The need to revise the Technical Guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition

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1. Introduction

The first guidance for applicants on how to assess toxigenic potential of species of the genus *Bacillus* was developed by the then Scientific Committee on Animal Nutrition (SCAN) and published in 2000 with the title *Opinion on the Safety of use of Bacillus species in Animal Nutrition* (EC, 2000). The SCAN guidance took 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 *Bacillus cereus* group. Since the SCAN Opinion was published it became apparent that the few reports of *B. cereus*-like enterotoxins occurring in species other than those of the *B. cereus* group and cited in the SCAN Opinion were 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 suggested an association with heat-stable surfactins and similar cyclic lipopeptides with surfactin activity rather than the enterotoxins typical of *B. cereus*. As hazards of this nature were not considered in the original SCAN Opinion, the FEEDAP Panel undertook a revision, also taking the opportunity to adopt this revision document as part of its technical guidance provided to applicants seeking authorisation of feed additives (EFSA FEEDAP Panel, 2011). The data requirements proposed for species belonging to the *B. cereus* group in the revised opinion was essentially unchanged other than requiring a full genome sequence analysis. The bulk of the changes introduced involved a substantial revision of the sections dealing with species other than those of the *B. cereus* group, with a shift to the detection of a capacity for the production of surfactins.

2. Surfactins and related cyclic lipopeptides

Surfactins represent a family of structurally similar cyclic lipopeptides which possess potent surfactant activity (Figure 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. 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).

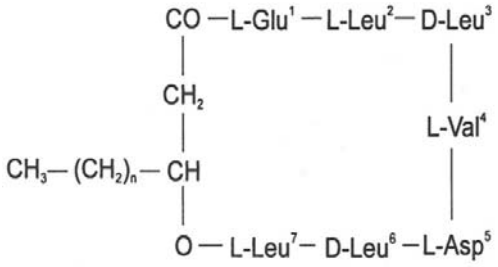

Figure 1: **Primary structure of surfactins (n = 9-11) (from Carrillo et al., 2003)**

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);
- 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 foodborne 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 foodborne 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).

Early data suggested that the surfactin-like cyclic peptides were produced by about 3-4 % of strains of *B. subtilis*, *B. licheniformis* and *B. pumilus* (Salkinoja-Salonen et al, 1999, From et al, 2005) and that virtually all were haemolytic. Consequently, the FEEDAP Panel concluded that the exclusion of such strains would be adequate to ensure consumer safety without precluding the use of *Bacillus* species in animal nutrition. Accordingly, the guidance available to applicants recommended an initial test for haemolysis followed by PCR detection of non-ribosomal peptide synthetase genes if the strain proved non-haemolytic. A positive PCR reaction was taken as indicative of a capacity to synthesise surfactins.

3. New evidence

Contrary to the original view that surfactin-like cyclic peptides were produced only by a small sub-set of bacilli, in a recent study, 53 strains of *B. licheniformis* isolated from different sources were all found to produce lichenysin (Madslien et al., 2013). The amount of production varied by more than two orders of magnitude, and the amount produced by some strains could only be detected by LC-MS/MS. However, the regulatory mechanisms controlling lichenysin production are unknown, so the amount produced by each strain may vary under different conditions. Relatives from the *B. subtilis* group are expected to behave similarly, and indeed this was found in a study from airborne bacteria in a subway station in Norway (Dybwad et al., 2012). By analysing for the presence of the genes making the surfactin-like toxins and analysing the different strains by using LC-MS/MS the picture seems to be very different than in the earlier studies.

In a similar exercise (Cocconcelli, personal communication) an examination of the published genomes of *B. subtilis* and related species was performed to detect the presence of genes coding for non-ribosomal peptide synthetases. The analysis was performed on a total of 26 complete whole genome sequences and 35 draft genomes of the species, *B. amyloliquefaciens, B. atrophaeus, B. subtilis B. licheniformis, B. mojavensis, B. sonorensis* and *B. vallismortis* (http://www.ncbi.nlm.nih.gov/genome). This *in silico* approach demonstrated that all the *Bacillus* strains for which the genome is available harbour at least one operon coding for more than one non-ribosomal peptide synthetase.

In now appears that the synthetic apparatus for the production of surfactin-like cyclic peptides is universally present in B. subtilis and the related species which represent the large majority of commercially important strains. The present position adopted by the Panel that any indication of a capacity to produce such compound represents a hazard and should be avoided now appears disproportionate to the risk posed. Consequently, the FEEDAP Panel proposes to revise its guidance.

REFERENCES

Apetroaie-Constantin C, Mikkola R, Andersson MA, Teplova V, Suominen I, Johanssonand T and Salkinoja-Salonen M, 2009. *Bacillus subtilis* and *B. mojavensis* strains connected to food poisoning produce the heat stable toxin amylopsin. Journal of Applied Microbiology ISSN 1364-5072.

Carrillo C, Teruel JA, Aranda FJ and Ortiz A, 2003. Molecular mechanism of membrane permeabilization by the peptide antibiotic surfactin. Biochimica et Biophysica Acta, 1611, 91-97.

Dybwad M, Granum PE, Bruheim P, Blatny JM (2012) Characterization of Airborne Bacteria at an Underground Subway Station. Appl Environ. Microbiol. 78, 1917-1929.

EC (European Commission), 2000, online. Opinion of the Scientific Committee on Animal Nutrition on the safety of use of *Bacillus* species in animal nutrition. Available from http://ec.europa.eu/food/fs/sc/scan/out41_en.pdf

EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), 2011. Technical Guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition. EFSA Journal 2011;9(11):2445. [13 pp.]

From, C., R. Pukall, P. Schumann, V. Hormazábal and P. E. Granum (2005). Toxin-producing ability among Bacillus spp. outside the Bacillus cereus group. Applied and Environmental Microbiology 71, 1178-1183

From C, Hormazabal V, and Granum PE, 2007a. Food poisoning associated with pumilacidin-producing *Bacillus pumilus* in rice. International Journal of Food Microbiology,115, 319-324.

From C, Hormazabal V, Hardy SP and Granum PE, 2007b. In vitro cytotoxicity in *Bacillus mojavensis* is abolished following loss of sfp and surfactin synthesis. International Journal of Food Microbiology 117, 43-49.

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 (3), 351-355.

Madslien E, Rønning H, Lindbäck T, Hassel B, Andersson M, Granum, PE (2013). Lichenysin is produced by most Bacillus licheniformis. Manuscript ID: JAM-2013-0877

Mikkola R, Andersson MA, Teplova V, Grigoriev P, Kuehn T, Loss, S, Tsitko I, Apetroaie C, Saris NEL, Veijalainen P and Salkinoja-Salonen MS, 2007. Amylosin from *Bacillus amyloliquefaeiens*, a K+ and Na+ channel-forming toxic peptide containing a polyene structure. Toxicon, 49, 1158-1171.

Nieminen T, Rintaluoma N, Andersson M, Taimisto A-M, Ali-Vehmas T, Seppälä A, Priha O and Salkinoja-Salonen M, 2007. Toxinogenic *Bacillus pumilus* and *Bacillus licheniformis* from mastitic milk. Veterinary Microbiology, 124, 329-339

Ongena M and Jacques P, 2008. *Bacillus* lipopeptides: versatile weapons for plant disease biocontrol. Trends Microbiol. 16, 3, 115-125.

Salkinoja-Salonen, M. S., R. Vuorio, M. A. Andersson, P. Kampfer, M. C. Andersson, T. Honkanen-Buzalski and A. C. Scoging (1999). Toxigenic strains of *Bacillus licheniformis* related to food poisoning. Applied and Environmental Microbiology 65, 4637-4645.