit of detection
LOQ	limit of quantification
MF	mutation frequencies
MIC	minimum inhibitory concentration
MLA	mouse lymphoma assay
MN	micronuclei
MPN	most probable number
NOAEL	no observed adverse effect level
OECD	Organisation for Economic Co-operation and Development
PCB	polychlorinated biphenyls
PCDD/F	polychlorinated dibenzo- <i>p</i> -dioxins and dibenzofurans
PCM	Polish Collection of Microorganisms
PCR	polymerase chain reaction
PFU	plaque forming unit
TEQ	toxic equivalent
XLD	xylose lysine deoxycholate
WHO	World Health Organization

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

In the current application authorisation is sought under Article 4(1) for a preparation of bacteriophages (Bafasal®) under the category/functional group 4(d) 'zootechnical additives'/other zootechnical additives', according to Annex I of Regulation (EC) No 1831/2003. Authorisation is sought for the use of the *feed additive* for all avian species.

According to the Applicant, the *feed additive* contains a preparation of four bacteriophages: 3sent1, 8sent65, 8sent1748 and 5sent1,⁴⁵ obtained from *Salmonella enterica* ser. Gallinarum 1 (accession number B/0011, Polish Collection of Microorganisms (PCM)). The *feed additive* is to be marketed in liquid form, containing equivalent amounts of the four bacteriophages, with a minimum concentration of each phage of 1.25×10^7 Plaque Forming Units (PFU)/mL, leading to a total concentration $\geq 5 \times 10^7$ PFU/mL. The *feed additive* is intended to be used directly in *water* and *liquid complementary feeds* at a minimum dose of 2×10^6 PFU/bird per day.

For the identification of the four bacteriophages 3sent1, 8sent65, 8sent1748 and 5sent1, the EURL recommends for official control the Phage-specific PCR method (BF-PCR) proposed by the Applicant.

For the enumeration of the four bacteriophages 3sent1, 8sent65, 8sent1748 and 5sent1 in *the feed additive*, *water* and *liquid complementary feeds*, the Applicant submitted a single-laboratory validated and further verified method based on a double agar overlay plaque assay. Based on the performance characteristics available, the EURL recommends this method for official control.

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, as last amended by Regulation (EU) 2015/1761), is not considered necessary.

⁴⁵ These are in-house identifiers corresponding to PCM F/00071, PCM F/00070, PCM F/00069 and PCM F/00097, respectively.