SCIENTIFIC OPINION

Guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition[1†]

EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP)[2,3]

European Food Safety Authority (EFSA), Parma, Italy

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ABSTRACT

Bacillus species are used in animal production directly as microbial feed additives or as the source of other feed additives, notably enzymes. The principal safety concern for consumers and, to a lesser extent livestock, associated with *Bacillus* is a capacity for toxin production. However, the capacity for toxin production and the nature of the toxins produced is unevenly distributed over the genus, occurring frequently in some species and more rarely in others. In principle, the selection of strains belonging to the *B. cereus* taxonomic group for direct use in animal production is considered inadvisable. If, however, they are proposed then the full genome should be sequenced and a bioinformatic analysis made to search for genes coding for enterotoxins and cereulide synthase. If there is evidence of homology, the non-functionality of the genes (e.g., mutation, deletion) must be demonstrated. For other species, concerns centre on the production of cyclic lipopeptides (surfactins) in amounts able to cause demonstrable cell disruption. A two-step approach to assessment is proposed. Firstly a test for haemolysis on sheep blood agar. If the strain proves to be β-haemolytic it is not recommended for use. If not then this should be followed by cytotoxicity tests made preferably with Vero cells using a concentrate of the supernatant. Two methods of concentration are recommended, the first optimized for protein toxins and the second for heat-stable peptides. If the strain proves to be cytotoxic it is not recommended for use.

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[†] This guidance document replaces the previous EFSA Technical Guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition, adopted in November 2011 (EFSA-Q-2009-00973). The document has been globally modified except for Section 3.3
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KEY WORDS

Bacillus species, enterotoxin production, surfactins, emetic toxin, cereulide, cyclic lipopeptides.

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BACKGROUND AS PROVIDED BY EFSA

Regulation (EC) No 1831/20031 establishes the rules governing the Community authorisation of additives for use in animal nutrition. Moreover, Article 7(6) of this Regulation provides for the European Food Safety Authority (EFSA) to publish detailed guidance to assist applicants in the preparation and presentations of applications.

EFSA has the responsibility to assess the safety of feed additives before an authorisation is granted. A considerable amount of feed additives are composed by micro-organisms. As a tool to simplify and harmonise within EFSA the assessment of micro-organisms used in food and feed, the Scientific Committee published in 2007 one opinion on the introduction of a Qualified Presumption of Safety (QPS) approach for the assessment of selected micro-organisms.

The list of micro-organisms included in such opinion and considered to qualify for the QPS approach to safety assessment is updated regularly by the BIOHAZ Panel. The last update is from 2012. The QPS approach is regularly used by the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) in the assessment of microbial products subject to a pre-authorisation assessment.

Bacillus species are widely used as feed additives, and several of them are considered to qualify for the QPS approach to safety assessment, provided that the qualification of the absence of food poisoning toxins, surfactant activity or enterotoxic activity is met. In 2000, the Scientific Committee for Animal Nutrition (SCAN) adopted an opinion on the safety of use of *Bacillus* species in animal nutrition. This opinion was revised in 2011 by the FEEDAP Panel in the form of the Technical Guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition, and updated according to the most recent scientific and technical developments. The aim of this document, which complements the QPS Opinion, is to provide applicants with proportionate and up-to-date guidance on how to conduct the safety assessment of *Bacillus*-based products.

This Guidance makes a clear difference between the *Bacillus cereus* group (including known human enteropathogens) and other *Bacillus* species. Any toxigenic potential of non-*B. cereus* species appears to be linked to the production of heat-stable toxins referred to as surfactins or cyclic non-ribosomal peptides.

Science evolves fast and since the Guidance document was issued, new information on the toxicity and prevalence of these toxins has become available. Therefore, the FEEDAP Panel in view of this and of the experience gained so far from the assessment of the toxigenic potential of products based on *Bacillus* species (other than *B. cereus*) is intended to produce an update of the Guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition. This output is aimed at highlighting the uncertainties and making proposals to address them in the context of the assessment of the dossiers of non-*Bacillus cereus* based products.

TERMS OF REFERENCE AS PROVIDED BY EFSA

The FEEDAP Panel is requested to update the Guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition.

1. Introduction

A number of strains of *Bacillus* species are used in animal production either directly as microbial feed additives or as the source of other feed additives, notably enzymes. Regulation (EC) No 1831/2003 requires that all feed additives, including micro-organisms, are assessed for safety before being placed on the market. The principal safety concern for consumers and, to a lesser extent livestock, associated with *Bacillus* (and related genera) is a capacity for toxin production. However, the capacity for toxin production is unevenly distributed over the genus, occurring frequently in some species and more rarely, if at all, in others. For this reason, the Scientific Committee on Animal Nutrition (SCAN), when first developing guidance in this area, recommended that the use of strains of the *Bacillus cereus* taxonomic group, a group containing many known pathogenic strains, be strongly discouraged. However, the Committee recognised that strains from other *Bacillus* species may be considered safe (EC, 2000). The FEEDAP Panel concurs with this general position.

The Qualified Presumption of Safety (QPS) approach to the safety assessment of micro-organisms adopted by EFSA is considered applicable to most of the commercially relevant *Bacillus* species (EFSA, 2007, 2012). This approach requires the unambiguous identification of the strain being assessed, a demonstration of susceptibility to clinically relevant antibiotics and, in particular, evidence that the strain lacks a capacity for toxin production. Any other strain of *Bacillus* or related genera not falling within the scope of the QPS approach would also require an assessment of toxigenic potential. This document is intended to provide technical guidance for the assessment of any toxigenic potential for strains of *Bacillus* intended to be used directly as a feed additive or indirectly as a source of such additives.

2. The scope of the guidance

Although a number of species earlier considered to belong to the genus *Bacillus* have been transferred to other genera, to date none has been the subject of a feed additive assessment. Since relatively little is known about the toxigenic capacity of the genera related to *Bacillus* (i.e. *Geobacillus, Aneurinibacillus* and *Paenibacillus*) and, consequently, whether the approach to safety assessment described would fully apply, it is considered prudent to restrict this guidance to bacterial strains belonging to *Bacillus sensu strictu.*

3. Safety concerns caused by *Bacillus* species

3.1. Identification

Characterisation of *Bacillus* strains according to Claus and Berkeley (1986) and Bergey's manual of Systematic Bacteriology (2009) must be completed by molecular methods to identify strains to the species level. This is essential as it determines whether the current guideline applies and, if so, the nature of the testing recommended. Partial sequences (approximately 500 bp) of the 16S rRNA gene can be amplified using methods described in Guinebretière et al. (2001) and From et al. (2005), and compared to sequences from databases. If the partial sequence does not provide a definitive identification, then the 16S rRNA gene should be fully sequenced (Guinebretière et al., 2001). To differentiate species within the *B. subtilis* group, partial sequences of the *gyrA* gene or *gyrB* genes may be needed in addition to the 16S rRNA gene sequences. These can be obtained using methods described in Chun and Bae (2000) and From et al. (2005) for *gyrA* and Wang et al. (2007) for *gyrB*.

3.2. Assessment of *Bacillus* species other than the *Bacillus cereus* group

Bacillus species other than members of the *B. cereus* group are a rare cause of foodborne diseases. The production of the *B. cereus*-like diarrhoeal enterotoxins by some strains of other *Bacillus* species was described in the SCAN opinion (EC, 2000), although such strains have so far not been associated with foodborne diseases. The current view is that the very few reports of *B. cereus*–like enterotoxins occurring in other species of *Bacillus* are likely to have resulted from a misidentification of the strain involved (From et al., 2005). The few incidents of food poisoning investigated where non-*B. cereus* group strains were determined to be the causative organism suggest an association with heat-stable surfactins and similar cyclic lipopeptides with surfactin activity. The capacity for surfactin production appears widely distributed and has been documented for strains of *B. subtilis* (Hwang et al., 2009, From et al., 2007a, Mikkola et al., 2007, Apetroaie-Constantin et al., 2009), *B. licheniformis* (Nieminen et al., 2007), *B. pumilus* (Taylor et al., 2005, From et al., 2007b) and by *B. mojavensis* (From et al., 2005). However, although commonly found in low concentrations, problems have been encountered only when cyclic lipopeptides are produced in amounts able to cause demonstrable cell disruption.

Accordingly the steps recommended for the assessment of non-*B. cereus* group species are:

a) A test for haemolysis on sheep blood agar at 30 °C, incubated for 48 hours. Suitable positive and negative controls should be included (B. subtilis ATCC 21332 is suggested as the positive control and the B. subtilis type strain as the negative control). If the strain proves to be β-haemolytic it is not recommended for use. If not then;

b) A cytotoxicity test made preferably with Vero cells using a concentrate of the supernatant. Two methods of concentration are recommended, the first optimized for protein toxins and the second for heat-stable peptides. Both should be tested. The protocol presented in the Appendix is recommended but the use of methods based on lactate dehydrogenase (LDH) release or propidium iodide uptake is considered a valid alternative. If the strain proves to be cytotoxic it is not recommended for use.

3.3. Assessment of species belonging to the *Bacillus cereus* group

A review of the virulence factors involved in the gastro-intestinal infections caused by *B. cereus* can be found in Stenfors-Arnesen et al. (2008):

- The role of hemolysin BL (Hbl) and of the non-hemolytic enterotoxin (Nhe) in diarrhoeal outbreaks has been confirmed (Stenfors-Arnesen et al., 2008). In particular the mode of action of Nhe on the cell membranes has been described (Lindbäck et al., 2010). Genes coding for Nhe, unlike those coding for Hbl, are present in most, if not all, strains of *B. cereus* (Guinebretière et al., 2010, Fagerlund et al., 2007) and the amount of Nhe produced at 32 °C by *B. cereus* strains was correlated with their cytotoxic activities (Moravek et al., 2006).
- The toxin previously named 'Enterotoxin K' has been characterised as a beta-barrel cytotoxin now called CytK (Lund et al., 2000). Two forms are distinguished (Fagerlund et al., 2004), CytK1 being more cytotoxic than CytK2.
- Enterotoxin T has now been identified as the result of a cloning artefact (Hansen et al., 2003) and should no longer be considered as a virulence factor.
- Enterotoxin FM has been identified as an endopeptidase (Tran et al., 2010) which does not show direct toxic activity on epithelial cells.
- Emetic toxin (cereulide) is still the only toxin identified in B. cereus causing the emetic disease. Its potent toxic effect on liver cells and various mammalian cell lines has been shown (Andersson et al., 2007). Fatal or very severe B. cereus emetic outbreaks have been reported since 2000 (Shiota et al., 2010; Posfay-Barbe et al., 2008; Dierick et al., 2005). The non-ribosomal peptide synthase producing cereulide has been identified (Ehling-Schulz et al., 2005) and characterised (Magarvey et al., 2006).

Other factors produced by *B. cereus* with various toxic activities have been characterised (Hemolysin II and several metalloproteases) but there is no evidence so far of their implication in gastro-intestinal diseases (Cadot et al., 2010). The toxic effect some of them show on macrophages may rather indicate a role in clinical infections.

In summary, diarrhoeal disorders produced by *B. cereus* result from the production of toxins Nhe, Hbl and CytK, alone or in combination in the intestine (Table 1). The emetic disease results from the production of cereulide by *B. cereus* cells in the food.

Table 1: *Bacillus cereus* toxins which can be considered as the causative agents of gastro-intestinal diseases (Stenfors-Arnesen et al., 2008)

Toxin	Genes/operons	Nature	Foodborne infection/intoxication
Nhe (non hemolytic enterotoxin)	*nhe*	Protein (three components)	diarrhoeal
Hbl (hemolysin BL)	*hbl*	Protein (three components*)	diarrhoeal
CytK (cytotoxin K)	*cytK*	Protein	diarrhoeal
Cereulide	*ces*	Cyclic peptide	emetic

* The production of a fourth component, whose role has not been elucidated, was shown by Clair et al., 2010.

In principle, the selection of strains belonging to the *B. cereus* taxonomic group for direct use in animal production is considered inadvisable.

If, however, they are proposed for use then the full genome (including chromosome and plasmids) should be sequenced and bioinformatic analysis made to search for genes coding for enterotoxins and cereulide synthase (Table 1). If there is evidence of homology, the non-functionality of the genes (e.g., mutation, deletion) should be demonstrated.

Strains harbouring a toxigenic potential should not be used as feed additives.

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APPENDIX

RECOMMENDED PROCEDURE FOR THE DETECTION OF CYTOTOXICITY USING VERO CELLS

1. Preparation of test substance:

a. Bacterial cells should be grown in brain heart infusion broth (BHI) at 32°C for six hours. Cells should be removed by centrifugation at room temperature. Supernatant should be concentrated a 10-fold by ammonium sulphate (80 %) precipitation. After ammonium sulphate removal toxicity is determined using 100 µL of concentrated supernatant in the Vero cells assay.

b. Bacterial cells should be grown for 10 days at 37 °C on Trypticase soy agar plates (TSA). Bacterial biomass (approximately 60 mg), equivalent to 10^9-10^{10} CFU, is extracted with 1.0 mL methanol and heated for 30 minutes at 80 °C. The dry pellet is resuspended in 0.5 mL of methanol, vortexed and centrifuged for 3 minutes at 13000 x g. The supernatant is collected and heated at 80 °C until complete evaporation (30 minutes). The dry residue is dissolved in 200 µL methanol and stored in dark glass vials at 4 °C before use.

2. Cell assay

Vero cells are grown in MEM medium supplemented with 5 % foetal calf serum. Cells are seeded into 24-well plates two-three days before testing. Before use, check that the growth of the Vero cells is confluent. If so, remove the medium and wash the cells once with 1 mL preheated (37 ºC) MEM medium.

- Add 1 mL preheated (37 ºC) low-leucine medium to each well and then add the test substance (1-100 µL of *Bacillus* supernatant), incubate the cells for 2 hours at 37 ºC.

- Remove the low-leucine medium with the toxin, wash each well once with 1 mL preheated (37 ºC) low-leucine medium. Mix 8 mL preheated low-leucine medium with 16 µL 14C-leucine and add 300 µL of this mixture to each well, incubate the cells for 1 hour at 37ºC.

- Remove the radioactive medium and add 1 mL 5 % trichloroacetic acid (TCA) to each well, incubate at room temperature for 10 minutes. Remove TCA, and wash the wells twice with 1 mL of 5 % TCA.

- After removing TCA, add 300 µL 0.1 M KOH and incubate at room temperature for 10 minutes. Transfer the content of each well to liquid scintillation tubes with 2 mL of liquid scintillation cocktail. Vortex the tubes, and count the radioactivity in a scintillation counter for 1 minute.

- Percentage inhibition of protein synthesis is calculated using the following formula: ((Neg. ctrl – sample)/Neg. ctrl) x 100; the negative control is Vero cells from wells without addition of sample. Below 20 % inhibition is considered negative.

An alternative method is to measure propidium iodide (PI) uptake in Vero cell suspensions using a spectrofluorimeter. Two-day-old confluent monolayers of Vero cells are used as described above. Cell suspensions with in a final concentration of about 10^6 cells in 2 mL EC buffer containing PI (5 µg/mL) are held in a thermostatically controlled (37 °C) 1 cm quartz cuvette to which the test substance is then added. Cells are continuously mixed by the use of a magnetic stirrer and 'flea'.

Fluorescence should be monitored every 30 seconds using excitation/emission wavelengths of 575/615 nm and 5 nm slits for both. Results are used without subtraction of background fluorescence.

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