SCIENTIFIC OPINION

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Safety and efficacy of a feed additive consisting of *Bacillus subtilis* FERM BP-07462, *Enterococcus lactis* FERM BP-10867 and *Clostridium butyricum* FERM BP-10866 (BIO-THREE®) for chickens for fattening, chickens reared for laying, turkeys for fattening, turkeys reared for breeding, all avian species for rearing/fattening to slaughter and all avian species reared for laying or breeding to point of lay (TOA BIOPHARMA Co., Ltd.)

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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 safety and efficacy of BIO-THREE® when used as a feed additive for chickens for fattening, chickens reared for laying, turkeys for fattening, turkeys reared for breeding, all avian species for rearing/fattening to slaughter and all avian species reared for laying or breeding to point of lay. The product under assessment is based on viable cells/spores of *Bacillus subtilis* FERM BP-07462, *Enterococcus lactis* FERM BP-10867 and *Clostridium butyricum* FERM BP-10866. Based on the tolerance study provided, the Panel concluded that the additive is safe for the target species under the conditions of use. The additive is safe for the consumers of products derived from animals receiving the additive. The additive is not irritant to skin and eyes. The additive is a respiratory sensitiser. No conclusions could be drawn on its potential to be a skin sensitiser. The use of the product as a feed additive is of no concern for the environment. The FEEDAP Panel was not in the position to conclude on the efficacy of BIO-THREE® for the target species. BIO-THREE® is compatible with diclazuril, decoquinate and halofuginone. No conclusions could be drawn on the compatibility of BIO-THREE® with monensin sodium, salinomycin sodium, narasin, robenidine hydrochloride and maduramicin ammonium.

Keywords: zootechnical additives, gut flora stabilisers, *Bacillus subtilis* FERM BP-07462, *Enterococcus lactis* FERM BP-10867, *Clostridium butyricum* FERM BP-10866, 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 TOA Biopharma Co., Ltd., Japan, represented in the European Union by TOA Biopharma Co., Ltd. Europe Representative Office,[2] for authorisation of the product containing *Bacillus subtilis* FERM BP-07462, *Enterococcus lactis*[3] FERM BP-10867 and *Clostridium butyricum* FERM BP-10866 (BIO-THREE®), when used as a feed additive for chickens for fattening, chickens reared for laying, turkeys for fattening, turkeys reared for breeding, all avian species for rearing/fattening to slaughter and all avian species reared for laying or breeding to point of lay[4] (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). The particulars and documents in support of the application were considered valid by EFSA as of 21 October 2020.

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 containing *Bacillus subtilis* FERM BP-07462, *Enterococcus lactis* FERM BP-10867 and *Clostridium butyricum* FERM BP-10866 (BIO-THREE®), when used under the proposed conditions of use (see Section 3.1.4).

1.2. Additional information

The product under assessment is based on viable spores/cells of *Bacillus subtilis* FERM BP-07462, *Enterococcus lactis* FERM BP-10867 and *Clostridium butyricum* FERM BP-10866 and is not 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 dossier[5] in support of the authorisation request for the use of the product containing *Bacillus subtilis* FERM BP-07462, *Enterococcus lactis* FERM BP-10867 and *Clostridium butyricum* FERM BP-10866 (BIO-THREE®) 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.[6]

[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] TOA Biopharma Co., Ltd., Japan represented in the EU by TOA Biopharma Co., Ltd. Europe Representative Office, Plaza Ausias March 1, ES-08195 Sant Cugat del Vallès, Barcelona, Spain.
[3] Originally designated as *Enterococcus faecium*.
[4] Following the request for Supplementary information dated 2 December 2021, the applicant clarified the target species for which the application was made.
[5] FEED dossier reference: FAD-2020-0058.
[6] The full report is available on the EURL website: https://joint-research-centre.ec.europa.eu/publications/fad-2020-0058_en#files

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of the product containing *Bacillus subtilis* FERM BP-07462, *Enterococcus lactis* FERM BP-10867 and *Clostridium butyricum* FERM BP-10866 (BIO-THREE®) is in line with the principles laid down in Regulation (EC) No 429/2008[7] and the relevant guidance documents: Guidance on studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012), Guidance on the identity, characterisation and conditions of use of feed additives (EFSA FEEEDAP Panel, 2017a), Guidance on the characterisation of microorganisms used as feed additives or as production organisms (EFSA FEEDAP Panel, 2018a), Guidance on the assessment of the safety of feed additives for the target species (EFSA FEEDAP Panel, 2017b), Guidance on the assessment of the safety of feed additives for the consumer (EFSA FEEDAP Panel, 2017c), Guidance on the assessment of the efficacy of feed additives (EFSA FEEDAP Panel, 2018b), Guidance on the assessment of the safety of feed additives for the environment (EFSA FEEDAP Panel, 2019).

3. Assessment

The product containing viable spores/cells of *Bacillus subtilis* FERM BP-07462, *Enterococcus lactis* FERM BP-10867 and *Clostridium butyricum* FERM BP-10866 (BIO-THREE®) is intended to be used as a zootechnical additive (functional group: gut flora stabilisers) in feed and water for drinking for chickens for fattening, chickens reared for laying, turkeys for fattening, turkeys reared for breeding, all avian species for rearing/fattening to slaughter and all avian species reared for laying or breeding to point of lay. It will be hereafter referred to with its trade name BIO-THREE®.

3.1. Characterisation

3.1.1. Characterisation of the active agents

The *B. subtilis*, *E. lactis*[8] and *C. butyricum* strains are deposited in the Japanese National Institute of Technology and Evaluation, Patent Microorganisms Depositary with the accession numbers FERM BP-07462, FERM BP-10867 and FERM BP-10866, respectively.[9]

3.1.1.1. *Bacillus subtilis* FERM BP-07462

The active agent *B. subtilis* FERM BP-07462 was isolated from potato skins. The taxonomic identification of the strain as *B. subtilis* was confirmed by whole genome sequence (WGS)-based analyses.[10] [redacted][11]

The susceptibility of the strain to the antibiotics recommended by the FEEDAP Guidance (EFSA FEEDAP Panel, 2018a) was tested by agar dilution method according to the Clinical Laboratory Standards Institute (CLSI). All the minimum inhibitory concentration (MIC) values determined fell below the FEEDAP cut-off values.[12] Therefore, *B. subtilis* FERM BP-07462 is susceptible to the tested antibiotics.

The WGS data of the strain were interrogated for the presence of antimicrobial resistance (AMR) genes [redacted][13] No hits of concern were identified.

[7] 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.
[8] Deposited as *Enterococcus faecium*.
[9] Technical dossier/Section II/Annexes_Sect_II/Annex_II_2_1_2a.
[10] Technical dossier/Section II/Annexes_Sect_II/Annex_II_2_1_2b and Annex_II_2_1_2e.
[11] Technical dossier/Section II/Annex_II_2_1_2b.
[12] Technical dossier/Section II/Annexes_Sect_II/Annex_II_2_2_2b.
[13] Technical dossier/Section II/Annexes_Sect_II/Annex_II_2_1_2e.

The toxigenic potential of *B. subtilis* FERM BP-07462 was assessed according to the FEEDAP Guidance (EFSA FEEDAP Panel, 2018a).[14] No lysis of Vero cells was detected. Therefore, *B. subtilis* FERM BP-07462 is considered to be nontoxigenic.

3.1.1.2. *Enterococcus lactis* FERM BP-10867

The active agent was isolated from healthy human gut contents and was originally identified as *Enterococcus faecium*. Additional bioinformatic analyses of the WGS data, provided to support the taxonomic identification, allocated the strain to the *Enterococcus lactis* species.[15] This identification was based on: [redacted]

The susceptibility of the strain to the antibiotics recommended by the FEEDAP Guidance (EFSA FEEDAP Panel, 2018a) was tested by an agar dilution method according to the CLSI.[16] The MIC values of the strain were compared with the defined EFSA cut-off values for the closest related species *E. faecium*. All MIC values fell below the FEEDAP cut-off values, except for [redacted]

The WGS data of the production strain, [redacted] were interrogated for the presence of AMR genes [redacted][17] (Isnard et al., 2013) [redacted] (Portillo et al., 2000; Singh et al, 2001). [redacted] this resistance raises no safety concerns since no acquired AMR genes were found in the WGS.

According to the FEEDAP guidance (EFSA FEEDAP Panel, 2018a), the *E. faecium* safety should be assessed demonstrating the absence of genetic markers typical of the clinical isolates *E. faecium* clade A (IS*16*, *esp*, *hyl*) and the susceptibility to ampicillin. Taking into consideration the allocation of clade B strains to *E. lactis* species (Belloso Daza et al., 2021), the FEEDAP Panel considers these criteria applicable also to *E. lactis* strains. *E. lactis* FERM BP-10867 proved to be susceptible to ampicillin. The WGS data of the active agent, [redacted], were interrogated for the presence of genes encoding virulence factors, including IS*16*, hyl_{Efm} and *esp* genes, [redacted][18] No relevant hits were identified. Moreover, the absence of the virulence factors IS*16*, hyl_{Efm} and *esp* genes in the chromosome [redacted] of FERM BP-10867 was confirmed [redacted] Therefore, the active agent FERM BP-10867 is free of the relevant virulence factors (IS*16*, hyl_{Efm} and *esp*).

3.1.1.3. *Clostridium butyricum* FERM BP-10866

The active agent *C. butyricum* FERM BP-10866 was isolated from healthy human gut contents. The taxonomic identification of the strain as *C. butyricum* was confirmed by WGS-based analyses.[19] This was based on [redacted]

[14] Technical dossier/Section II/Annexes_Sect_II/Annex_II_2_2_2a.
[15] Technical dossier/Supplementary information February 2022/Annex_1.
[16] Technical dossier/Section II/Annexes_Sect_II/Annex_II_2_2_2b and Spontaneous information November 2020/Annexes/Annex_II_2_2_2d.
[17] Technical dossier/Section II/Annexes_Sect_II/Annex_II_2_1_2f and Annex_II_2_2_2c.
[18] Technical dossier/Section II/Annexes_Sect_II/Annex_II_2_1_2f.
[19] Technical dossier/Section II/Annexes_Sect_II/Annex_II_2_1_2d and Annex_II_2_1_2g.

[redacted]

The susceptibility of the strain to the antibiotics recommended by the FEEDAP Guidance (EFSA FEEDAP Panel, 2018a) was tested by agar dilution method following the method of the CLSI.[12] [redacted]

To elucidate the nature of the resistance [redacted] the applicant interrogated the WGS data of the active agent, [redacted] for the presence of AMR genes [redacted][20] No hits of concern were detected. [redacted] since no acquired AMR genes were found in the WGS, these resistances do not raise safety concerns. Moreover, anaerobes are usually resistant to aminoglycosides, which is thought to be due to the lack of cytochrome-mediated transport (Rasmussen et al., 1997).

The WGS data of the active agent, [redacted] were interrogated for the presence of toxin and virulence factors genes [redacted][20] No hits of concern were identified. Moreover, the WGS data of the active agent were searched for the presence of neurotoxins [redacted] No hits were identified.

3.1.2. Characterisation of the additive

BIO-THREE® is intended to be marketed in two formulations, one with dry potato starch for use in feed and one with lactose for use in water.

[redacted][21] [redacted] The excipient represents approximately [redacted] of the final additive. According to the applicant, no antimicrobial substances are used during the manufacturing of the additive.[22]

The guaranteed minimum total concentration of viable spores/cells of the active agents in the product is 1.0×10^8 CFU *B. subtilis* FERM BP-07462/g; 1.0×10^9 CFU *E. lactis* FERM BP-10867/g and 1.0×10^8 CFU *C. butyricum* FERM BP-10866/g additive, respectively.[23] Six batches of the formulation with dry potato starch were analysed for the batch-to-batch variation: *B. subtilis* FERM BP-07462 was on average 1.2×10^8 CFU/g (range $0.7-2.2 \times 10^8$ CFU/g); *E. lactis* FERM BP-10867 was on average 0.5×10^9 CFU/g (range $0.4-0.6 \times 10^9$ CFU/g) and *C. butyricum* FERM BP-10866 was on average 1.0×10^8 CFU/g (range $0.8-1.1 \times 10^8$ CFU/g).[24] The Panel notes that the counts of *E. lactis* FERM BP-10867 fell below the specifications set by the applicant.

Three batches of the formulation with dry potato starch were analysed for chemical and microbiological contamination.[25] The analyses for chemical contaminants included arsenic, cadmium, lead and mercury which were below their corresponding limits of quantification (LOQs).[26] The analyses

[20] Technical dossier/Section II/Annexes_Sect_II/Annex_II_2_1_2g.
[21] Technical dossier/Section II/Annexes_Sect_II/Annex_II_3_1 and Supplementary information July 2021/2_EFSA_SIn_18Dec2020_reply and 4_Annexes/Annex_3_1.
[22] Technical dossier/Supplementary information July 2021/4_Annexes/Annex_4_1.
[23] Technical dossier/Supplementary information July 2021/2_EFSA_SIn_18Dec2020_reply and 4_Annexes/Annex_4_1.
[24] Technical dossier/Supplementary information July 2021/2_EFSA_SIn_18Dec2020_reply and 4_Annexes/Annex_1_1.
[25] Technical dossier/Section II/Annexes_Sect_II/Annex_II_1_4.
[26] Technical dossier/Supplementary information July 2021/4_Annexes/Annex_5_1. LOQ in mg/kg were 0.1 for arsenic and 0.05 for cadmium, lead and mercury.

for mycotoxins included deoxynivalenol, aflatoxins B1, B2, G1 and G2, ochratoxin A and zearalenone which were below their corresponding LOQs[27] and HT-2 toxin and T-2 toxin which were not detected.[28] Polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/F) ranged from 0.079 to 0.083 ng TEQ/kg. The sum of PCDD/F and dioxin-like polychlorinated biphenyls (PCBs) ranged from 0.086 to 0.090 ng TEQ/kg. Non-dioxin like PCBs (ICES-6) were also analysed and ranged from 0.052 to 0.055 μg/kg. Analysis of microbial contamination indicated that presumptive *Bacillus cereus*, *Enterobacteriaceae*, yeasts and moulds were below their corresponding LOQ (< 10 CFU/g), while *Salmonella* spp. was not detected in 25 g.[29]

The amounts of the above detected impurities do not represent a safety concern.

The applicant provided data on physical properties of the additive formulated with dry potato starch and lactose as excipients (pilot batches). BIO-THREE® in dry potato starch excipient has a tap density[30] of 986 kg/m^3 and a bulk density[31] measured in three batches of 799 kg/m^3 (range 793−804 kg/m^3). Its dusting potential (three batches) using the Stauber-Heubach method was 725 mg/m^3.[32] The same three batches were analysed for particle size distribution using laser diffraction, and particles below 100, 50 and 10 μm amounted up to 99.9, 76 and 0.6%, respectively.[33] Three batches of BIO-THREE® in lactose excipient were measured for bulk density and showed an average value of 730 kg/m^3 (range 730−731 kg/m^3).[34] The dusting potential was measured in the same three batches (Stauber-Heubach method) and the values ranged between 1,230 and 1,935 mg/m^3. The same three batches were analysed for particle size using laser diffraction, and particles below 100, 50, 10 and 1 μm amounted up to 58.2, 39.4, 14.6 and 1.5%, respectively.

3.1.3. Stability and homogeneity

Studies on shelf-life, stability in premixtures and feedingstuffs, as well as homogeneity in feedingstuffs were performed with the additive in dry potato starch excipient. Stability and homogeneity studies in water for drinking were performed with the additive in lactose excipient (pilot batches).[35]

The shelf-life of the additive was determined by monitoring three batches stored at 25°C in double zip-lock plastic bags for a period of 24 months.[36] The results showed no losses (< 0.5 log) in the numbers of bacilli, enterococci and clostridia after 24 months storage.

The stability of BIO-THREE® (one batch) in a vitamin and mineral premixture for poultry (without choline chloride) was studied when added at 500 g/kg premixture and stored at 25°C for 7 months in double zip-lock plastic bags.[37] No losses (< 0.5 log) were observed for bacilli and clostridia, whilst the number of enterococci was 1.1 log CFU/g less after 7 months storage.

The stability of BIO-THREE® (one batch) during feed processing was studied when added at 4,000 mg/kg feed to mash feed for poultry (based on maize and soybean meal, with 200 mg choline chloride/kg feed), and pelleted at 65°C.[38] The results showed negligible losses (< 0.5 log) for bacilli and clostridia, whilst losses of 2.5 log CFU/g were observed for enterococci.

The stability of two batches of BIO-THREE® in mash feed was studied when added in two different feeds for poultry: one based on maize and soybean meal (containing 200 mg choline chloride/kg feed) supplemented at 4,000 mg BIO-THREE®/kg and the other on wheat and Hipro soya (with about 3,000 mg choline chloride/kg feed) supplemented at 200 mg BIO-THREE®/kg feed.[39] The samples were stored in double zip-lock plastic bags at room temperature for 3 months. When added at 4,000 mg/kg feed, the results showed that no losses were observed for bacilli, whilst the numbers of

[27] Technical dossier/Supplementary information July 2021/4_Annexes/Annex_5_1. LOQ in μg/kg were 100 for deoxynivalenol, 0.5 for aflatoxins B1 and G1 and ochratoxin A, 0.2 for aflatoxins B2 and G2, 0.1 for fumonisin B1 and B2 and 20 for zearalenone.
[28] Technical dossier/Supplementary information July 2021/4_Annexes/Annex_5_1. LOQ and LOD in μg/kg for HT-2 and T-2 Toxin were 20 and 10, respectively.
[29] Technical dossier/Supplementary information July 2021/4_Annexes/Annex_5_1. Limit of detection (LOD) for *Salmonella* spp. detection was 5-10 CFU/25 g.
[30] Technical dossier/Supplementary information July 2021/4_Annexes/Annex_2_4.
[31] Technical dossier/Section II/Annexes Sect.II/Annex_II_1_5a and Annex_II_1_5c.
[32] Technical dossier/Section II/Annexes Sect.II/Annex_II_1_5d.
[33] Technical dossier/Section II/Annexes Sect.II/Annex_II_1_5a and Annex_II_1_5b.
[34] Technical dossier/Supplementary information July 2021/4_Annexes/Annex_2_3.
[35] Technical dossier/Supplementary information July 2021/ 2_EFSA_SIn_18Dec2020_reply.
[36] Technical dossier/Section II/Annexes Sect.II/Annex_II_4_1a.
[37] Technical dossier/Section II/Annexes Sect.II/Annex_II_4_1c.
[38] Technical dossier/Section II/Annexes Sect.II/Annex_II_4_2.
[39] Technical dossier/Section II/Annexes Sect.II/Annex_II_4_1d.

enterococci and clostridia were 0.6 log CFU/g less after 3 months storage. When added at 200 mg/kg feed, the results showed no losses for bacilli, whilst the numbers of enterococci and clostridia were 1 and 0.6 log CFU/g less after 3 months storage, respectively.

The stability of BIO-THREE® (one batch) in pelleted feed was studied when added via premixture at 4,000 mg/kg feed in a mash feed for poultry (based on maize and soybean meal, with 200 mg choline chloride/kg feed), pelleted at 65°C and stored in double zip-lock plastic bags at room temperature for 4 months.[40] Negligible losses (< 0.5 log) were observed in the numbers of bacilli, enterococci and clostridia after 4 months storage.

The stability of BIO-THREE® (one batch) in water (100 mg/L water) was studied with and without agitation when stored at room temperature (20−25°C) for up to 48 h.[41] The results showed no losses (< 0.5 log) for bacilli and clostridia, whilst the losses of enterococci with and without agitation were < 0.6 and < 2 log CFU/mL less after 24 and 48 h, respectively.

The homogeneous distribution of the additive in mash and pelleted feed was investigated when added at 4,000 mg/kg to poultry feed based on maize and soybean meal (with choline chloride).[38] Ten subsamples of the mash and of the pelleted feeds were analysed for bacilli, enterococci and clostridia counts. The coefficients of variation (CV) in mash feed were 0.6, 2.4 and 2.6% for bacilli, enterococci and clostridia, respectively. The CVs in pelleted feed were 3.4, 10.1 and 1.6% for bacilli, enterococci and clostridia, respectively.

The homogeneous distribution of the additive in water for drinking was investigated when added at 100 mg/L.[42] Ten subsamples were analysed for bacilli, enterococci and clostridia counts immediately after preparation and after 2 h (with or without agitation prior to analysis). The coefficients of variation were < 1.4% for bacilli, enterococci and clostridia under all the experimental conditions.

3.1.4. Conditions of use

The product is proposed for use in feed and water for drinking for chickens for fattening, chickens reared for laying, turkeys for fattening, turkeys reared for breeding, all avian species for rearing/fattening to slaughter and all avian species reared for laying or breeding to point of lay at a minimum inclusion level of 2.0×10^7 *B. subtilis* FERM BP-07462 CFU/kg, 2.0×10^8 *E. lactis* FERM BP-10867 CFU/kg and 2.0×10^7 *C. butyricum* FERM BP-10866 CFU/kg complete feed (dry potato starch formulation) and of 1.0×10^7 *B. subtilis* FERM BP-07462 CFU/L, 1.0×10^8 *E. lactis* FERM BP-10867 CFU/L and 1.0×10^7 *C. butyricum* FERM BP-10866 CFU/L of water for drinking (lactose formulation).[43]

The applicant requests for the simultaneous use of the additive with the following coccidiostats: decoquinate, diclazuril, halofuginone, monensin sodium, maduramicin ammonium, narasin, robenidine and salinomycin sodium.

3.2. Safety

3.2.1. Safety of the active agents

The strain *B. subtilis* FERM BP-07462 belongs to a species considered eligible for the qualified presumption of safety (QPS) approach to safety assessment (EFSA, 2007; EFSA BIOHAZ Panel, 2020). This approach requires the identity of the strain to be conclusively established and evidence that the strain lack toxigenic potential and does not show acquired resistance to antibiotics of human and veterinary importance. The FEEDAP Panel noted that the identity of FERM BP-07462 has been unambiguously established. Evidence was provided on the lack of toxigenic potential of the strain and on the absence of antimicrobial resistance genes. Therefore, *B. subtilis* FERM BP-07462 does not raise safety concerns for the target species, consumers of products derived from animals fed the additive and the environment.

Regarding the other two active agents, *E. lactis* FERM BP-10867 and *C. butyricum* FERM BP-10866, both strains belong to taxonomic units which are not eligible for the QPS assessment. The strains *E. lactis* FERM BP-10867 and *C. butyricum* FERM BP-10866 were shown not to harbour acquired AMR genes and, based on the WGS data provided, are not expected to be virulent or to produce any toxic

[40] Technical dossier/Section II/Annexes Sect.II/Annex_II_4_1e.
[41] Technical dossier/Section II/Annexes Sect.II/Annex_II_4_1f and Supplementary information February 2022/Annex_2.
[42] Technical dossier/Section II/Annexes Sect.II/Annex_II_4_1f.
[43] Technical dossier/Supplementary information July 2021/2_EFSA_SIn_18Dec2020_reply and Supplementary information February 2022/2_APPENDIX_Applicant_response_Jan22.

compound. The strains are also not expected to produce antimicrobial compounds of relevance for human and animal health.

3.2.2. Safety for the target species

3.2.2.1. Safety for chickens for fattening

A total of 960 1-day-old male chickens for fattening (Ross 308) were weighed and distributed to 48 pens allocated to four dietary treatments (12 replicate pens per treatment).[44] Two basal diets (starter, from day 1 to 21; grower, from day 22 to 35) based on wheat, soya and maize were either not supplemented (control) or supplemented with BIO-THREE® to provide 2.0×10^7 *B. subtilis* FERM BP-07462 CFU, 2.0×10^8 *E. lactis* FERM BP-10867 CFU and 2.0×10^7 *C. butyricum* FERM BP-10866 CFU per kg feed (1× minimum recommended level), 2.0×10^8 *B. subtilis* FERM BP-07462 CFU, 2.0×10^9 *E. lactis* FERM BP-10867 CFU and 2.0×10^8 *C. butyricum* FERM BP-10866 CFU per kg feed (10×) or 2.0×10^9 *B. subtilis* FERM BP-07462 CFU, 2.0×10^{10} *E. lactis* FERM BP-10867 CFU and 2.0×10^9 *C. butyricum* FERM BP-10866 CFU per kg feed (100×) (confirmed by analysis). Diets were offered ad libitum in mash form for 35 days. Mortality and health status were checked daily and dead animals were weighed and necropsied. Average pen body weight and feed intake were recorded on days 1, 21 and 35 and average daily feed intake, average daily gain and feed to gain ratio calculated and corrected for mortality. At day 35, blood samples were obtained from two birds per pen and analysed for haematology and blood biochemistry.[45] An analysis of variance (ANOVA) was done with the mortality, performance and blood parameters data and considering the treatment as the effect. The experimental unit considered was the pen for the performance and mortality data, and the individual animal for blood parameters. Group means were compared with the Tukey test. Significance level was set at 0.05.

The design of the study and results are presented in Tables 1 and 2, respectively (trial 3; see Section 3.3.1). Mortality was below 2.1% in all treatments without statistical differences between them.[46] No adverse effects were observed in any of the performance parameters due to the supplementation with BIO-THREE® at 100× the minimum recommended level. Regarding the haematological parameters, no relevant differences were observed between the overdose groups and the control.

The BIO-THREE® supplementation up to 100-fold the recommended level did not adversely affect the health and performance of chickens for fattening.

3.2.2.2. Conclusions on the safety for the target species

The FEEDAP Panel concludes that BIO-THREE® is safe for chickens for fattening at the recommended level of 2.0×10^7 *B. subtilis* FERM BP-07462 CFU, 2.0×10^8 *E. lactis* FERM BP-10867 CFU and 2.0×10^7 *C. butyricum* FERM BP-10866 CFU per kg feed with a margin of safety of 100. The conclusion is extended to the use of the additive in water for drinking at 1.0×10^7 *B. subtilis* FERM BP-07462 CFU/L, 1.0×10^8 *E. lactis* FERM BP-10867 CFU/L and 1.0×10^7 *C. butyricum* FERM BP-10866 CFU/L. The conclusion is extended to chickens reared for laying and extrapolated to turkeys for fattening, turkeys reared for breeding, all avian species for rearing/fattening to slaughter and all avian species reared for laying or breeding to point of lay.

3.2.3. Safety for the consumer

3.2.3.1. Toxicological studies

Bacterial reverse gene mutation assay

In order to investigate the potential of BIO-THREE® to induce gene mutations in bacteria, the Ames test was performed according to OECD Test Guideline (TG) 471 (OECD, 1997) and following Good

[44] Technical dossier/Supplementary information July 2021/Annex_12_1.

[45] Total red blood concentration, packed cell volume, haemoglobin, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, white blood cells (and formula: lymphocytes, heterophils, monocytes, eosinophils, basophils), thrombocytes, prothrombin time and fibrinogen, sodium, potassium, chlorine, calcium, phosphorus, magnesium, total protein, albumin, globulin, glucose, urea/uric acid, cholesterol, triglycerides, creatinine, bile acid, bilirubin, amylase, serum amyloid A, alpha-1 acid glycoprotein, alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase, gamma-glutamyltransferase, alkaline phosphatase and creatine kinase.

[46] Technical dossier/Supplementary information July 2021/Annex_10_1.

Laboratory Practice (GLP) in *Salmonella* Typhimurium strains TA98, TA100, TA1535, TA1537 and TA102.[47] The test item (providing per gram 1.3×10^8 *B. subtilis* FERM BP-07462 CFU, 4.2×10^8 *E. lactis* FERM BP-10867 CFU and 1.0×10^8 *C. butyricum* FERM BP-10866 CFU) was tested in the form of a DMSO extract at 2 g/mL. The top concentration volume was the one compatible with the test system, i.e. 100 and 50 μL/plate of DMSO initial extract in the conditions without and with pre-incubation, respectively. Four lower concentration volumes chosen according to a geometrical (half-log) ratio were also tested in the presence and absence of metabolic activation. Appropriate positive and negative controls were evaluated concurrently. All positive control chemicals induced significant increases in revertant colony numbers, confirming the sensitivity of the tests and the efficacy of the S9-mix. Precipitate and toxicity were not observed. No increase in the mean number of revertant colonies was observed at any tested concentration in any tester strains with or without S9-mix. The Panel concludes that the test item in the form of a DMSO extract did not induce gene mutations in bacteria under the experimental conditions employed in this study.

In vitro micronucleus test

To evaluate the potential of BIO-THREE® to induce chromosome damage, an *in vitro* micronucleus test was performed in TK6 lymphoblastoid human cells according to OECD TG 487 (OECD, 2014) and following GLP.[49] The test item (providing per gram 1.3×10^8 *B. subtilis* FERM BP-07462 CFU, 4.2×10^8 *E. lactis* FERM BP-10867 CFU and 1.0×10^8 *C. butyricum* FERM BP-10866 CFU)[48] was tested in the form of a DMSO extract at 2 g/mL. The top dose volume was the one compatible with the test system, i.e. 1% of DMSO initial extract. Two lower concentrations (0.5 and 0.25% DMSO initial extract) were also analysed. A short treatment (3 + 24 h of recovery) with and without S9-mix and a continuous treatment (27 + 0 h recovery) without S9-mix were the experimental conditions applied. Appropriate positive and negative control chemicals were used, and the results obtained confirmed that the experimental system was sensitive and valid. No significant cytotoxicity was induced by treatment with the test item as measured by relative population doubling. The frequency of micronuclei in mononucleated cells was comparable in treated and negative control cultures both in the presence and absence of metabolic activation. The Panel concludes that the test item in the form of a DMSO extract did not induce micronuclei in mammalian cells under the experimental conditions employed in this study.

Subchronic toxicity study

Crl:CD (SD) rats (10/sex per group) received by oral gavage the individual active agents (*B. subtilis* FERM BP-07462 (7.0×10^7 CFU/g), *E. lactis* FERM BP-10867 (8.0×10^9 CFU/g), *C. butyricum* FERM BP-10866 (3.0×10^8 CFU/g)) or a mixture of the three active agents (providing per gram 3.0×10^6 *B. subtilis* FERM BP-07462 CFU, 5.0×10^7 *E. lactis* FERM BP-10867 CFU and 2.0×10^7 *C. butyricum* FERM BP-10866 CFU) at dose levels of 0 (control) or 3,000 mg /kg body weight (bw) per day for 90 consecutive days.[50] Additional groups of 10 males and 10 females were given the mixture of the three active agents at a dose level of 1,500 mg/kg bw per day. The Panel notes that while the CFU/g of each active agent when tested individually showed compliance with the specifications proposed for the additive, the mixture showed lower counts than the one present in the additive. The study was conducted following the GLP principles and broadly in compliance with OECD TG 408, except for an investigation of sensory reactivity, motor activity, grip strength and thyroid hormones.

No treatment-related clinical signs were observed. All animals survived the treatment period.

A statistically significant increase in body weight was reported in females given the mixture of the three active agents at 1,500 mg/kg bw per day and at 3,000 mg/kg bw per day, of approximately 9% and 7%, respectively, when compared to control. Occasionally, a statistically significant increase of food consumption was reported in females given *E. lactis* FERM BP-10867 (days 43–50 and 74), *C. butyricum* FERM BP-10866 (days 50–53, 67 and 74), or the mixture of the three active agents at 1,500 mg/kg group sporadically from day 25 and at 3,000 mg/kg group on days 43, 50 and 74. In the absence of a clear dose response, these effects were considered not treatment-related and not considered adverse.

[47] Technical dossier/Section III/Annexes_Sect_III/Annex_III_2_2_2a.
[48] Technical dossier/Supplementary information July 2021/4_Annexes/Annex_1_1.
[49] Technical dossier/Section III/Annexes_Sect_III/Annex_III_2_2_2b.
[50] Technical dossier/Section III/Annexes_Sect_III/Annex_III_2_2_3.

A statistically significant increase of mean corpuscular haemoglobin concentration (MCHC) was reported in males given *C. butyricum* FERM BP-10866 and a decrease of lactate dehydrogenase was showed in males given *B. subtilis* FERM BP-07462. A statistically significant decrease of creatine kinase was noted in females given *E. lactis* FERM BP-10867, *C. butyricum* FERM BP-10866 or *B. subtilis* FERM BP-07462. However, since all individual values were in the range of the concurrent control, this was not considered of toxicological significance. In the females in the same group, a statistically significant decrease of monocyte ratio was reported. Considering the absence of correlated changes in white blood cell count (WBC) and the marginal magnitude of change, this find was considered incidental and of no toxicological significance. A statistically significant decrease in the monocyte (−31%) and basophil ratios (−25%) was noted in females treated with the mixture of the three active agents at 1,500 mg/kg group; however, since no changes of these parameters were reported at the highest dose level, they were considered not treatment-related. In females in the group given the mixture of the three active agents at 3,000 mg/kg, statistically significant decrease of red blood cell count (RBC) (−5%), mean corpuscular volume (MCV) increase (+3%) and mean corpuscular haemoglobin (MCH) increase (+3%) were noted. However, given the absence of treatment-related haemoglobin or erythrocyte effects, and since the individual values were within the lower limit of the laboratory historical control data, changes in RBC and erythrocyte indices were considered not treatment-related and of no toxicological significance.

Pathology and histopathology revealed a decrease in the relative kidney weight not associated with histopathological findings in females of the *E. lactis* FERM BP-10867 group. An increase in relative pituitary gland weight in males and a decrease of relative ovary weight in females given *B. subtilis* FERM BP-07462 were recorded. In the groups given the mixture of the three active agents, decreases in the relative weights of some organs (ovaries at 1,500 and 3,000 mg/kg, in the brain of females at 1,500 and 3,000 mg/kg and in the submandibular glands in females at 3,000 mg/kg) were recorded. However, no histopathological alterations were reported for these organs. One male of the *B. subtilis* FERM BP-07462 group showed dilatation of the pelvis in the kidney, also confirmed by histopathology. In one female in the group given the mixture of the three active agents at 3,000 mg/kg, a mass in the subcutis (left cervical) was reported following clinical observation. This mass was evaluated to be a carcinoma arising from the submandibular glands, after histopathological evaluation. According to the study authors, this is a spontaneous rare tumour observed in rats and was not considered treatment-related. In another female of this group, the thyroid was unilaterally deficient, but no histopathological lesions were observed in the other thyroid.

Based on the results of the study, no adverse effects were observed at any of the doses tested.

3.2.3.2. Conclusions on the safety for the consumer

The results obtained in the genotoxicity studies and the subchronic oral toxicity study do not indicate any cause for concern arising from the use of BIO-THREE® as an additive in animal feed.

The FEEDAP Panel concludes that the use of BIO-THREE® in animal nutrition under the proposed conditions of use is safe for the consumers.

3.2.4. Safety for user

3.2.4.1. Effects in the respiratory system

Based on the dusting potential of the additive (in dry potato starch excipient up to 725 mg/m^3 and in lactose up to 1,935 mg/m^3), the FEEDAP Panel considered that exposure by inhalation is likely. Due to the proteinaceous nature of its active agents, the additive is considered a respiratory sensitiser.

3.2.4.2. Effect on skin and eyes

The skin irritation potential of BIO-THREE® in dry potato starch excipient was tested in a GLP study performed according to OECD TG 439, which showed that it is not a skin irritant.[51]

The eye irritation potential of BIO-THREE® in dry potato starch excipient was tested in a GLP study performed according to OECD TG 492, which showed that it is not an eye irritant.[52]

No information on skin sensitisation potential was provided; therefore, the FEEDAP Panel cannot conclude on the skin sensitisation potential of the additive.

[51] Technical dossier/Section III/Annexes_Sect_III/Annex_III_3_1_2.
[52] Technical dossier/Supplementary information July 2021/4_Annexes/Annex_8_1.

The FEEDAP Panel considered that the conclusions reached from the studies conducted with the dry potato starch formulation, would be extended to the lactose formulation.

3.2.4.3. Conclusions on safety for the user

BIO-THREE® is non-irritant to the skin and eyes but is a respiratory sensitiser. No conclusions can be drawn on the potential of the additive to cause skin sensitisation.

3.2.5. Safety for the environment

The additive BIO-THREE® is based on viable cells/spores of *B. subtilis* FERM BP-07462, *E. lactis* FERM BP-10867 and *C. butyricum* FERM BP-10866. *B. subtilis*, *E. lactis* and *C. butyricum* are naturally present in soils, plants or gastrointestinal tract of animals. Moreover, the strain *B. subtilis* FERM BP-07462 qualifies for the QPS approach to safety assessment. Therefore, the use of *B. subtilis* FERM BP-07462, *E. lactis* FERM BP-10867 and *C. butyricum* FERM BP-10866 as BIO-THREE® in animal nutrition is not expected to pose a risk for the environment.

3.3. Efficacy

3.3.1. Efficacy for chickens for fattening

To support the efficacy in chickens for fattening, the applicant made available a publication (Inatomi, 2015) and submitted six efficacy trials. However, the study described in the publication and two[53] of the efficacy trials were not further considered since the husbandry conditions under which the birds were raised (regarding the stocking density and bedding used, respectively) do not reflect the conditions in which the birds should be raised in the EU and were not in line with Directive 2007/43/EC.[54] The other four efficacy trials[55] were conducted in the same trial site, two studies in 2019 and the other two in 2020. The trials conducted within the same year were conducted at the same time, had the same trial design and used almost identical diets. The FEEDAP Panel considered the trials conducted at the same time as not independent and the data from those trials were pooled as a single trial (trial 1[56] and trial 2[57] in the text below).

The Panel also considered the tolerance trial (see Section 3.2.2) to be relevant for the assessment of the efficacy (trial 3 in the text below). The Panel notes that the trial was conducted in the same trial site as the other efficacy trials considered. The study design and main results of the trials considered in the assessment are presented in Tables 1 and 2, respectively.

In all the trials, 1-day-old male birds (Ross 308) were raised in pens in groups of 30 (trial 1 and 2) or 20 (trial 3) birds. In the three trials, there was a control group with birds fed starter/grower non-supplemented diets and one experimental group with chickens fed starter/grower diets containing the BIO-THREE® at 2.0×10^7 *B. subtilis* FERM BP-07462 CFU, 2.0×10^8 *E. lactis* FERM BP-10867 CFU and 2.0×10^7 *C. butyricum* FERM BP-10866 CFU per kg feed (the minimum recommended concentration). Other levels were also considered in trial 1 (double the minimum recommended level in CFU/kg feed) and trial 3 (ten or hundred times the minimum recommended level in CFU/kg feed). In all cases, the counts (CFU/kg feed) of each strain contained in the additive were confirmed by analysis. The diets were administered on ad libitum basis and in mash form for a total of 42 days (studies 1 and 2) or 35 days (study 3). Health and mortality were monitored daily throughout the study and the body weight and feed intake were recorded. Mortality-adjusted feed to gain ratio was calculated. An analysis of variance was done with the data from each single study using the pen as the experimental unit. Group means were compared with Tukey test. Significance level was set at 0.05.

[53] Technical dossier/Section IV/Annex IV_3_3, Annex IV_3_4 and Annex IV_3_5.
[54] Council Directive 2007/43/EC of 28 June 2007 laying down minimum rules for the protection of chickens kept for meat production, OJ L 182 12.7.2007, p. 19.
[55] Technical dossier/Section IV/Annex IV_3_1 and Annex IV_3_2 and Spontaneous information/Annex IV_3_6 and Annex IV_3_7.
[56] Technical dossier/Section IV/Annex IV_3_1 and Annex IV_3_2.
[57] Technical dossier/Spontaneous information/Annex IV_3_6 and Annex IV_3_7.

Table 1: Trial design and use levels in the trials in chickens for fattening

Trial	Total No of animals (animals per replicate) replicates per treatment	Breed sex (duration)	Composition feed (form)	Groups (×10^7 CFU *B. subtilis*/× 10^8 CFU *E. lactis*/× 10^7 CFU *C. butyricum* per kg feed))		
				Intended	Starter analysed	Grower analysed
1[(a)]	1,560 (30) 16/18	Ross 308 Males (42 d)	Maize and soya bean (Mash)	0/0/0	< 0.002/< 0.0002/< 0.001	< 0.002/< 0.0002–0.01/< 0.001
				2.0/2.0/2.0	0.2–1.6/0.4–1.6/2.8–3.0	1.0–2.0/0.2–0.42/4.0–5.3
				4.0/4.0/4.0	2.0–40/1.0–2.0/4.0–4.2	60–76/0.68–2.0/6.3–7.3
2[(b)]	1,560 (30) 26	Ross 308 Males (42 d)	Maize and soya (Mash)	0/0/0	22–60/0.0073–0.053/< 0.004	41–62/0.0073–0.022/< 0.004
				2.0/2.0/2.0	1.9–2.4/0.93–0.94/1.4–1.6	1.7–1.9/0.72–0.8/1.2–1.3
3[(c)]	960 (20) 12	Ross 308 Males (35 d)	Wheat, soya and maize (Mash)	0/0/0	< 0.1–0.66/< 0.01–0.057/< 0.1	< 0.1–0.47/< 0.01/< 0.1
				2.0/2.0/2.0	1.0–3.7/0.59–1.9/2.6–2.9	1.0–3.2/0.42–0.55/2.1–2.5
				20/20/20	16–26/4.8–20/19–26	20–26/3.5–6.0/20–26
				200/200/200	180–230/41–57/200–240	190–210/39–70/190

(a): Technical dossier/Section IV/Annex IV_3_1 and Annex IV_3_2.
(b): Technical dossier/Spontaneous information/Annex IV_3_6 and Annex IV_3_7.
(c): Technical dossier/Supplementary information July 2021/Annex 12.1

Table 2: Effects of BIO-THREE® on the performance of chickens for fattening

Trial	Groups (*B. subtilis*/*E. lactis*/*C. butyricum* (CFU/kg feed))	Daily feed intake (g)	Final body weight (g)	Average daily gain (g/bird)	Feed to gain ratio	Mortality and culling (%)[(a)]
1	0/0/0	116.4	2,581[b]	60.2[b]	1.93[b]	3.12
	$2.0 \times 10^7/2.0 \times 10^8/2.0 \times 10^7$	114.5	2,565[b]	59.9[b]	1.91[b]	1.85
	$4.0 \times 10^7/4.0 \times 10^8/4.0 \times 10^7$	115.6	2,694[a]	62.9[a]	1.84[a]	2.22
2	0/0/0	110.2	2,572[b]	60.1[b]	1.83[b]	2.2
	$2.0 \times 10^7/2.0 \times 10^8/2.0 \times 10^7$	108.9	2,611[a]	61.0[a]	1.78[a]	2.3
3	0/0/0	99.6	1,995[a]	55.7[a]	1.79[b]	1.7
	$2.0 \times 10^7/2.0 \times 10^8/2.0 \times 10^7$	99.7	2,112[b]	59.1[b]	1.69[a]	0.8
	$2.0 \times 10^8/2.0 \times 10^9/2.0 \times 10^8$	99.1	2,091[b]	58.4[b]	1.70[a]	2.1
	$2.0 \times 10^9/2.0 \times 10^{10}/2.0 \times 10^9$	100.5	2,062[ab]	57.5[ab]	1.75[b]	2.1

[a,b]: Mean values within a trial and within a column with a different superscript are significantly different $p < 0.05$.
(a): Technical dossier/Supplementary information July 2021/Annex 10.1.

In trial 1, the birds receiving BIO-THREE® at double the minimum recommended level (in CFU/kg feed) showed a significantly higher body weight and average daily gain and better feed to gain ratio compared to control birds, but there were not significant differences between the control and the group receiving the minimum recommended level.

In trials 2 and 3, the birds fed BIO-THREE® at the minimum recommended level showed a significantly higher body weight and average daily gain and better feed to gain ratio compared to control birds.

Overall, three studies showed positive effects of the supplementation with BIO-THREE® on the performance of chickens for fattening. However, the Panel notes that the three studies were done in the same trial location, which does not comply with the requirements of Regulation (EC) No 429/2008 and the Guidance on the assessment of the efficacy of feed additives (EFSA FEEDAP Panel, 2018a). Therefore, the Panel is not in the position to conclude on the efficacy of the additive for the target species.

3.3.2. Compatibility with coccidiostats

The applicant provided *in vitro* studies to support the compatibility of *B. subtilis* FERM BP-07462, *E. lactis* FERM BP-10867 and *C. butyricum* FERM BP-10866 with diclazuril, decoquinate and halofuginone; and *in vitro* and *in vivo* studies to support the compatibility of the three strains with monensin sodium, salinomycin sodium, narasin, robenidine hydrochloride and maduramicin ammonium.[58]

MIC values of these coccidiostats against the individual active agents were assessed *in vitro* using the broth microdilution method in aerobic (*B. subtilis* FERM BP-07462, *E. lactis* FERM BP-10867) or anaerobic (*C. butyricum* FERM BP-10866) conditions. The MIC values for diclazuril (> 4.8 mg/kg), decoquinate (> 160 mg/kg) and halofuginone (> 12 mg/kg) were greater than four times the maximum authorised level of these coccidiostats in feed (1.2, 40 and 3 mg/kg, respectively).[59] The MIC values[60] for monensin sodium (< 31.5 mg/kg), salinomycin sodium (< 17.5 mg/kg), narasin (< 17.5 mg/kg), robenidine hydrochloride (< 9 mg/kg) and maduramicin ammonium (< 1.5 mg/kg) were below four times their maximum authorised dose (125, 70, 70, 36 and 6 mg/kg, respectively).

The results indicate that BIO-THREE® is compatible with diclazuril, decoquinate and halofuginone; however, as the MIC values for monensin sodium, salinomycin sodium, narasin, robenidine

[58] Technical dossier/Section II/Annexes_Sect_II/Annex_II_4_4a and Spontaneous information November 2020/5_Annexes/Annex_II_4_4b.
[59] Maximum authorised levels: diclazuril 1 mg/kg (chickens for fattening, guinea fowls, chickens reared for laying and turkey for fattening); decoquinate 40 mg/kg; (chickens for fattening); halofuginone 3 mg/kg (chickens for fattening and turkeys); monensin sodium 125 mg/kg (chickens for fattening and chickens reared for laying) and 100 mg/kg (turkeys); salinomycin sodium 70 mg/kg (chickens for fattening) and 50 mg/kg (chickens reared for laying); narasin 70 mg/kg (chickens for fattening); robenidine hydrochloride 36 mg/kg (chickens for fattening); maduramicin ammonium 6 mg/kg (chickens for fattening) and 5 mg/kg (turkeys).
[60] Data shown is for the most susceptible strain, *C. butyricum* FERM BP-10866 except for robenidine hydrochloride for which the most susceptible strains were *B. subtilis* FERM BP-07462 and *E. lactis* FERM BP-10867.

hydrochloride and maduramicin ammonium were less than four times the maximum authorised level for these coccidiostats, an *in vivo* study was submitted.

In the *in vivo* study, a total of 420 chickens were distributed into seven treatments (6 pens of 10 birds per treatment).[61] The duration of the trial was 21 days. The birds were fed the basal feed (maize and Hipro soya) which was supplemented with BIO-THREE® at a level ensuring the microbiological content at 2.6×10^7 *B. subtilis* FERM BP-07462 CFU, 1.6×10^8 *E. lactis* FERM BP-10867 CFU and 3.2×10^7 *C. butyricum* FERM BP-10866 CFU per kg feed. The feed was either not supplemented or supplemented with the corresponding coccidiostat at the maximum authorised level: monensin sodium (125 mg/kg feed), salinomycin sodium (70 mg/kg), narasin (70 mg/kg), robenidine hydrochloride (36 mg/kg feed) or maduramicin ammonium (6 mg/kg feed). The analysed dose of each coccidiostat was close to its maximum authorised level. A group not supplemented with BIO-THREE® nor coccidiostats was included to represent the regular ileal microbiota.

At the end of the trial, 12 birds per treatment were killed and their ileal contents were sampled to determine *B. subtilis* FERM BP-07462, *E. lactis* FERM BP-10867 and *C. butyricum* FERM BP-10866 counts. The analysis of the samples was also performed with heat treatment in order to differentiate between the vegetative cells and spores for *B. subtilis* FERM BP-07462 and *C. butyricum* FERM BP-10866.

Detailed results are given in Table 3. The differences between the groups were statistically analysed with Tukey's test, comparing each group against the control diet (supplemented with the strain but without coccidiostat). The results are presented as medians.

Table 3: Effect of coccidiostats on the counts of ileal contents of chickens fed with *B. subtilis* FERM BP-07462, *E. lactis* FERM BP-10867 and *C. butyricum* FERM BP-10866

Treatment	Ileum counts (Log_{10} CFU/g)				
	***B. subtilis* FERM BP-07462**		***E. lactis* FERM BP-10867**	***C. butyricum* FERM BP-10866**	
	+ Heat treatment	**– Heat treatment**	**– Heat treatment**	**+ Heat treatment**	**– Heat treatment**
Negative control	2.7	2.7	5.7	1.8	1.7
BIO-THREE® (control)	3.4	3.4	5.5	1.7	1.7
BIO-THREE® + 125 mg Monensin sodium/kg feed	3.6	3.3	5.4	1.7	1.7
BIO-THREE® + 70 mg Salinomycin sodium/kg feed	3.4	3.5	4.5	1.7	1.7
BIO-THREE® + 36 mg Robenidine hydrochloride/kg feed	3.7	3.7	4.9	1.7	1.7
BIO-THREE® + 6 mg Maduramicin ammonium/kg feed	4.0	3.9	5.3	1.7	1.7
BIO-THREE® + 70 mg Narasin/kg feed	3.5	3.5	5.4	1.7	1.7

The colonies of *B. subtilis* in the samples tested were identified at species level using an internally developed PCR method and at strain level using RAPD-PCR. The profiles obtained confirmed the correspondence of the colonies with *B. subtilis* FERM BP-07462. The counts for *B. subtilis* FERM BP-07462 in the groups supplemented with the additive and coccidiostats were equal or higher than the counts in the control group.

Regarding the colonies of *C. butyricum*, those were identified at species level in 24 out of 83 samples tested using an internally developed PCR method. The colonies were further identified at strain level using RAPD-PCR. However, only in 11 samples, the profiles obtained confirmed the correspondence of the colonies with *C. butyricum* FERM BP-10866. The Panel notes that the ileal counts for *C. butyricum* FERM BP-10866 in the groups supplemented with the additive alone and the additive plus coccidiostats were very low. The rapid transit time in the ileum and the microaerophilic conditions of the ileum might have prevented the germination of the spores in that section of the intestine. Therefore, no conclusions can be drawn from the data related to this strain.

[61] Technical dossier/Spontaneous information November 2020/Annexes/Annex_II_4_4b.

Regarding the colonies of *E. lactis*, those were first isolated on KFSA medium (KF *Streptococcus* Agar with triphenyltetrazolium chloride) supplemented with kanamycin (800 μg/mL) and then identified using GP ID cards followed by RAPD-PCR. The colonies were preliminarily identified as belonging to the *E. faecium* species and none of the profiles obtained by RAPD-PCR corresponded to the one of *E. lactis* FERM BP-10867. Therefore, the correspondence of the colonies in the samples with *E. lactis* FERM BP-10867 was not confirmed. The Panel notes that the active agent *E. lactis* FERM BP-10867 proved to be susceptible to all the aminoglycosides tested, including kanamycin,[16] and no data showing the ability of the strain to grow on KFSA medium supplemented with kanamycin (800 μg/mL) were provided. Therefore, uncertainties remain on the suitability of the medium used to isolate the colonies of *E. lactis* FERM BP-10867 from the samples tested.

Therefore, the Panel cannot conclude on the compatibility of BIO-THREE® with monensin sodium, salinomycin sodium, narasin, robenidine hydrochloride and maduramicin ammonium.

3.3.3. Conclusions on efficacy

The FEEDAP Panel is not in the position to conclude on the efficacy of BIO-THREE® for the target species.

The FEEDAP Panel concludes that BIO-THREE® is compatible with diclazuril, decoquinate and halofuginone. No conclusions can be drawn on the compatibility of BIO-THREE® with monensin sodium, salinomycin sodium, narasin, robenidine hydrochloride and maduramicin ammonium.

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[62] and Good Manufacturing Practice.

4. Conclusions

BIO-THREE® is considered safe for chickens for fattening, chickens reared for laying, turkeys for fattening, turkeys reared for breeding, all avian species for rearing/fattening to slaughter and all avian species reared for laying or breeding to point of lay at the recommended level of 2.0×10^7 *B. subtilis* FERM BP-07462 CFU, 2.0×10^8 *E. lactis* FERM BP-10867 CFU and 2.0×10^7 *C. butyricum* FERM BP-10866 CFU per kg feed and at 1.0×10^7 *B. subtilis* FERM BP-07462 CFU/L, 1.0×10^8 *E. lactis* FERM BP-10867 CFU/L and 1.0×10^7 *C. butyricum* FERM BP-10866 CFU/L of water for drinking with a wide margin of safety.

The additive is safe for the consumers of products derived from animals fed with the additive.

The additive is not irritant to skin and eyes but is considered a respiratory sensitiser. No conclusions can be drawn on its potential to be a skin sensitiser.

The use of the product as a feed additive is of no concern for the environment.

The FEEDAP Panel is not in the position to conclude on the efficacy of BIO-THREE® for the target species. BIO-THREE® is compatible with diclazuril, decoquinate and halofuginone. No conclusions can be drawn on the compatibility of BIO-THREE® with monensin sodium, salinomycin sodium, narasin, robenidine hydrochloride and maduramicin ammonium.

Documentation as provided to EFSA/Chronology

Date	Event
29/07/2020	Dossier received by EFSA. BIO-THREE® (*Bacillus subtilis* TO-A (BS), *Enterococcus faecium* T-110 (EF), *Clostridium butyricum* TO-A (CB)) for chickens for fattening, chickens reared for laying, turkeys for fattening, turkeys reared for breeding, minor poultry species. Submitted by TOA BIOPHARMA Co., Ltd.
12/08/2020	Reception mandate from the European Commission
21/10/2020	Application validated by EFSA – Start of the scientific assessment
18/11/2020	Reception of spontaneous information from the applicant
09/12/2020	Reception of the Evaluation report of the European Union Reference Laboratory for Feed Additives

[62] 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.

Date	Event
18/12/2020	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. *Issues: characterisation, user safety, efficacy*
07/01/2021	Clarification teleconference during risk assessment
22/01/2021	Clarification teleconference during risk assessment
22/01/2021	Comments received from Member States
15/02/2021	Clarification teleconference during risk assessment
03/02/2021	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. *Issues: target species safety*
29/03/2021	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*
29/07/2021	Reception of supplementary information from the applicant - Scientific assessment re-started
14/10/2021	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, efficacy*
02/12/2021	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*
01/02/2022	Reception of supplementary information from the applicant - Scientific assessment re-started
29/03/2022	Reception of spontaneous information from the applicant
05/04/2022	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. *Issues: target species safety, efficacy*
12/04/2022	Reception of supplementary information from the applicant - Scientific assessment re-started
04/05/2022	Opinion adopted by the FEEDAP Panel. End of the Scientific assessment

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Abbreviations

ANOVA	analysis of variance
ANI	average nucleotide identity
bw	body weight
CLSI	Clinical Laboratory Standards Institute
CV	coefficients of variation
CFU	colony forming unit
ENA	European Nucleotide Archive
EURL	European Union Reference Laboratory
FEEDAP	Panel on Additives and Products or Substances used in Animal Feed
GLP	Good Laboratory Practice
LOQs	limits of quantification
MCHC	mean corpuscular haemoglobin concentration
MCV	mean corpuscular volume
MCH	mean corpuscular haemoglobin
PCDD/F	Polychlorinated dibenzo-p-dioxins and dibenzofurans
PCB	polychlorinated biphenyls
PFGE	Pulsed Field Gel Electrophoresis
QPS	qualified presumption of safety
RBC	red blood cell count
VFDB	virulence factor database
WBC	white blood cell count
WGS	whole genome sequence

Annex A – Executive Summary of the Evaluation Report of the European Union Reference Laboratory for Feed Additives on the Method(s) of Analysis for *Bacillus subtilis* TO-A,[63] *Enterococcus faecium* T-110[64] and *Clostridium butyricum* TO-A[65]

In the current application an authorisation is sought under Article 4(1) for a preparation containing *Bacillus subtilis* TO-A, *Enterococcus faecium* T-110 and *Clostridium butyricum* TO-A (*BIO-THREE®*), under the category/functional group 4(b) 'zootechnical additives'/'gut flora stabilisers', according to Annex I of Regulation (EC) No 1831/2003. The authorisation is sought for the use of the *feed additive* for chickens for fattening, chickens reared for laying, turkeys for fattening, turkeys reared for breeding and all minor avian species to slaughter or to point of lay.

According to the Applicant, the *feed additive* contains as active agents viable spores of the non-genetically modified microorganism (non-GMM) *B. subtilis* TO-A, non-GMM *E. faecium* T-110 and viable spores of non-GMM *C. butyricum* TO-A. The feed additive is intended to be marketed as dry or liquid preparations containing a minimum content of 1×10^7 colony forming unit (CFU) B. subtilis TO-A /g, 1×10^8 CFU *E. faecium* T-110/g and 1×10^7 CFU *C. butyricum* TO-A /g and to be used directly in complete *feedingstuffs* or in complementary feeds and in *water* at a minimum dose of 2.0×10^6 CFU *B. subtilis* TO-A; 2.0×10^7 CFU *E. faecium* T-110 and 2.0×10^6 CFU *C. butyricum* TO-A /kg complete *feedingstuffs* and of 1.0×10^6 CFU *B. subtilis* TO-A, 1.0×107 CFU *E. faecium* T-110 and 1.0×10^6 CFU *C. butyricum* TO-A /l of *water*.

For the identification of the three target strains the EURL recommends for official control Pulsed Field Gel Electrophoresis (PFGE), a generally recognised methodology for the genetic identification of bacterial strains.

For the enumeration of *Bacillus subtilis* TO-A and *Enterococcus faecium* T-110 in the *feed additive, premixtures, feedingstuffs* and *water* the Applicant proposed the ring-trial validated spread plate methods EN 15784 and EN 15788, respectively, while for the enumeration of *Clostridium butyricum* TO-A in the *feed additive, premixtures feedingstuffs* and *water*, the Applicant proposed the pour plate method ISO 15213. Furthermore, the Applicant provided evidences of the suitability of all the mentioned methods for the enumeration of these microorganisms in the mentioned matrices.

Based on the performance characteristics reported and the applicability evidences provided by the Applicant, the EURL recommends for official control the internationally recognised standard methods EN 15784, EN 15788 and ISO 15213 for the enumeration of *Bacillus subtilis* TO-A, *Enterococcus faecium* T-110 and *Clostridium butyricum* TO-A respectively in the feed additive, *premixtures, feedingstuffs* and *water*.

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.

[63] In-house name for *B. subtilis* FERM BP-07462.
[64] In-house name for *E. lactis* FERM BP-10867. During the assessment the strain was identified as *Enterococcus lactis*.
[65] In-house name for C. *butyricum* FERM BP-10866.