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Safety and efficacy of a feed additive consisting of *Bacillus subtilis* strains CNCM I-4606, CNCM I-5043 and CNCM I-4607 and *Lactococcus lactis* CNCM I-4609 for all animal species (Nolivade)

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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 preparation of *Bacillus subtilis* CNCM I-4606, *B. subtilis* CNCM I-5043, *B. subtilis* CNCM I-4607 and *Lactococcus lactis* CNCM I-4609 when used as a technological additive (hygiene condition enhancer) for all animal species. The product is intended for use in compound feeds and feed materials for all animal species at a minimum inclusion level of 1×10^9 CFU *B. subtilis* and 1×10^9 CFU *L. lactis* per kg or litre. The two bacterial species are considered by EFSA to be eligible for the qualified presumption of safety (QPS) approach. As the identity of the strains has been clearly established and they did not show acquired resistance to antibiotics of human and veterinary importance, the use of these strains in animal nutrition is considered safe for the target species, consumers and the environment. The additive is not irritant to skin and eyes or a skin sensitiser. Given the proteinaceous nature of the active agents, the additive should be considered a respiratory sensitiser. The Panel is not in the position to conclude on the efficacy of the additive to significantly reduce the growth of either *Salmonella* Typhimurium or *Escherichia coli* in feed.

Keywords: technological additive, hygiene condition enhancer, *Bacillus subtilis* CNCM I-4606, *Bacillus subtilis* CNCM I-5043, *Bacillus subtilis* CNCM I-4607, *Lactococcus lactis* CNCM I-4609, efficacy

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1. Introduction

1.1. Background and Terms of Reference

Regulation (EC) No 1831/2003[1] establishes the rules governing the Community authorisation of additives for use in animal nutrition. In particular, Article 4(1) of that Regulation lays down that any person seeking authorisation for a feed additive or for a new use of a feed additive shall submit an application in accordance with Article 7.

The European Commission received a request from Nolivade[2] for authorisation of a preparation of *Bacillus subtilis* CNCM I-4606, *Bacillus subtilis* CNCM I-5043, *Bacillus subtilis* CNCM I-4607 and *Lactococcus lactis* CNCM I-4609, when used as a feed additive for all animal species (category: technological additive; functional group: hygiene condition enhancers).

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). EFSA received directly from the applicant the technical dossier in support of this application. The particulars and documents in support of the application were considered valid by EFSA as of 14 April 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 preparation of *Bacillus subtilis* CNCM I-4606, *Bacillus subtilis* CNCM I-5043, *Bacillus subtilis* CNCM I-4607 and *Lactococcus lactis* CNCM I-4609 (MixBaLac), when used under the proposed conditions of use (see Section 3.1.4).

1.2. Additional information

This additive is a preparation containing viable spores of three strains of *B. subtilis* (CNCM I-4606, CNCM I-5043 and CNCM I-4607) and live cells of *L. lactis* (CNCM I-4609). It has not been previously authorised as a feed or food 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[3] in support of the authorisation request for the use of a preparation of *B. subtilis* CNCM I-4606, *B. subtilis* CNCM I-5043, *B. subtilis* CNCM I-4607 and *L. lactis* CNCM I-4609 as a feed additive.

EFSA has verified the European Union Reference Laboratory (EURL) report as it relates to the methods used for the control of the active agent in animal feed. The Executive Summary of the EURL report can be found in Annex A.[4]

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of a preparation of *B. subtilis* CNCM I-4606, CNCM I-5043, CNCM I-4607 and *L. lactis* CNCM I-4609 is in line with the principles laid down in Regulation (EC) No 429/2008[5] 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 FEEDAP Panel, 2017a), 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 characterisation of microorganisms used as feed

[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] Nolivade, 2/4 avenue de Ker Lann CS 17228, 35172, BRUZ Cedex, France.
[3] FEED dossier reference: FAD-2019-0090.
[4] The full report is available on the EURL website: https://ec.europa.eu/jrc/sites/default/files/finrep-fad-2019-0090-mixbalac.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.

additives or as production organisms (EFSA FEEDAP Panel, 2018a), Guidance on the assessment of the efficacy of feed additives (EFSA FEEDAP Panel, 2018b) and Guidance on the assessment of the safety of feed additives for the environment (EFSA FEEDAP Panel, 2019).

3. Assessment

The present additive is based on a preparation containing viable spores of three strains of *B. subtilis* (CNCM I-4606, CNCM I-5043 and CNCM I-4607) and viable cells of *L. lactis* (CNCM I-4609). It will be hereafter referred to as MixBaLac. MixBaLac is intended to be used as a technological additive (functional group: hygiene condition enhancers) in feed for all animal species to reduce the microbiological level of enteropathogens such as *E. coli* and *Salmonella* spp.[6]

3.1. Characterisation

3.1.1. Characterisation of the active agents

The four bacterial strains (three *B. subtilis* and one *L. lactis* strains) were originally isolated from the environment of poultry farms and are deposited in the Collection Nationale de Cultures de Microorganismes (CNCM) Institut Pasteur in France with the accession numbers CNCM I-4606, CNCM I-5043, CNCM I-4607 and CNCM I-4609, respectively.[7] They have not been genetically modified.

The taxonomic identification of the strains as *B. subtilis* and *L. lactis* was confirmed based on the whole genome sequence (WGS). [redacted]

The susceptibility of the bacterial strains to the battery of antibiotics recommended by the FEEDAP Panel (EFSA FEEDAP Panel, 2018a) was tested [redacted] for the strains *B. subtilis* CNCM I-4606, CNCM I-5043 and CNCM I-4607,[12] [redacted] for *L. lactis* CNCM I-4609.[13] All the minimum inhibitory concentration (MIC) values were equal to or fell below the corresponding cut-off values and, therefore, the strains are considered susceptible to the relevant antibiotics.

The [redacted] was used for mass screening of contigs for antimicrobial resistance genes using the [redacted] databases plus an in-house database.[14] [redacted] The screening did not identify any hits of concern for any of the bacterial strains.

Cytotoxicity of the supernatant obtained from the three *B. subtilis* strains was screened with Vero-cells (LDH assay) according to the FEEDAP guidance (EFSA FEEDAP Panel, 2018a).[15] No cytotoxic activity was detected.

3.1.2. Characterisation of the product

[redacted] to reach a minimum declared content of 6.7×10^8 colony forming units (CFU)/g for each individual *B. subtilis* strain (2×10^9 CFU/g of all *B. subtilis* strains) (2–6%) and 2×10^9 CFU/g of *L. lactis* (1–8%). The applicant declared that no antimicrobials are used during the manufacturing of the additive.[16]

[6] Technical dossier/Supplementary information February 2021/FAD-2019-0090_SIn_27.10.20.
[7] Technical dossier/Section II/Annex_II_2_10.
[8] Technical dossier/Section II/Annex II_2.1.
[9] Technical dossier/Section II/Annex II_2.2.
[10] Technical dossier/Section II/Annex II_2.3.
[11] Technical dossier/Section II/Annex II_2.7.
[12] Technical dossier/Section II/Annex II_2.20.
[13] Technical dossier/Section II/Annex II_2.21.
[14] Technical dossier/Annexes II_2_1, II_2_2 and II_2_3.
[15] Technical dossier/Section II/Annex II_2_24.
[16] Technical dossier/Section II.

The specifications of active agents were confirmed by the analysis of five batches with a mean value of 8.4×10^9 CFU/g (range 6.6×10^9–1.1×10^{10} CFU/g) for all *B. subtilis* strains, and a mean value of 5.9×10^9 CFU/g (range 3.3×10^9–7×10^9 CFU/g) for the lactic acid bacterium.[17] Enumeration of the individual *B. subtilis* strains was based [redacted].[18] Five batches showed a mean value of 1.5×10^9 CFU/g (range 9.2×10^8–2.9×10^9 CFU/g) for *B. subtilis* CNCM I-4606, a mean value of 1.2×10^9 CFU/g (range 7.3×10^8–1.8×10^9 CFU/g) for *B. subtilis* CNCM I-5043 and a mean value of 1.3×10^9 CFU/g (range 8.5×10^8–2.1×10^9 CFU/g) for *B. subtilis* CNCM I-4607.

Microbial contaminants were analysed in three different batches.[19] In all cases, the results were below the specifications (Enterobacteriaceae < 10 CFU/g, total coliforms < 10 CFU/g, *E. coli* < 10 CFU/g, *Salmonella* spp. no detection in 25 g, *B. cereus* < 100 CFU/g, coagulase-positive staphylococci < 100 CFU/g, sulphite-reducing anaerobes < 10 CFU/g, *Clostridium perfringens* < 10 CFU/g and yeasts and filamentous fungi < 10^3 CFU/g).

Three different batches of the additive were examined for the presence of arsenic, lead, cadmium, mercury, fluorine, dioxins, dioxins and dioxin-like-polychlorinated biphenyls (PCBs) and non-dioxin-like-PCBs.[20] Results showed mean values of 0.370 mg/kg for arsenic (range: 0.341–0.391 mg/kg), 1.298 mg/kg for lead (range: 1.140–1.448 mg/kg), 25.7 mg/kg for fluorine (range: 18.6–29.8 mg/kg), 0.027 ng/kg for dioxins (range: 0.024–0.031 ng/kg), 0.038 ng/kg for dioxins and dioxin-like-PCBs (range: 0.034–0.044 ng/kg) and 0.048 μg/kg for non-dioxin-like-PCBs (range: 0.043–0.055 μg/kg); and levels below the limits of quantification (LOQs) for cadmium (<0.045 mg/kg) and mercury (< 0.023 mg/kg).

Three batches of MixBaLac were also analysed for mycotoxins concentration. The results showed the following mean values for deoxynivalenol (0.275 mg/kg, range: 0.265–0.295 mg/kg), zearalenone (0.12 mg/kg, range: 0.08–0.14 mg/kg), fumonisins B1 + B2 (0.25 mg/kg, range: 0.17–0.38 mg/kg) and T-2+HT-2 toxins (0.023 mg/kg, range: 0.01–0.035 mg/kg). Values for aflatoxin B1 and B2 and ochratoxin A were < LOQ.[21,22]

None of the above analysed impurities are considered of concern.

Three batches of the additive (carriers not specified) were examined for particle size distribution by laser diffraction and dusting potential by the Stauber–Heubach method.[23] Results showed that 37% by volume of the additive consists of particles with diameters below 100 μm, 21% below 50 μm and 5% below 10 μm. The average dusting potential was 5.4 g/m^3 (4.9–5.8 g/m^3). The average bulk density and tapped density were 600 kg/m^3 and 700 kg/m^3, respectively.

3.1.3. Stability

The shelf-life of MixBaLac was tested on three batches stored at –25°C, 5°C and 25°C/60% relative humidity (RH).[24] Total bacilli and lactic acid bacteria counts after 6 months at –25°C and 3 months at 5°C showed no losses (< 0.5 Log). The applicant declared that the product did not prove to be stable at 25°C, and thus the results of these analyses were not provided. In a second study, the shelf-life of the additive was tested on other three batches stored at –25°C for 12 months.[25] No losses were observed (< 0.5 Log). Although no monitoring of individual strains of *B. subtilis* was done, in the absence of any significant reduction of total bacilli counts and based on the spore-forming nature of the strains, these are expected to behave similarly.

The applicant states that MixBaLac is not intended to be used in commercial premixtures, but it could be used in specific premixtures based on mineral ingredients such as calcium carbonate, sepiolite

[17] Technical dossier/Section II/Annex_II_1_4 and Annex II_1_6.
[18] Technical dossier/Supplementary information September 2020/FAD-2019-0090_SIn_01.07.20/Annex SI_2 and Annex SI_3.
[19] Technical dossier/Section II/Annex_II_1_9.
[20] Technical dossier/Section II/Annex_II_1_8.
[21] Technical dossier/Section II/Annex_II_1_10.
[22] Limits of quantification: T-2 + HT-2 toxins: 0.01 mg/kg, deoxynivalenol: 0.01 mg/kg, zearalenone: 0.01, fumonisins B1 + B2: 0.01 mg/kg, aflatoxin B1 and B2: 0.001 mg/kg and ochratoxin A: 0.001 mg/kg.
[23] Technical dossier/Section II/Annex_II_1_11.
[24] Technical dossier/Section II/Annex_II_4_1 and Annex_II_1_4.
[25] Technical dossier/Supplementary information September 2020/FAD-2019-0090_SIn_01.07.20/Annex SIn 4.

and/or wheat feed to facilitate its incorporation in solid feedingstuffs. The stability of the additive (one batch) was tested when incorporated into a premixture containing [redacted], and a second premixture containing [redacted], and stored in heat-sealed opaque bags at −25°C and 5°C and 25°C/60% RH.[26] After 6 months no losses of *Bacillus* spp. counts were observed (< 0.5 Log units) in samples stored at −25°C and 5°C or of *L. lactis* counts (< 0.5 Log units) in samples stored at −25°C for both premixtures. The applicant declared a malfunctioning of the cooling chamber leading to unreliable results of *L. lactis* counts in samples stored at 5°C. Moreover, the applicant also declared that the additive proved not to be stable in these premixtures at 25°C/60% RH, and therefore, the results of the analysis were not submitted.

3.1.4. Conditions of use

The additive is intended for use in compound feeds and feed materials for all animal species at the minimum inclusion level of 1×10^9 total CFU *B. subtilis* and 1×10^9 CFU *L. lactis* per kg or litre.[27]

The applicant states that in case of dry feed, the additive is to be added to feed materials or compound feeds during the manufacturing process and/or when there is a risk of high moisture content and/or of contamination. In case of liquid feed, the additive should be added and mixed to dry feed just before the liquid preparation or directly in the liquid feed. MixBaLac is not intended to be used in commercial premixtures containing trace elements or preservatives.

3.2. Safety

3.2.1. Safety for the target species, consumers and environment

The species *B. subtilis* and *L. lactis* are considered by EFSA to be suitable for the Qualified Presumption of Safety (QPS) approach to safety assessment (EFSA, 2007; EFSA BIOHAZ Panel, 2020). This approach requires the identification of the strains to be conclusively established and evidence that they do not show acquired resistance to antibiotics of human and veterinary importance, and in the case of the *B. subtilis* strains, these do not show toxigenic potential. In the view of the FEEDAP Panel the identity of the strains was unambiguously established as *B. subtilis* and *L. lactis* and the other applicable qualifications have been met. Consequently, *B. subtilis* CNCM I-4606, *B. subtilis* CNCM I-5043, *B. subtilis* CNCM I-4607 and *L. lactis* CNCM I-4609 are presumed safe for the target species, consumers and the environment. Since no risks are expected from other components of the additive, MixBaLac is also presumed to be safe for the target species, consumers and the environment.

3.2.2. Safety for the user

The potential of MixBaLac was tested in a valid *in vitro* study performed according to OECD guideline 439, which showed that it is not irritant to the skin.[28]

The eye irritation potential of MixBaLac was tested in a valid *in vitro* study performed according to OECD guideline 438, and no prediction could be made for eye irritation.[29] Following the inconclusive results of the *in vitro* study and considering that MixBaLac is not skin irritant, an *in vivo* study was performed according to OECD guideline 405, which showed that the additive is not eye irritant/ corrosive.[30]

A valid skin sensitisation *in vivo* study following OECD guideline 442B showed that MixBaLac does not have any skin sensitisation potential.[31]

The dusting potential (5.4 g/m^3) suggests that exposure by inhalation is possible. Given the proteinaceous nature of the active agents, the additive should be considered a respiratory sensitiser.

The applicant listed several anticaking agents and carriers which would allow multiple formulations of the additive to be produced, and consequently, not all forms can be directly tested for user safety. However, for assessing the safety for the user of the additive, the active agents are the principal concern provided that other components do not introduce safety issues. For this specific product, the excipients used in the preparation of the final formulation do not introduce additional risks.

[26] Technical dossier/Supplementary information July 2020/Annex_II_4_2.
[27] Technical dossier/Supplementary information February 2021.
[28] Technical dossier/Section III/Annex_III_3.1.
[29] Technical dossier/Section III/Annex_III_3.2.
[30] Technical dossier/section III/Annex_III_3.3.
[31] Technical dossier/Section III/Annex_III_3.4.

3.2.2.1. Conclusions on safety for the user

MixBaLac is not irritant to skin and eyes or a skin sensitiser but should be considered a respiratory sensitiser.

3.3. Efficacy

The additive is intended to improve the hygienic characteristics of the feed by reducing the microbiological level of enteropathogens such as *E. coli* and *Salmonella* spp. To support the efficacy as a hygiene condition enhancer, 13 *in vitro* studies were conducted. However, six[32] of these were not further considered since the feed matrices used were sterilised prior to the inoculation of the additive and target organisms, and thus, these are considered not to represent the standard farming practices and conditions of use.

The remaining seven *in vitro* studies showed a common design and investigated the effects of MixBaLac in a range of feed matrices (Table 1). [redacted]

Table 1: Summary of *the in vitro* trials of different feed matrices inoculated with *S.* Typhimurium [redacted] or *E. coli* [redacted] and treated with MixBaLac

Study	Feed matrix (% dry matter) inoculant	Treatment MixBaLac (CFU *Bacillus subtilis*/l or kg and *Lactococcus lactis*/l or kg)	*Salmonella* spp. or *E. coli* counts (log_{10} CFU/mL or g) at days of the experiment					
			0	1	2	3	5	7
1[(a)]	[redacted]	Control	[redacted]	–	[redacted]	–	–	–
		1×10^9 1×10^9	[redacted]	–	[redacted]	–	–	–
		Δ	[redacted]	–	[redacted]	–	–	–
2[(b)]	[redacted]	Control	[redacted]	–	[redacted]	–	–	[redacted]
		1×10^9 1×10^9	[redacted]	–	[redacted]	–	–	[redacted]
		Δ	[redacted]		[redacted]			[redacted]
3[(c)]	[redacted]	Control	[redacted]	–	–	[redacted]	[redacted]	–
		1×10^9 1×10^9	[redacted]	–	–	[redacted]	[redacted]	–
		Δ	[redacted]			[redacted]	[redacted]	

[32] Technical dossier/Section IV/Annex_IV_2_1, Annex IV.2.2, Annex IV.2.3, Annex_IV_2_4, Annex_IV_2_5 and Annex_IV_3_1.
[33] From the Institut Pasteur culture collection.
[34] From NOLIVADE culture collection [redacted].

Study	Feed matrix (% dry matter) inoculant	Treatment MixBaLac (CFU *Bacillus subtilis*/l or kg and *Lactococcus lactis*/l or kg)	*Salmonella* spp. or *E. coli* counts (log_{10} CFU/mL or g) at days of the experiment					
			0	1	2	3	5	7
4[(d)]	[redacted]	Control	[redacted]	[redacted]	[redacted]	–	–	–
		1×10^9 1×10^9	[redacted]	[redacted]	[redacted]	–	–	–
		Δ	[redacted]	[redacted]	[redacted]			
5[(e)]	[redacted]	Control	[redacted]	[redacted]	[redacted]	–	–	–
		1×10^9 1×10^9	[redacted]	[redacted]	[redacted]	–	–	–
		Δ	[redacted]	[redacted]	[redacted]			
6[(f)]	[redacted]	Control	[redacted]	[redacted]	–	[redacted]	–	–
		1×10^9 1×10^9	[redacted]	[redacted]	–	[redacted]	–	–
		Δ	[redacted]	[redacted]		[redacted]		
7[(g)]	[redacted]	Control	[redacted]	[redacted]	[redacted]	–	–	–
		1×10^9 1×10^9	[redacted]	[redacted]	[redacted]	–	–	–
		Δ	[redacted]	[redacted]	[redacted]			

CFU: colony forming unit.
a, b: Means in the same column within study are significantly different compared to control with $p \leq 0.05$.
Δ: *Salmonella* spp. or *E. coli* counts (log_{10} CFU/mL) between control and MixBaLac; –: not measured.
(a): Technical dossier/Section IV/Annex_IV_4_1 and Supplementary information April 2021/FAD-2019-0090_SIn_28.04.2021 and Annex_SIII_1 and Annex_SIII_8.
(b): Technical dossier/Section IV/Annex_IV_5_1 and Supplementary information April 2021/FAD-2019-0090_SIn_28.04.2021 and Annex_SIII_2 and Annex_SIII_8.
(c): Technical dossier/Section IV/Annex_SII_2 and Supplementary information April 2021/FAD-2019-0090_SIn_28.04.2021 and Annex_SIII_3, Annex_SIII_8 and Annex_SIII_9.
(d): Technical dossier/Section IV/Annex_SII_3 and Supplementary information April 2021/FAD-2019-0090_SIn_28.04.2021/ Annex_SIII_4, Annex_SIII_8 and Annex_SIII_9.
(e): Technical dossier/Section IV/Annex_SII_4 and Supplementary information April 2021/FAD-2019-0090_SIn_28.04.2021/ Annex_SIII_5, Annex_SIII_8 and Annex_SIII_9.
(f): Technical dossier/Section IV/Annex_SII_5 and Supplementary information April 2021/FAD-2019-0090_SIn_28.04.2021/ Annex_SIII_6, Annex_SIII_8 and Annex_SIII_9.
(g): Technical dossier/Section IV/Annex_SII_6 and Supplementary information April 2021/FAD-2019-0090_SIn_28.04.2021/ Annex_SIII_7, Annex_SIII_8 and Annex_SIII_9.

In study 1 with dry feed, no statistically significant differences were observed in *Salmonella* spp. counts between the treated soya meal and the control after 2 days of storage.

In study 2, treatment of a high moisture soya meal with the additive resulted in a statistically and biologically significant/relevant reduction of *Salmonella* spp. counts compared to control after 7 days of storage. While *Salmonella* spp. counts slightly increased in the control feed over time, these remained stable in the treated feed.

In study 3, treatment of a maize kernels with the additive resulted in a statistically and biologically significant/relevant reduction of *Salmonella* spp. counts compared to control after 3 days of storage; however, this difference was not maintained at the end of the experiment (5 days) as *Salmonella* spp. counts declined in both the control and the treated feed.

In studies 4 and 5, treatment of mash feed for ducks resulted in limited but statistically significant lower counts of *Salmonella* spp. and *E. coli,* respectively compared to control at all the timepoints monitored.

In the two studies with liquid feeds (6 and 7), a statistically and biologically significant/relevant reduction of *Salmonella* spp. counts in treated feeds compared to control was observed in all timepoints monitored.

The Panel notes that the marginal reduction of counts observed in studies 4 and 5 (< 1 log) leads to results of questionable biological/practical value. Moreover, the timepoints at which significant results were observed in studies 2 and 7 may be questionable from the practical viewpoint, which

might limit the extrapolation of the results to practical use conditions. In addition, the Panel notes that the additive has been tested against only one strain of *S.* Typhimurium. Thus, no conclusions can be drawn on the efficacy of the additive against a diversity of *Salmonella enterica* in feeds.

3.3.1. Conclusions on efficacy

In two studies, the addition of MixBaLac showed the potential to limit the growth of *Salmonella* Typhimurium in feed with a dry matter (DM) range of 13–23%. In other two studies, the addition of MixBaLac showed the potential to maintain or decrease the growth of *S.* Typhimurium in feed with a DM range of 49–58% compared to the control. In a fifth study, the addition of MixBaLac showed the potential to decrease *S.* Typhimurium counts, but the persistence of the effect observed was not maintained over time.

The marginal reduction of counts observed between treatment groups in one study is questionable from the biological/practical viewpoint and the Panel has reservations on the relevance of the effects observed in other two studies under practical use conditions. Therefore, the Panel is not in the position to conclude on the efficacy of MixBaLac to significantly reduce the growth of *S.* Typhimurium in feed.

In the absence of three positive studies showing significant and biologically relevant results, the Panel is not in the position to conclude on the efficacy of MixBaLac to significantly reduce the growth of *E. coli* in feed.

4. Conclusions

The additive consisting of *B. subtilis* strains CNCM I-4606, CNCM I-5043 and CNCM I-4607 and *L. lactis* CNCM I-4609 is considered safe for the target species, consumers and the environment.

The additive is not irritant to skin and eyes and is not a skin sensitiser but should be considered a respiratory sensitiser.

The Panel is not in the position to conclude on the efficacy of the additive to significantly reduce the growth of *S.* Typhimurium or *E. coli* in feed.

5. Documentation as provided to EFSA/Chronology

Date	Event
18/12/2019	Dossier received by EFSA. MixBaLac (*Bacillus subtilis* CNCM I-4606, CNCM I-5043, CNCM I-4607 and *Lactococcus lactis* CNCM I-4609) as a hygiene condition enhancer for all animal species. Submitted by Nolivade.
22/01/2020	Reception mandate from the European Commission
14/04/2020	Application validated by EFSA – Start of the scientific assessment
01/07/2020	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 of the additive*
17/07/2020	Reception of the Evaluation report of the European Union Reference Laboratory for Feed Additives
22/09/2020	Reception of supplementary information from the applicant - Scientific assessment re-started
14/10/2020	Comments received from Member States
27/10/2020	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. *Issues: efficacy*
10/03/2021	Reception of supplementary information from the applicant - Scientific assessment re-started
28/04/2021	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. *Issues: efficacy*
26/05/2021	Reception of supplementary information from the applicant - Scientific assessment re-started
30/09/2021	Opinion adopted by the FEEDAP Panel. End of the Scientific assessment

References

EFSA (European Food Safety Authority), 2007. Opinion of the Scientific Committee on a request from EFSA on the introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA. EFSA Journal 2007;587, 16 pp. https://doi.org/10.2903/j.efsa.2007.587

EFSA BIOHAZ Panel (EFSA Panel on Biological Hazards), Koutsoumanis K, Allende A, Alvarez-Ordóñez A, Bolton D, Bover-Cid S, Chemaly M, Davies R, De Cesare A, Hilbert F, Lindqvist R, Nauta M, Peixe L, Ru G, Simmons M, Skandamis P, Suffredini E, Cocconcelli PS, Fernández Escámez PS, Maradona MP, Querol A, Suarez JE, Sundh I, Vlak J, Barizzone F, Correia S and Herman L, 2020. Scientific Opinion on the update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA (2017–2019). EFSA Journal 2020;18(2):5966, 56 pp. https://doi.org/10.2903/j.efsa.2020.5966

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2012. Guidance on studies concerning the safety of use of the additive for users/workers. EFSA Journal 2012;10(1):2539, 5 pp. https://doi.org/10.2903/j.efsa.2012.2539

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), Rychen G, Aquilina G, Azimonti G, Bampidis V, Bastos ML, Bories G, Chesson A, Cocconcelli PS, Flachowsky G, Gropp J, Kolar B, Kouba M, López-Alonso M, López Puente S, Mantovani A, Mayo B, Ramos F, Saarela M, Villa RE, Wallace RJ, Wester P, Anguita M, Galobart J and Innocenti ML, 2017a. Guidance on the identity, characterisation and conditions of use of feed additives. EFSA Journal 2017;15(10):5023, 12 pp. https://doi.org/10.2903/j.efsa.2017.5023

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), Rychen G, Aquilina G, Azimonti G, Bampidis V, Bastos ML, Bories G, Chesson A, Cocconcelli PS, Flachowsky G, Gropp J, Kolar B, Kouba M, López-Alonso M, López Puente S, Mantovani A, Mayo B, Ramos F, Saarela M, Villa RE, Wallace RJ, Wester P, Anguita M, Galobart J, Innocenti ML and Martino L, 2017b. Guidance on the assessment of the safety of feed additives for the target species. EFSA Journal 2017;15(10):5021, 19 pp. https://doi.org/10.2903/j.efsa.2017.5021

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), Rychen G, Aquilina G, Azimonti G, Bampidis V, Bastos ML, Bories G, Chesson A, Cocconcelli PS, Flachowsky G, Gropp J, Kolar B, Kouba M, López-Alonso M, López Puente S, Mantovani A, Mayo B, Ramos F, Saarela M, Villa RE, Wallace RJ, Wester P, Anguita M, Dujardin B, Galobart J and Innocenti ML, 2017c. Guidance on the assessment of the safety of feed additives for the consumer. EFSA Journal 2017;15(10):5022, 17 pp. https://doi.org/10.2903/j.efsa.2017.5022

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), Rychen G, Aquilina G, Azimonti G, Bampidis V, Bastos ML, Bories G, Chesson A, Cocconcelli PS, Flachowsky G, Gropp J, Kolar B, Kouba M, López-Alonso M, López Puente S, Mantovani A, Mayo B, Ramos F, Saarela M, Villa RE, Wallace RJ, Wester P, Glandorf B, Herman L, Kärenlampi S, Aguilera J, Anguita M, Brozzi R and Galobart J, 2018a. Guidance on the characterisation of microorganisms used as feed additives or as production organisms. EFSA Journal 2018;16(3):5206, 24 pp. https://doi.org/10.2903/j.efsa.2018.5206

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), Rychen G, Aquilina G, Azimonti G, Bampidis V, Bastos ML, Bories G, Chesson A, Cocconcelli PS, Flachowsky G, Gropp J, Kolar B, Kouba M, López-Alonso M, López Puente S, Mantovani A, Mayo B, Ramos F, Saarela M, Villa RE, Wallace RJ, Wester P, Anguita M, Galobart J, Innocenti ML and Martino L, 2018b. Guidance on the assessment of the efficacy of feed additives. EFSA Journal 2018;16(5):5274, 25 pp. https://doi.org/10.2903/j.efsa.2018.5274

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), Bampidis V, Bastos ML, Christensen H, Dusemund B, Kouba M, Kos Durjava M, López-Alonso M, López Puente S, Marcon F, Mayo B, Pechová A, Petkova M, Ramos F, Sanz Y, Villa RE, Woutersen R, Brock T, Knecht J, Kolar B, Beelen P, Padovani L, Tarrés-Call J, Vettori MV and Azimonti G, 2019. Guidance on the assessment of the safety of feed additives for the environment. EFSA Journal 2019;17(4):5648, 78 pp. https://doi.org/10.2903/j.efsa.2019.5648

Abbreviations

ANI	average nucleotide identity
CFU	colony forming unit
CLSI	Clinical and Laboratory Standards Institute
CV	coefficient of variation
DM	dry matter
EURL	European Union Reference Laboratory
FEEDAP	EFSA Panel on Additives and Products or Substances used in Animal Feed
MIC	minimum inhibitory concentration
OECD	Organisation for Economic Co-operation and Development
PCB	polychlorinated biphenyl
qPCR	quantitative real-time PCR
QPS	Qualified Presumption of Safety
RH	relative humidity
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 MixBaLac

In the current application an authorisation is sought under Article 4(1) for the preparation of *Bacillus subtilis* CNCM I-4606, *Bacillus subtilis* CNCM I-5043, *Bacillus subtilis* CNCM I-4607 and *Lactococcus lactis* CNCM I-4609 (MixBaLac) under the category/functional group 1(n) 'technological feed additive'/'hygiene condition enhancer', according to Annex I of Regulation (EC) No 1831/2003. The authorisation is sought for the use of the feed additive for all animal species.

According to the Applicant, the feed additive contains as an active substance the viable spores of *Bacillus subtilis* (CNCM I-4606, CNCM I-5043, CNCM I-4607) and live cells of *Lactococcus lactis* (CNCM I-4609). The feed additive contains a minimum content of 2×10^9 Colony Forming Units (CFU) of total *Bacillus subtilis*/g feed additive and a minimum content of 2×10^9 CFU of *Lactococcus lactis*/g feed additive.

The feed additive is intended to be used directly into feedingstuffs or through premixtures at a minimum dose of 1×10^9 CFU of total *Bacillus subtilis*/kg (or L) complete feedingstuffs and at a minimum dose of 1×10^9 CFU of *Lactococcus lactis*/kg (or L) complete feedingstuffs.

For the identification of *Bacillus subtilis* CNCM I-4606, *Bacillus subtilis* CNCM I-5043, *Bacillus subtilis* CNCM I-4607 and *Lactococcus lactis* CNCM I-4609, the EURL recommends for official control Pulsed Field Gel Electrophoresis (PFGE), a generally recognised methodology for genetic identification of bacterial strains.

For the enumeration of the total *Bacillus subtilis* (CNCM I-4606, CNCM I-5043 and CNCM I-4607) in the feed additive, premixtures and feedingstuffs the Applicant submitted the ring-trial validated spread-plate CEN method EN 15784.

Based on the performance characteristics available, the EURL recommends this method for official control for the enumeration of the total *Bacillus subtilis* (CNCM I-4606, CNCM I-5043 and CNCM I-4607) in the feed additive, premixtures and feedingstuffs.

For the enumeration of *Lactococcus lactis* (CNCM I-4609) in the feed additive, premixtures and feedingstuffs the Applicant proposed the ISO 15214 pour-plate method dedicated for the enumeration of mesophilic lactic acid bacteria in food and feed.

Based on acceptable applicability data of the method, the EURL recommends it for official control for the enumeration of *Lactococcus lactis* (CNCM I-4609) in the feed additive, premixtures and feedingstuffs.

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.