SCIENTIFIC OPINION

Technical Guidance on the assessment of the toxigenic potential of *Bacillus* and related genera 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

ABSTRACT

KEY WORDS

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[3] Acknowledgement: The Panel wishes to thank the members of the Working Group on Micro-organisms including Atte von Wright, Per Einar Granum and Christophe Nguyen-thé for the preparation of this opinion.

Suggested citation: EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP); **Error! Reference source not found.**. EFSA Journal 20xx; volume(issue):xxxx. [12 pp.]. doi:10.2903/j.efsa.20NN.NNNN. Available online: www.efsa.europa.eu

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BACKGROUND AS PROVIDED BY EFSA

Regulation (EC) No 1831/2003[4] 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 one opinion on the introduction of a Qualified Presumption of Safety (QPS) approach for the assessment of selected micro-organisms (EFSA, 2007). This last opinion has been updated on a yearly basis, and will be continuously updated in the future. The QPS approach is currently 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.

One of the genera included in this opinion is *Bacillus*, which is commonly found in a number of feed additives. The QPS opinion imposes, for *Bacillus*, the qualification that the absence of food poisoning toxins, surfactant activity or enterotoxic activity. In 2000 the Scientific Committee for Animal Nutrition (SCAN) adopted an opinion on the safety of use of *Bacillus* species in animal nutrition.

Since the adoption of the SCAN opinion on this matter, new scientific data have become available that call for a revision of the taxonomy and toxin production of the *Bacillus* species. Therefore, the FEEDAP Panel considers it necessary to update this document in order to provide applicants with proportionate and up-to-date guidance on how to conduct the safety assessment of these products.

TERMS OF REFERENCE

The FEEDAP Panel is requested produce a Technical Guidance on the safety of use of *Bacillus* species in animal nutrition taking into account the opinion from the Scientific Committee for Animal Nutrition (SCAN) on the safety of use of *Bacillus* species in animal nutrition and any new scientific data available.

[4] OJ L 268, 18.10.2003, p. 29.

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. Approximately half of the present production of bulk enzymes derives from strains of this or related genera. Regulation (EC) No 1831/2003 requires that all feed additives, including microorganisms, 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) recommended that the use of strains of the *Bacillus cereus* taxonomic group, a group containing many known pathogenic strains, be strongly discouraged, but 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 microorganisms adopted by EFSA is considered applicable to most of the commercially relevant *Bacillus* species. This approach requires the unambiguous identification of the strain being assessed, a demonstration of a lack of antibiotic resistance determinants 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. To date, applicants seeking regulatory approval for a novel strain of *Bacillus* have relied for guidance on how to assess toxigenic potential on the SCAN Opinion on the safety of use of *Bacillus* species in animal nutrition published in 2000. The SCAN guidance takes as its basis the then existing knowledge on the structure and biogenesis of toxins produced by *B. cereus*, assuming that toxins found in other *Bacillus* species would have sufficiently similar properties to be detected by the methods developed for the *B. cereus* group. Subsequent to the SCAN opinion there have been significant developments both in the knowledge of toxins prevalent in the *B. cereus* group and, more importantly, in the toxigenic potential of other *Bacillus* species and their prevalence. These developments are reviewed below. In addition, more refined methods of genetic analysis are now more widely available.

In view of the need to update the guidance contained within the original SCAN opinion, the FEEDAP Panel takes the opportunity to adopt the revision as part of the technical guidance provided to applicants seeking authorisation of feed additives.

2. Taxonomy

2.1. Current taxonomy and phylogeny of *Bacillus* and related genera (other than *B. cereus* group)

Since the first edition of the Bergey's Manual of Systematic Bacteriology, the structure and content of the genus *Bacillus* have been substantially modified (Claus and Berkeley, 1986; Ash *et al*., 1993; Priest, 1993). It was recognised as being very heterogeneous both genetically and phenotypically and Ash *et al.*, (1991) proposed a division into five phylogenetic groups based on the 16S rRNA gene sequence. Subsequently, several new genus were proposed which contain some species previously allocated in the *Bacillus* genus. For instance, *B. stearothermophilus* is now considered part of the genus *Geobacillus,* strains of *Bacillus brevis* are part of the genus *Brevibacillus* or the genus *Aneurinibacillus* and *Bacillus polymyxa* belong to the genus *Paenibacillus*.[5] Both genera *Bacillus* and *Geobacillus* are part of the *Bacillaceae* family, the genus *Paenibacillus* being part of the *Paenibacillacea* family.

Most strains of Gram-positive, spore forming bacteria that have been or are used as feed additives

[5] List of Prokaryotic names with Standing in Nomenclature, http://www.bacterio.cict.fr/

belong to species included in the new restricted definition of the genus *Bacillus*. Within the *Bacillus* genus, *B. subtilis* is divided in sub-species,[6] some pigmented strains have been regrouped in the species *B. atrophaeus* (Nakamura 1989), two species, *B. mojavensis* (Roberts *et al.*, 1994), and *B. vallismortis* (Roberts *et al.,* 1996), were defined from *B. subtilis* like strains isolated from soil. Together with *B. amyloliquefaciens* (Priest *et al.*, 1987) and *B. licheniformis* they form the "*B. subtilis* group" (Chun and Bae 2000), differing by few or no phenotypic characters and having very high similarities of their 16S rRNA sequences. *B. sonorensis,* very close to *B. licheniformis* (Palmisano *et al*., 2001) was more recently defined. The sequence of the gyrase A (Chun and Bae 2000) or gyrase B genes (Wang *et al*., 2007) can be very useful when discriminating between strains of these species.

A phylogenetic analysis of several species from the *Bacillus* and related genera distinguished ten phylogenetic groups (Xu and Côté 2003). *B. subtilis* group and *B. licheniformis* clustered in Group VI, *B. megaterium* and *B. pumilus* in Group II, *B. coagulans* and *Geobacillus stearothermophilus* in Group I. The species implicated so far in foodborne poisoning (see 3.1) and able to produce peptides with toxic activites (*B. subtilis* group, *B. licheniformis*, *B. pumilus*) are therefore not restricted to a single phylogenetic group.

2.2. Current taxonomy and phylogeny of the *Bacillus cereus* group

Bacillus cereus causes two types of gastro-intestinal disorders (diarrhoeic and emetic) as described in the SCAN opinion (EC, 2000). *B. cereus* also causes a wide range of non gastro-intestinal infections which are presumably not linked to the presence of *B. cereus* in the food or feed chain (EFSA 2007, 2008).

The *B. cereus* group contains several species: *B. cereus* senso stricto, *B. thuringiensis*, *B. anthracis*, *B. mycoides*, *B. pseudomycoides* and *B. weihenstephanensis* (EC, 2000; Stenfors-Arnesen *et al.*, 2008). Since 2000, investigations of the *B. cereus* group showed that several of these species are not true genomic species. The "Hypercat" database (Tourasse e*t al.,* 2010) provides a global view of the phylogenetic structure of the *B. cereus* group, established by the combination of several approaches (multi locus sequence typing, amplified fragment length polymorphism and multi locus enzyme electrophoresis).[7] This database is organised according to the seven (I-VII) phylogenetic groups of the *B. cereus* cluster defined by Guinebretière *et al.* (2008).

All strains so far isolated from emetic disease belong to phylogenetic group III. Phylogenetic group VII contains strain NVH391/98, which carries a particularly potent form of toxin (CytK1, Fagerlund *et al.,* 2004) which caused the only known fatal diarrhoeal *B. cereus* outbreak (Lund et al. 2000). In contrast, phylogenetic group VI (which regroups *B. weihenstephanensis* and *B. mycoides*) does not include any strains associated with an outbreak of foodborne diseases (Guinebretière *et al.,* 2008). *B. anthracis* appears as a lineage included in one of these phylogenetic groups (group III) and cases of anthrax have been caused by *B. cereus* strains carrying genes of anthrax virulence factors (Hoffmaster *et al.*, 2004). It is worth noting that *B. thuringiensis* strains can occur in the same phylogenetic clusters as *B. cereus* strains implicated in foodborne outbreaks.

3. Safety concerns caused by *Bacillus* species

3.1. Virulence factors found in *Bacillus* and related genera (other than *B. cereus* group)

Bacillus species other than *B. cereus* group are a rare cause of foodborne diseases as outlined in the SCAN opinion (EC, 2000). Production of heat stable toxins referred to as surfactins has been shown for some strains of *B. subtilis, B. licheniformis, B. pumilus* (Taylor *et al.,* 2005) and by *B. mojavensis* (From *et al*., 2005).

[6] List of Prokaryotic names with Standing in Nomenclature, http://www.bacterio.cict.fr/
[7] http://mlstoslo.uio.no/cgi-bin/mlstdb/mlstdbnet4.pl?dbase=hyperdb&page=hyperindex&file=bcereusgrp_isolates.xml

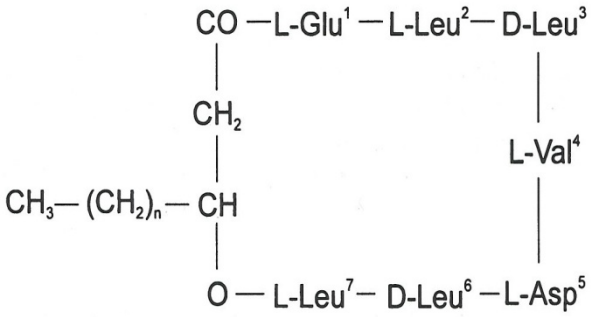

Figure 1. Primary structure of surfactins (n = 9-11) (Carrillo *et al.*, 2003).

Surfactins represent a family of structurally similar cyclic lipopeptides which possess potent surfactant activity (Fig. 1). The biosynthesis of these microbial lipopeptides is accomplished non-ribosomally by large multienzyme systems that are composed of catalytic domains that catalyse all steps in peptide biosynthesis including the selection and ordered condensation of amino acid residues. Genes coding for surfactins can be detected by polymerase chain reaction (PCR). To detect strains able to produce the lipopeptides, a new PCR screening approach has been developed using degenerated primers based on the intraoperon alignment of adenylation and thiolation nucleic acid domains of all enzymes implicated in the biosynthesis of each lipopeptide family (Tapi *et al*., 2009). All the *Bacillus* strains producing these lipopeptides are haemolytic, and a number of surveys have shown that about 5-6 % of *Bacillus* strains have the capacity to produce surfactins (From *et al*., 2005; Salkinoja-Salonen *et al.*, 1999). It is know that these surfactins create pores in epithelial cells (From *et al*., 2007a; From *et al*., 2007b) and are toxic to sperm cells (Salkinoja-Salonen *et al.*, 1999).

Some examples of toxic peptides produced by *Bacillus* species are:

- amylosin produced by *B. amyloliquefaciens*, a member of the *B. subtilis* group (Mikkola *et al.*, 2007);
- the lipopeptides fengycin and surfactin from *B. subtilis* and *B. mojavensis* (Hwang *et al*., 2009, From *et al.*, 2007a);
- pumilacidin from *B. pumilus* (From *et al.*, 2007b);
- lichenysin from *B. licheniformis* (Nieminen *et al.*, 2007).

Pumilacidin was associated with a food borne poisoning outbreak linked to rice (From *et al.*, 2007b). Lichenysin was produced by *Bacillus* sp. isolated from mastitis in dairy cows. Surfactin and amylosin were proposed to be the origin of the cytotoxic activities found in some strains of *B. mojavensis* implicated in food borne poisoning (From *et al.*, 2007a, Apetroaie-Constantin *et al*., 2009). All the above described peptides have toxic activities on cell lines and sperm cells, as seen with cereulide, the emetic toxin of *B. cereus*. However, these toxins have a structure and biogenesis distinct from that of cereulide. They are lipopeptides which confer their surfactant properties (Ongena and Jacques 2008). The presence of the *ces* gene coding for cereulide also has been reported in one strain of *Bacillus pumilus* (Parvathi *et al.*, 2009). However the production of cereulide was not demonstrated.

Production of the *B. cereus*-like diarrhoeal enterotoxins by some strains of other *Bacillus* species was described in SCAN (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* and related genera are likely to have resulted from a misidentification of the strain involved (From *et al.*, 2005).

3.2. Virulence factors of *Bacillus cereus* responsible for gastro intestinal diseases

A recent 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 *Bacillus 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 named "Enterotoxin K" in SCAN (2000) 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 reported in the SCAN Opinion 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 (Table 1), diarrhoeal disorders produced by *B. cereus* result from the production of toxins Nhe, Hbl and CytK, alone or in combination in the intestine. 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 recently shown by Clair *et al.*, 2010.

4. Methods to detect toxigenic potential

Characterisation of *Bacillus* strains according to Claus and Berkeley (1986) 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), 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 from the *B. subtilis* group, as defined in section 2.1 of the present guidelines, partial sequences of the *gyrA* gene or *gyrB* genes are needed in addition to the partial 16s rRNA sequences, which can be obtained using methods described in Chun and Bae (2000), From *et al*. (2005) for *gyrA* and Wang *et al.* (2007) for *gyrB*.

4.1. *Bacillus* and related genera (other than *B. cereus* group)

The SCAN guidance took as its basis that toxins produced by species other than members of the *B. cereus* taxonomic group would have sufficiently similar properties to the known *B. cereus* toxins to be detected by the methods developed for the *B. cereus* group. It now seems unlikely that *B. cereus*-like enteroxins are produced in species other than the *B. cereus* group. Any toxigenic potential in such species appears far more likely to arise from the production of surfactins. Consequently, the recommended approach to the safety assessment of strain other than *B. cereus* has changed its focus, concentrating on the capacity to produce surfactins. However, a single cytotoxicity assay is retained to confirm the expected absence of *B. cereus*-like enterotoxins.

The steps recommended are:

1. A test for haemolysis on blood agar (30° C, incubated for 3 days). 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 haemolytic it is not recommended for use. If not then;

2. PCR detection of non-ribosomal peptide synthase genes following the method of Tapi *et al*. (2009), including a negative and positive controls (see 1) should be done followed by a cytotoxicity test made preferably with Vero cells and following the protocol in the annex using a concentrate of the supernatant. If the strain proves cytotoxic it is not recommended for use.

   A positive PCR reaction would normally be taken as indicative of a capacity to synthetise surfactins. However, peptides produced by non-ribosomal routes which do not share the properties of surfactins but may produce a positive PCR have been reported. If this is suspected then further work would be needed to establish the structure and properties of the peptide(s).

If other methods are considered appropriate by an applicant, these should be fully described and justified.

4.2. *Bacillus cereus* taxonomic group

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 (see Table 1). If there is evidence of homology, the non functionality of the genes (mutation, deletion, etc) should be demonstrated.

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

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ANNEX

Recommended procedure for the detection of cytotoxicity using Vero cells

Vero cells are grown in MEM medium supplemented with 5 % fetal 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 (37ºC) MEM medium.

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

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

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

4. After removing the 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 liquid scintillation cocktail. Vortex the tubes, and count the radioactivity in a scintillation counter for 1 minute.

5. 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.

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 contained 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 toxin 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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