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Safety and efficacy of *Bacillus amyloliquefaciens* (NCIMB 30229) as a silage additive for all animal species

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Abstract

The additive under assessment is a preparation of *Bacillus amyloliquefaciens* NCIMB 3022 intended for use as a technological additive in forages for all animal species at a proposed minimum dose of 5×10^7 CFU/kg fresh materials. The species *B. amyloliquefaciens* is considered by EFSA to be suitable for the qualified presumption of safety approach to safety assessment. This approach requires the identification of the strain and evidence that it is not toxigenic and does not show acquired resistance to relevant antibiotics. In a previous assessment, the identity and susceptibility to clinically relevant antibiotics of the active agent was established but the lack of toxigenic potential could not be demonstrated. Therefore, the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) could not conclude on the safety of the product for target animals, consumers, users or the environment. In the same opinion, the potential of the additive to improve aerobic stability of silages at the proposed dose was not convincingly demonstrated. A new cytotoxicity study was conducted using methanol extracts. The methanol extract was cytotoxic at the inclusion level of 5%, while no cytotoxicity was observed at lower concentrations. The current guidance on *Bacillus* requires absence of toxic effect using the non-concentrated supernatant. Since the applicant was unable to determine the correlation between the methanol extracts and the *Bacillus* supernatant concentrations, the FEEDAP Panel is unable to conclude on the toxigenic potential of the strain based on the current data. An additional efficacy study with a similar protocol to the ones previously assessed was conducted. The study showed an improved aerobic stability when added to forage at the proposed dose. However, the FEEDAP Panel remains unable to conclude on the efficacy of the additive to improve the ensiling process on the basis of a single positive result.

Keywords: technological additive, silage additive, *Bacillus amyloliquefaciens*, safety, cytotoxicity, methanol extract, efficacy

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1. Introduction

1.1. Background and Terms of Reference as provided by the requestor

Regulation (EC) No 1831/2003[1] establishes the rules governing the Community authorisation of additives for use in animal nutrition and in particular, Article 9 defines the terms of the authorisation by the Community.

The applicant, SILAC EEIG, is seeking a Community authorisation of *Bacillus amyloliquefaciens* MBS-BS-01/NCIMB 30229 to be used as a silage additive for all animal species (Table 1).

Table 1: Description of the substances

Category of additive	Technological additive
Functional group of additive	Silage additive
Description	*Bacillus amyloliquefaciens* MBS-BS-01/NCIMB 30229
Target animal category	All species and categories
Applicant	SILAC EEIG
Type of request	New opinion

On 11 December 2012, the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) of the European Food Safety Authority ('Authority'), in its opinion on the safety and efficacy of the product, concluded that the use of the strain in production of silage presents a hazard for consumers, users and environment, and potentially also to the target animals. The Authority also concluded that the potential of the additive to improve aerobic stability of silages at the proposed dose of 5.0×10^7 CFU/kg fresh materials was not convincingly demonstrated.

The Commission gave the possibility to the applicant to submit complementary information in order to complete the assessment on the safety and efficacy of the product and to allow a revision of the Authority's opinion.

The Commission has now received new data on the safety and efficacy of *Bacillus amyloliquefaciens* MBS-BS-01/NCIMB 30229.

In view of the above, the Commission asks the Authority to deliver a new opinion on the safety and efficacy of *Bacillus amyloliquefaciens* MBS-BS-01/NCIMB 30229 as a silage feed for all species based on the additional data submitted by the applicant.

1.2. Additional information

In 2011, the European Food Safety Authority (EFSA) was requested by the European Commission to re-evaluate the product when used as a technological additive (functional group: silage additive) in feed for all animal species (EFSA FEEDAP Panel, 2013). In that opinion the FEEDAP Panel was unable to conclude either on the safety of the product for consumers of products derived from animals fed the treated silage, users, environment and target animals or on the efficacy of the product.

The species *B. amyloliquefaciens* is considered by EFSA to be suitable for the Qualified Presumption of Safety (QPS) approach to safety assessment (EFSA, 2007; EFSA BIOHAZ Panel, 2017). This approach requires the identity of the strain to be conclusively established and evidence that the strain is not toxigenic and does not show acquired resistance to antibiotics of human and veterinary importance.

2. Data and methodologies

2.1. Data

The present assessment is based on data submitted by the applicant in the form of additional information[2] to a previous application on the same product.[3]

[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] FEED dossier reference: FAD-2015-0015.
[3] FEED dossier reference: FAD-2010-0192.

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of *Bacillus amyloliquefaciens* MBS-BS-01/NCIMB 30229 is in line with the principles laid down in Regulation (EC) No 429/2008 and the relevant guidance documents: Guidance on technological additives (EFSA FEEDAP Panel, 2012), Guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition (EFSA FEEDAP Panel, 2014).

3. Assessment

The additive under assessment is a preparation of *Bacillus amyloliquefaciens* MBS-BS-01/NCIMB 30229 with a minimum specification of 2×10^{10} CFU/g additive, intended for use as a technological additive (functional group: silage additive) in forages for all animal species. It is intended for use with forages at ensiling at a proposed minimum dose of 5×10^{7} CFU/kg fresh materials for the improvement of the aerobic stability in easy and moderately difficult to ensile material, as specified by Regulation (EC) No 429/2008.

In the previous assessment, the identity and susceptibility to clinically relevant antibiotics of *Bacillus amyloliquefaciens* NCIMB 3022 was established. The toxigenic potential was assessed following the recommendation of the Technical Guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition (EFSA FEEDAP Panel, 2011). Although the strain proved not to be haemolytic on blood agar, a polymerase chain reaction (PCR)-DNA sequencing approach showed the presence of genes involved in the synthesis of the non-ribosomal peptides surfactin, fengycin and bacillomycin (a member of iturin family). A mass spectrometry analysis (matrix-assisted laser desorption/ionisation time of flight (MALDI-TOF)), performed on a vegetative culture of *B. amyloliquefaciens* (NCIMB 30229), detected the peptides with a mass corresponding to surfactin or pumilacidin, fengycin, iturin A or mycosubtilin. The Panel requested a cytotoxicity assay made according to the latest version of the applicable guidance document (EFSA FEEDAP Panel, 2014), but the applicant failed to produce it. Consequently, the Panel concluded that '*B. amyloliquefaciens* (NCIMB 30229) produces cyclic lipopeptides which possess potent surfactant activity and are known to exert toxicity on mammalian cells (From et al., 2007a,b; Hwang et al., 2009). Although the strain is intended for use only in the production of silage, as a spore former it will survive the ensiling process and be ingested by the target animals. The spores will also survive passage through the gastrointestinal tract of animals and be a potential source of contamination of food of animal origin and of the environment. The greatest risk would be to those handling the product on farm following oral, dermal or respiratory exposure. In this context, it should be noted that the dusting potential of the commercial formulations tested was high'.

3.1. Toxigenic potential of *Bacillus amyloliquefaciens* MBS-BS-01/NCIMB 3022

A new cytotoxicity test using Vero cells was conducted according to the recommendation of the former version of the Technical Guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition (EFSA FEEDAP Panel, 2011).[4] The focus of the applicant was on the potential production of iturin A. After cultivation and incubation, the bacterium was extracted into methanol and the resulting extract was analysed by nuclear magnetic resonance. Results were compared with those of a commercial iturin A solution (range 1–500 μg/mL).

The methanol extract was cytotoxic at the inclusion level of 5%, while no cytotoxicity was observed at lower concentrations (0.1%, 0.5% and 1%). Iturin A was cytotoxic in the concentration range 7.2–24 μM (5% inclusion of iturin A solutions 150–500 μg/mL) while no cytotoxicity was observed at lower concentrations (0.048–4.8 μM (5% inclusion of iturin A solutions 1–100 μg/mL)).

The current guidance on *Bacillus* requires the absence of toxic effect using the non-concentrated supernatant. The FEEDAP Panel WG could have accepted the results of the cytotoxicity test provided that the ratio methanol/*Bacillus* supernatant were supplied and would equate to the testing of the supernatant alone. However, the applicant stated that no correlation on concentration could be made since the cells were harvested from plates and not from a liquid culture. Since non-ribosomal peptides are released in the supernatant, if cells are collected from agar surface, the detection of a secreted

[4] Technical dossier/Annex IIa.

compound is limited. Therefore, the FEEDAP Panel is unable to conclude on the toxigenic potential of *Bacillus amyloliquefaciens* MBS-BS-01/NCIMB 3022 based on the current data.

3.2. Safety for the target species, consumers, users and environment

In the absence of evidence of lack of toxigenic potential of the strain, the Panel does not have a basis to modify its previous conclusions and remains unable to conclude on the safety of the product for target animals, consumers of products derived from animals fed the treated silage, users, or environment.

3.3. Efficacy[5]

In the previous opinion, three experiments with laboratory-scale silos were carried out using samples of forage of differing dry matter and water-soluble carbohydrate content, representing material easy and moderately difficult to ensile. As described in the previous opinion, no significant effects of the additive on any of the ensiling parameters determined or on aerobic stability were observed in any of the studies, and therefore, the Panel concluded that the potential of the additive to improve aerobic stability of silages at the proposed dose of 5.0×10^7 CFU/kg fresh material was not convincingly demonstrated.

A new experiment has been submitted, with a similar protocol to the ones previously assessed.[6] The study is described in the dossier.

Throughout the 7 days of the aerobic stability test, the average temperature of treated silages was significantly lower than that of control silages (23.3 vs 35.4°C, $p < 0.05$). With treated silage, a rise of 3°C above ambient temperature was more than 2 days longer than that shown by untreated, and the difference was statistically significant (3.75 vs 1.47 days, $p < 0.05$). No other parameter measured was significantly affected by the treatment.

Although the new submitted study showed improved aerobic stability, the Panel is unable to conclude on the efficacy of the product on the basis of a single positive result.

4. Conclusions

The FEEDAP Panel remains unable to conclude either on the safety of the product for consumers of products derived from animals fed the treated silage, users, environment and target animals or on the efficacy of the product.

Documentation provided to EFSA

1) Supplementary information on *Bacillus amyloliquefaciens* (MBS-BS-01NCIMB 30229). April 2015. Submitted by FEFANA asbl.
2) Supplementary information on *Bacillus amyloliquefaciens* (MBS-BS-01NCIMB 30229). Supplementary information. February 2017. Submitted by FEFANA asbl.

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[5] This section has been edited following the provisions of Article 8(6) and Article 18 of Regulation (EC) No 1831/2003.
[6] Technical dossier/Annex XIII.

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Hwang YH, Kim MS, Song IB, Park BK, Lim JH, Park SC and Yun HI, 2009. Subacute (28 day) toxicity of surfactin C, a lipopeptide produced by *Bacillus subtilis*, in rats. Journal of Health Science, 55, 351–355.

Abbreviations

CFU	colony forming unit
DM	dry matter
FEEDAP	EFSA Panel on Additives and Products or Substances used in Animal Feed
MALDI-TOF	matrix-assisted laser desorption/ionisation time of flight
PCR	polymerase chain reaction
QPS	Qualified Presumption of Safety
WSC	water-soluble carbohydrate