European Food Safety Authority

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TECHNICAL REPORT

Outcome of the public consultation on the draft Guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition[1]

European Food Safety Authority[2,3]

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ABSTRACT

The European Food Safety Authority (EFSA) carried out a public consultation to receive input from the scientific community and all interested parties on the update of the Guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition. The draft Guidance was prepared by the EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP Panel) and endorsed for public consultation at its Plenary meeting on 18 June 2013. The written public consultation for this document was open from 26 June to 30 September 2013. EFSA received comments from 12 interested parties. EFSA and its FEEDAP Panel wish to thank all stakeholders for their contributions. The current report summarises the outcome of the public consultation and includes a brief summary of the comments received and how these were addressed. The FEEDAP Panel prepared an updated version of the Guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition taking into account the questions/comments received. This guidance was discussed and adopted at the FEEDAP Plenary meeting on 8 April 2014, and is published in the EFSA Journal.

KEY WORDS

guidance, feed additives, *Bacillus*, safety, toxigenic potential

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BACKGROUND

The European Food Safety Authority (EFSA) asked the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) to update the guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition. In line with EFSA's policy on openness and transparency and in order for EFSA to receive comments from the scientific community and stakeholders on its work, EFSA engages in public consultations on key issues. Accordingly, the draft guidance, which was endorsed by the FEEDAP Panel on 18 June 2013, was released for public consultation from 26 June to 30 September 2013. Stakeholders were informed and invited to submit comments.

TERMS OR REFERENCE

The FEED Unit is requested to produce a Technical report summarising the outcome of the public consultation on the draft Guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition.

CONSIDERATION

1. Introduction

The European Food Safety Authority (EFSA) asked the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) to update the guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition. The FEEDAP Panel endorsed the draft guidance on 18 June 2013. In line with EFSA's policy on openness and transparency, and in order for EFSA to receive comments on its work from the scientific community and stakeholders, EFSA engages in public consultations on key issues. Accordingly, the draft guidance was published on EFSA's website and open for public consultation between 26 June 2013 and 30 September 2013. The comments received were considered by the FEEDAP Panel and an updated guidance was discussed and adopted at the FEEDAP Plenary meeting on 8 April 2014, and is published in the EFSA Journal (EFSA FEEDAP Panel, 2014). EFSA is committed to publishing the comments received during the public consultation, as well as a short report on the outcome of the consultation.

2. Screening of the comments received

EFSA received 18 comments from 12 interested parties, a public organisation, two industry associations, a professional association, four consultant organisations and four Universities.

The comments received are listed in the Appendix. All comments were subject to evaluation and assessment. Where considered appropriate, the guidance document has been modified to take account of the comments.

3. Summary of the main comments received and EFSA's considerations

Some of the comments referred to a need for an overarching Guidance document covering areas other than feed additives. The areas mentioned (e.g., feed additives, food supplements) fall under different legal frameworks. The FEEDAP Panel has produced this Guidance document to assist applicants in the preparation and the presentation of applications for *Bacillus*-based feed additives as foreseen in Article 7(6) of Regulation (EC) No 1831/2003.

Some stakeholders questioned the approach of using an *in vitro* cytotoxicity study as the only means to discriminate between hazardous and safe strains. The Panel recognises the inherent uncertainty of the use of cytotoxicity assays. However, at present more reliable alternative methods are not available.

The use of laboratory animal models to test enteropathogenicity was suggested. However, there are no currently validated animal methods available for bacilli.

Some stakeholders suggested a case-by-case approach to assess the safety of a given strain. The Panel notes that this would require a complete assessment including demonstration of safety for target animals, consumers of products derived from animals fed the microbial additives, users and the environment and would be contrary to EFSA's action to develop the qualified presumption of safety approach to safety assessment.

Some more details on the methodology proposed to test cytotoxicity were requested. More information and/or references have been added in the Guidance.

Some comments challenged the link between cytotoxic lipopeptides and haemolysis and between positive *in vitro* haemolysis reaction and *in vivo* toxicity. The Panel accepts the concerns related to haemolysis as a proof of cytotoxicity of bacilli and has removed this requirement from the Guidance.

One stakeholder highlighted that safety is a function of metabolism and not of nomenclature. Therefore, in the case when strains considered and tested as *Bacillus* and shown to be toxin free are subsequently transferred to a different genus, the safety assessment should stand. The Panel agrees with this statement but does not see the need to specify this in the Guidance.

Several comments challenged the association between toxigenic potential of non-*Bacillus cereus* species and the production of heat-stable toxins or surfactins/cyclic non-ribosomal peptides and made reference to the Japanese traditional product "natto", known to contain surfactins without causing apparent ill effects in consumers. The Panel recognises the uncertainty and has only suggested that there may be an association between surfactin like-lipopeptides and food intoxication.

One stakeholder stated: "*However, the recent scientific evidences that classify the Bacillus cereus group strains in seven phylogenetic subgroups in accordance with the level of risk to cause food poisoning and the scientifically recognised safety and long history of safe use of some Bacillus cereus strains used in animal nutrition allow the Committee to recognise that strains from Bacillus cereus group and from other Bacillus species may be considered safe. The FEEDAP Panel concurs with this general position*" The FEEDAP Panel does not agree with this text and considers it a misquotation of the Guidance. Furthermore, long history of use is not as such a proof of safety.

One stakeholder stated that it can be assumed that an anticipated cytotoxic activity caused by lipopeptides is directly related to an interaction with the cell membrane. Therefore, an assay for cytotoxicity based on inhibition of protein synthesis (as suggested) would not be the logical choice but rather an endpoint directly related to membrane damage. This could be the propidium iodide uptake assay, as mentioned in the guidance or a neutral red uptake. Unfortunately, none of these assays are validated against these surfactin like-lipopeptides. The Panel considers that this will be an end-point that is independent of the mechanism of toxicity which is basically what the Panel favours given the uncertainty surrounding the role of surfactin like-lipopeptides in food intoxication.

A stakeholder raised a concern on the fact that methods for the preparation of the test item in the cytotoxicity study and alternative to that based on ^{14}C–leucine uptake inhibition have not been standardised and validated. The Panel agrees with this comment and has removed this alternative method from the Guidance. However, although the Panel recognises the inherent uncertainty of the use of cytotoxicity assay with Vero cells, at present alternative methods of proven relevance are not available.

EFSA thanks all stakeholders for their contribution.

REFERENCES

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2014. Guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition. EFSA Journal 2014;12(5):3665, 13 pp. doi:10.2903/j.efsa.2014.3665

APPENDIX

Comments received during the public consultation on the draft Guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition

This list contains the comments submitted to EFSA via the public consultation held from 26 June 2013 to 30 September 2013. Comments submitted formally on behalf of an organisation appear with the name of the organisation. Comments received from different stakeholders are grouped.

#	Organization	Chapter	Comment
1	Pen & Tec Consulting Switzerland (PTCS) and UK (PTUK) and EFFSACO - European Federation of Food Safety Consultants AVC - Association of Veterinary Consultants Acumentia - food chain/beverages/pharma/feed/life science consultants AVI - Association of Veterinarians in Industry	Background / Introduction/ Safety concerns caused by *Bacillus* species	Lines 49-52: We note that *Bacillus* spp. are used in food supplements, foods, & as food enzyme producer strains. The same *Bacillus* strains may be used in feeds & foods. Member States, responsible for safety control under food hygiene & food supplement laws look to EFSA for strain safety guidance. Hence we suggest joint publication of *Bacillus* safety guidance by EFSA food/feed panels. This will attract inputs from medical, veterinary & food microbiologists, toxicologists, & epidemiologists, thus enhancing scientific quality & robustness. This is important, since the same *Bacillus* strains may be used as both feed additives and food supplements. Lines 70-71: We note that many safe *Bacillus* produce surfactins show haemolysis on sheep blood agar (SBA) & give positive results in Vero cell cytotoxicity. Surfactins were implicated in food poisoning, yet are not harmful in vivo at high concentrations. Consideration of Koch's postulates & use of laboratory animal models to test enteropathogenicity will help decide if *Bacillus* strains in food-poisoning outbreaks are enteropathogens. Most enteropathogenic strains produce enteritis in laboratory animal challenge models. In vitro tests alone are inadequate to establish pathogenicity. Lines 74-76: We suggest that since *Bacillus* strains have been used safely in the food chain for decades, & yet may give "toxic" results in vitro, a case-by-case approach is indicated, examining the body of evidence, including in vitro data, history of safe use, & in vivo safety data. For *Bacillus* strains with no history or in vivo data, it is sufficient to submit a combined TAS (target animal safety), dose-response & efficacy study. This would not add many more animal studies to an EFSA-compliant dossier, since applicants have to supply at least 3 efficacy studies, & at least 4 for meta-analysis. We would appreciate flexibility in consulting EFSA on the best approach, case-by-case. Lines 99-101: We consider that neither in vitro test proposed improves current QPS procedures due to the lack of international validation of such tests & the high rate of false positive outcomes. We suggest the EFSA consider each strain on a case-by-case basis, taking into account QPS, safety in use, strain identity & genomic data, & any existing *in vivo* data. Lines 127-129: We propose that suspect strains from food-poisoning incidents are investigated in vivo, before concluding that surfactins are toxins. Published data suggest that many surfactins are beneficial & have a high NOEL when tested in laboratory animals. Line 136: We support the principle that in vitro tests be used to reduce, refine & replace in vivo testing, but Vero cell cytotoxicity & other in vitro safety tests can give false positives, depending on source of cells & other laboratory condit*ions. They are not suitable, in isolation, to assess Bacillus safety. For example different outcomes have been reported when testing the same Bacillus* strain in Vero cell cytoxicity tests, using different commercial Vero cell suppliers. Lines 137-140: We consider that using β-haemolysis & other *in vitro* tests as sole indicators of pathogenicity is not coherent with other EFSA approaches, e.g. genotoxicity testing, where in vitro studies are used as screening tests, prior to any in vivo tests. Lines 139-140: This statement seems disproportionate to the risk of pathogenicity. Many safe food & feed strains are β-haemolytic on SBA.

#	Organization	Chapter	Comment
			We suggest to consider the body of data available, including safe use history & any pre-existing in vivo safety data. We are not in favour of requiring many in vivo toxicity studies, & the QPS procedure is adequate for Bacillus spp. A combined TAS/efficacy/dose response study is sufficient to support strain safety within an EFSA-compliant dossier. Line 141: We propose that in vitro safety tests be internationally validated. Regulation (EC) Nº 429/2008 states: "The use of in vitro methods … shall be encouraged. Such methods shall be of the same quality & provide the same level of assurance as the method they aim to replace." Well-validated, reliable markers of virulence should be sought & used for determining strain safety.
2	University of Bradford	Background	Lines 67-69: There seems to be a tacit assumption a positive haemolysis reaction in vitro corresponds to in vivo toxicity; this has been challenged From et al. (2007). The complexities of interpreting in vitro data and relating it to in vivo data are well illustrated by Trapecar et al., 2011 and serve as a further reminder that in vitro data does not necessarily predict in vivo toxicity. Lines 70-76: It is true that the science is evolving. A recent excellent review of *B. cereus* (Ceuppens et al. 2013) demonstrated that surfactins can be produced by strains that can be considered safe, despite showing haemolytic activity on sheep blood agar and giving positive results in some cell cytotoxicity screens. Surfactins are not considered harmful in vivo in low concentrations, despite sometimes being "positive" in in vitro toxicity screens. It is important to allow sponsors to be able to use appropriate laboratory animal models to properly screen for toxicity.It has also been shown that cytotoxic activity levels of culture filtrates and toxin distributions varies according to the phylogenetic group within the *Bacillus cereus* group, suggesting that these groups are of different clinical significance (Guinebretiere et al., 2010); this consequently raises the question whether all should be subject to the same screening protocol, this is relevant to the comments re lines 125 – 146.
3	University of Bradford	The scope of the guidance	Lines 103-108: I do believe that there is an opportunity here to make some additional points about strains that have the taxonomic status changed. As stated, a number of species earlier considered to belong to the genus *Bacillus* have been transferred to other genera. For strains that are considered and tested as Bacillus and shown to be toxin free and are then subsequentally transferred to a different genus, the safety assessment should stand. This could be written into the guidance as safety is not a function of nomenclature but a function of metabolism.
4	University of Bradford	Assessment of Bacillus species other than the *Bacillus cereus* group	Lines 125-146: This comment follows on from that made re lines 67-69 in that the link between cytotoxic lipopeptides and haemolysis is not absolutely clear; haemolysis on blood is probably not the best discriminatory characteristic. It can give rise to false negative and false positive results (Madslien et al., 2013; Plaza et al., 2006, Walter et al., 2010) and other compounds can cause haemolysis (Walter et al., 2010). It is imperative that sponsors are allowed to use additional and appropriate test systems as well as those listed if they have evidence that the listed tests are not predictive of in vivo testing. One of the reasons for this is that there is evidence suggesting that many surfactins are beneficial and have a high NOEL when tested in laboratory animals. Line 144: There is no reference to the methods that are based on lactate dehydrogenase (LDH)
5	University of Bradford	Appendix	Lines 338-339 The most important issue is how the results should be interpreted and this is not described, for example what is the threshold for a positive toxic reaction? Lines 342-344 This reference only mentions the ^{14}C-leucine method, other methods such as the propidium iodide and LDH method, are not referenced; this needs to be addressed. Full details of test protocols or full published references of agreed protocols need to be provided otherwise there will not be a level regulatory playing field. Published work in this area is ambiguous in part and so as this is such a pivotal part of EFSA decision making there needs to be agreement of detailed protocols.
6	FEFANA – EU Association of	Background	Lines 63-69: Lipopeptide concentrations causing hemolysis are not toxic in vivo. Juola, Kinnunen, Fog Nielsen & Wright (2013) have recently shown that *Bacillus subtilis* var *natto* strains isolated from the traditional Japanese breakfast health product Natto as well as extracts

#	Organization	Chapter	Comment
	Specialty Feed Ingredients and their Mixtures AMFEP – EU Association of Manufacturers and Formulators of Enzyme Products		from Natto products was clearly hemolytic on sheep blood agar after 2 days and toxic in a boar sperm motility test. Additional internal tests Natto strains isolated from Japanese Natto products shows beta- hemolysis and 1 alpha-hemolysis. As Natto is a widely eaten breakfast product in Japan it can also be concluded that surfactins at concentrations as found in Natto and resulting in hemolysis cannot be considered to be toxic to humans. Also From et al. (2007) states that no final conclusions should be drawn from in vitro toxicity tests to *in vivo* toxicity. If EFSA insist that lipopeptides are toxicogenic, we would like to draw the attention to the scientific justification for using beta- hemolysis on blood agar as sole and definitive rejection criteria for Bacillus strains: 1) the hemolysis assay was developed to identify known pathogens, not to show that an organism is pathogenic. 2) β-Hemolysis can be caused by protease activity, not only surfactins. A cell test will show if the enzyme activity can be considered as a safety issue. We will also draw attention to the general strategy in other safety guidelines (including EFSA) which requires data from in vitro assays. If there is a positive result in any of the in vitro studies an appropriate in vivo study shall be conducted to assess whether the in vitro data actually give rise to any safety concern or not. A risk assessment should not be made solely on the basis of data from in vitro studies.
7	FEFANA/AMFEP Newcastle University	Introduction	Line 91: The recent scientific evidences that classify the *Bacillus cereus* group strains in seven phylogenetic subgroups in accordance with the level of risk to cause food poisoning (Guinebretière et al., 2010) and the scientifically recognised safety and long history of safe use of some *Bacillus cereus* strains used in animal nutrition (Trapecar et al., 2011, Ceuppens et al., 2013) allow the Committee to recognise that strains from *Bacillus cereus* group and from other *Bacillus* species may be considered safe. The FEEDAP Panel concurs with this general position.
8	FEFANA/AMFEP	Assessment of *Bacillus* species other than the *Bacillus cereus* group	Lines 127-129: One of the important speculations from Apetroaie-Constantin's work is the functionality of surfactin as a signalling molecule inducing the newly-found toxin amylosin in food poisoning-associated *Bacillus*. They show that the surfactin-containing fraction of cell extracts from these food poisoning-associated *Bacillus* showed no toxicity by boar sperm motility test. The signal-inducing function of surfactin is widely known from various scientific articles. These evidences seem to be overlooked in this guidance which concludes that surfactin itself is the toxin. Lines 133-140: The link between cytotoxic lipopeptides and hemolysis is not clear and using hemolysis on blood as discrimination criteria for safety of Bacillus strains not the method suggested in recent literature. β-hemolysis can be very difficult to distinguish from α-hemolysis and it is the industry experience that the reference strains (negative and positive) do not always react as expected. Hemolysis is not an appropriate method for lipopeptide production giving rise to a lot of false negative and false positive results (Madslien et al., 2013; Plaza et al., 2006, Walter et al., 2010). Other compounds can cause hemolysis as e.g. lytic enzymes (so lipopeptide negative strains show hemolysis) (Walter et al., 2010). Formation of clearing zones can be inhibited although lipopeptides are produced (Madslien et al., 20137, Walter et al., 2010). According to Madslien et al. 2013 75% of the 52 *Bacillus* strains tested showed β-hemolysis. Proteases can cause the same hemolysis as surfactins and in this case should not be used as discrimination criteria for safety of the strain. Internal experimental data shows that proteases produced by *Bacillus* species like *B. subtilis* and *B. amyloliquefaciens* have a significant influence on the apparent hemolytic activity. It is further shown that even in the absence of the biosynthetic capacity to produce surfactin an increased protease production of microbial strains results in hemolysis clearing zones comparable to those of the surfactin producing strain. Hemolytic clearing zones on blood agar caused by proteases containing solutions from different sources can be reduced or completely abolished after heat-treatment or treatment with protease inhibitors. Lines 137-140: Hong et al. have isolated *Bacillus subtilis* strains from the gastro-intestinal tract of healthy humans. They found that all isolates were hemolytic. As such, hemolytic *B. subtilis* strains are naturally present in the gastro- intestinal tract without causing adverse events (*Bacillus subtilis* isolates comprised in this study even one quarter of the faecal isolates). Moreover, they could not make a correlation between the hemolytic activity of strains and surfactin production, since strains that produced no surfactin produced complete hemolysis as well. As such, using this hemolysis assay as the criterion for testing the safety of *B. subtilis* is not recommended and safety

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			should be investigated in vivo. No published scientific papers to our knowledge use hemolysis test as a sole method of evaluating toxigenic potential of Bacillus. Hemolytic potential, as well as the genetic potential of producing cyclic lipopeptides, is widely conserved across Bacillus species. Dybwad et al. showed recently that 76 strains out of 125 strains of airborne Bacillus were beta-hemolytic on sheep blood agar. Only 44 strains of these hemolytic strains possessed genes coding nonribosomal peptide synthetases. Of these 10 strains produced no cyclic lipopeptides detectable by LC-MS. Two important speculations from their study are; 1) hemolytic potential is common in *Bacillus species*, and 2) hemolysis test is not a reliable way of screening cyclic lipopeptide-producing strains. Hemolytic potential is also detected in some Bacillus strains in Natto product, which has been safely consumed in Japan and other countries with no reported incidence of food-poisoning. Lines 144-145: It is mentioned here that methods based on lactate dehydrogenase (LDH) release is a valid alternative but no reference to a protocol is given. Lines 180-186: Based on the comment to line 91 we suggest the following update: In principle, the selection of strains belonging to the Bacillus cereus phylogenetic groups III, VII, IV and II for direct use in animal production is not advisable due to the high risk of food poisoning associated with such strains. 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 184 cereulide synthase (Table 1). If there is evidence of homology, the non-functionality of the genes (e.g., mutation, deletion, lack of translation, differences in aas composition) should be demonstrated. Strains harbouring a genetical and phenotipical toxigenic potential should not be used as feed additives.
9	FEFANA/AMFEP	Appendix	We welcome the range of cell tests accepted by EFSA for safety testing and mainly see the challenge restricted to a detailed assay description, validation, control strains and end points considered as safe by EFSA. Additional information will be welcomed. Our experience to date shows that samples of the proposed control strains produced in accordance with these instructions do not give the expected results in either a NRU- or a MTT-assay. The positive control strain does not exhibit cytotoxic activity. This suggests that the preparation and use of these concentrates in tests for cytotoxicity have not been tested thoroughly and calls for a revision. The sample preparation described (10 x concentration) is arbitrary and acceptance criteria for the result is completely lacking. Line 302: It is not given how much of this should be used as test substance. Lines 333-338: It can be assumed that an anticipated cytotoxic activity caused by lipopeptides is directly related to an interaction with the cell membrane. Therefore, an assay for cytotoxicity based on inhibition of protein synthesis (as suggested) would not be the logical choice but rather an endpoint directly related to membrane damage. This could be the propidium iodide uptake assay, as mentioned in this guidance or a Neutral Red Uptake (NRU-) assay. Unfortunately, none of these assays are validated against these lipopeptides. Lines 338-339: It is not described how the data from the fluorescence monitoring should be managed or how the results should be interpreted. No threshold is given for when the test is showing toxicity. Lines 342-344: This reference is insufficient to stand alone for references to the Vero cell tests requested in the Guidance document. It only mentions the ^{14}C-leucine method which is already described in detail in the Guidance, however, neither the propidium iodide method nor LDH method mentioned in line 144 is mentioned. Few laboratories perform the Vero cell test as requested in the Guidance document. If applicants should be able to perform the tests themselves, it requires references to thoroughly described protocols and a description on how to interpret the data, e.g. in the form of threshold values as given for the 14C-leucine assay.
10	Newcastle University	Background	Lines 57-65: The scientific committee of EFSA published an opinion on the Qualified Presumption of Safety (QPS) approach for assessing

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			the safety of selected microorganisms for use in food and feed. Several Bacillus species has previously been accepted for use in food and feed on the basis that "the qualification of the absence of food poisoning toxins, surfactant activity or enterotoxic activity are met". This statement raises the important issue of the scientific evidence that is required to identify food poisoning toxins and enterotoxic activity. More specifically, it raises the issue as to the justification of including the absence of surfactant activity as a qualifying criterion. Lines 66-69: The Guidance makes an unsubstantiated statement that "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". This statement implicates surfactins and cyclic non-ribosomal peptides as heat-stable toxins without any qualification. Conflating these terms throughout the draft Guidance document seriously weakens its conclusions. As far as I am aware, there is no scientific evidence for such a broad statement and any case not all non-ribosomal peptides have surfactin-like activity. Most, if not all strains of *B. subtilis* and other non-*B. cereus* group strains encode genes for the synthesis of cyclic lipopeptides. The reference to these compounds in the Guidance document as "cytotoxins" (a substance having a specific toxic effect on certain cells) in the context of pathogenesis (e.g. food poisoning), rather than "cytolysins" (a substance that produces cytolysis), is not justifiable. A review of the literature by Edberg (1991) failed to reveal the production of authenticated toxins by *B. subtilis*. Although members of the non-*B. cereus* group have been associated with outbreaks of food poisoning (Gilbert et al., 1981 and Kramer et al., 1982 as cited by Logan, 1988), the exact nature of their involvement has not been rigorously established.
11	Newcastle University	Terms of reference	Lines 103-108: It is not clear why "surfactant activity" should be included alongside "food poisoning toxins" and "enterotoxins". The document states: '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'. I have two issues with this statement: (i) it repeats the error that "cyclic non-ribosomal lipopeptides" and "surfactins" as synonymous; (ii) it states unambiguously that surfactins are heat-stable toxins without providing evidential support. In my opinion currently there is no such evidence. The Guidance document seems to imply that if any cyclic (or other) lipopeptide is identified as being responsible for food poisoning, then all such compounds must be considered to be cytotoxins.
12	Newcastle University	Safety concerns caused by Bacillus species	Lines 127 -128: The guidance document indicates that the few incidents of food poisoning that have been investigated in which non-*B. cereus* group strains were the likely causative agent suggest an association with heat-stable surfactins and similar cyclic lipopeptides with surfactin activity. No evidence is given in support and since most such strains (including those used to the production of Natto) are able to synthesise surfactin and/or related lipopeptides, at first sight it is surprising that such cases of food poisoning are not common. Bacterial pathogenesis is a highly complex, multifactorial phenomenon and pathogenic strains encode a variety of virulence factors that enables them to, for example, (i) target specific sites infection (e.g. adhesins), (ii) evade the immune system (innate and acquired), (iii) modulate host cell signalling pathways, (iv) invade host cells, (v) damage host cells, (vi) respond to chemotactic and other host signals, (vii) resist unfavourable physiological conditions (e.g. low pH, high salt, digestive enzymes, antimicrobial peptides, reactive oxygen species) and many more. It is therefore relevant that, in contrast to pathogenic strains of *B. cereus*, whole genome analysis of *B. subtilis* and other non-*B. cereus* group strains does not reveal the presence of well-established virulence factors. Lines 136-146: Laboratory-based tests such as hemolysis and the Vero cell cytotoxicity assay were developed to detect known virulence factors in well-studied pathogens, in some cases to distinguish the diseases they are likely to cause, as in the case of enterotoxigenic (ETEC), enteropathogenic (EPEC) and enterohemorrhagic (EHEC) E. coli. They were not developed, as implied by the EFSA guidance document, as assays to identify potentially pathogenic strains. Indeed, neither of these assays is specific enough for this purpose since they give positive results to a range of chemical compounds that have never been implicated in pathogenesis. For example, the ability of surfactin to lyse cells in vitro is a feature of its surfactant activity1, and is a property that is shared with the widely used anionic detergent, sodium laural sulphate (SLS), present in many household products. To put its "toxicity" into perspective, the feeding of oral doses of surfactin C at concentrations ranging from 0 to 500mg/kg bw/day to pregnant ICR mice resulted in no maternal toxicity, fetotoxicity or teratogenicity. In

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			contrast, toxicity studies of SLS in mice and rabbits, using oral doses ranging from 0.2 to 600 mg/kg bw/day, resulted in total resorption of foetuses, increased litter loss and/or abortion at 600 mg/kg bw/day in the presence of severe maternal toxicity. At 300 mg/kg bw/day no developmental toxicity was observed, although slight-to-moderate maternal toxicity was observed. Finally, in contrast to its implied role in toxicity, the physiological role of surfactin has been extensively studied and is well understood. It is, in fact, involved in colonisation and distribution, rather than virulence. Undomesticated strains of *B. subtilis* form robust, multicellular communities that exhibit complex architectural features that include multicellular, aerial structures that resemble fungal fruiting bodies. Such strains undertake a multicellular behaviour known as "swarming motility" a social form of surface locomotion. Surfactin is essential for this form of behaviour by creating a transparent watery zone that preceded the advancing swarm front. This role is consistent with it physiological/surfactant characteristics.
13	University of Eastern Finland	Safety concerns caused by Bacillus species	Lines 128 – 129: 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. A critical reading of the publications describing the aforesaid association indicates that only a small minority (3 – 5 %) of strains isolated from food poisoning cases were phenotypically potential surfactin producers (Salkinoja-Salonen et al. 1999; Apetroaie-Constantin et al. 2009). The existing toxicological data indicates a very low (practically non existent) rodent toxicity of purified surfactins, whether administered orally, intravenously or intraperitoneally, or in single or multiple doses (Kikuchi and Hasumi, 2002; Hwang et al. 2009; Sun et al. 2012). Although there might be species differences in the sensitivity to these types of compounds, the data on the Japanese traditional product "natto", fermented by specific *Bacillus subtilis* strains, indicate that standard servings can contain as much as 0.1 g of surfactins without causing apparent ill effects in consumers (Juola, Kinnunen, Fog Nielsen, von Wright, manuscript submitted for publication). Thus surfactins (and other similar lipopeptides) appear to be very unlikely causes of any food poisonings, and the focus of the guidance appears to be misplaced.
14	University of Eastern Finland	Assessment of Bacillus species other than the Bacillus cereus group	Lines 137 – 140: 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. The haemolysis test suggested is unspecific, since haemolysis can be caused by several factors, including a strong proteolytic activity of the strain. Also strains having an established safety record, such as "natto" starters, are clearly beta-haemolytic in these conditions. Haemolysis as such, therefore, should not be considered as a definite exclusion criterion. Regarding the suggested controls, *B. subtilis* ATCC 6051 (type strain) is clearly beta-haemolytic at 37 °C and can give variable results at 30 °C. Lines 141 – 146: 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. The use of alternative test methods, instead of that based on ^{14}C – leucine uptake inhibition, is welcome. However, since the tests have not been standardized and validated, more detailed recommendations are needed, in particular on the testing of methanol extracts (how much of the solvent the various alternative test methods will tolerate). Proper positive and negative control strains should be also indicated. Finally, this test does not differentiate between surfactins, cereulide-like compounds and amylosin. Since surfactins are apparently innocuous, and the role of amylosin in food poisonings is unclear, even the vero-cell cytotoxicity should not be considered as a definitive exclusion criterion.

#	Organization	Chapter	Comment
			In conclusion, since the real causes of food poisonings associated with *Bacillus* species (other than those belonging to the *B. cereus* cluster) are unknown, and many types of substances (innocuous and potentially harmful) can cause both haemolysis and vero-cell cytotoxicity, there are no simple phenotypic tests that could differentiate between safe and suspect strains. Therefore the guidance should be reconsidered giving more emphasis on case-by case approach, and – in cases of reevaluation of existing products – on the exisiting safety record
15	Danish Veterinary and Food Administration	Assessment of Bacillus species other than the Bacillus cereus group	Line 121: The revised guidelines acknowledge that the (genetic) capacity for cyclic lipopeptide production is widely distributed in *B. subtilis*, *B. licheniformis* and other non-*B. cereus* group organisms and thus using this as criterion during assessment is not recommended. We support this view. The guidelines do however suggest using actual in vitro production of cyclic lipopeptides during assessment of specific strains. Strains found to produce sufficient cyclic lipopeptide to result in haemolysis (on sheep blood agar) and/or cytotoxicity (Vero cell assay) should according to the guidelines not be recommended for use. Thus the guidelines do not provide threshold concentrations for cyclic lipopeptides but rely on specified biological assays. We find the new focus on cyclic lipopeptides during assessment of Bacillus strains to be problematic for the following reasons: 1) In a very recent paper (Madslien et al. 2013) the in vitro production of Lichenysin (a cyclic lipopeptide) was demonstrated in all 53 tested B. lichenisformis strains from various sources. In 54% of these a cytotoxic effect in Vero cells was also attributed to the strain, which did not always match observed food-poisoning strains. The authors comment that environmental factors are known to affect lichenysin production and the amounts of lichenysin detected from each strain might therefor differ from the situation in vivo and in foods. We agree with the view that actual in vivo production of cyclic lipopeptide is difficult to extrapolate from in vitro assays. 2) Although cyclic lipopeptides are known to have a cytotoxic effect in e.g. Vero cell assays, there is lacking scientific knowledge regarding any negative effect in vivo in the gastrointestinal tract. Some cyclic lipopeptides, such as surfactin found in the traditional far-eastern product "Natto", are known to be very well tolerated. It appears that there are still considerable data-gaps in the link between food poisoning and cyclic lipopeptides. We agree with the conclusion made by Madslien et al. 2013 that the reason for the very few reports of B. licheniformis associated disease is probably that, even for the most potent lichenysin producers, a very high number of cells are required in order to synthesize sufficient lichenysin to cause toxicity in humans. This is probably also true for other cyclic lipopeptides. A safe track-record over decades of use is associated with many already marketed Bacillus-based products (non-B. cereus). These products may or may not be able to produce sufficient amounts of cyclic lipopeptides to be detected by the mentioned assays. We suggest that a pragmatic approach be taken towards already marketed products that reflect their safe record.
16	Danish Veterinary and Food Administration	Assessment of species belonging to the Bacillus cereus group	Line 147: Assessment of species belonging to the Bacillus group We agree that use of any B. cereus group strain in animal nutrition warrants caution due to the omnipresence of genes encoding enterotoxins within this group. The requirement for full genome sequencing and bioinformatics analysis to detect toxin genes is in our view justified for the *B. cereus* group. It should be noted that proving the non-functionality of toxin genes based on sequence information alone may be difficult. Strains belonging to the *B. cereus* group may harbor genes (Nhe and/or Hbl) encoding enterotoxins but not produce these in vivo at biologically significant concentrations. An example of this is Toyocerin, which has been marketed for many years without any documented or recorded negative effects. It should be considered to implement an option to waive the suggested requirements for *B. cereus* products, which have demonstrated an excellent safety record after many years of use in animal nutrition.
17	Danish Veterinary and Food Administration	Appendix	Line 287: The assays which are suggested to be used to determine cytotoxicity of cyclic lipopeptides are fairly complex and not necessarily easy to setup. In our opinion, there should be options to use alternative assays as well.

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18	Royal Holloway University of London	Safety concerns caused by Bacillus species	I have observed recent discussions at EFSA regarding the safety of *Bacillus* species as food supplements especially with regard to the production of hemolysins and surfactants. It is recognized by EFSA that a number of Bacillus species are in current use as food staples (e.g., Natto) or as probiotics (*B. subtilis*, *B. licheniformis*). EFSA correctly identifies such strains as QPS. Despite this, the logic and rationale for how strains are classified as safe seems overly complicated. In the USA self-affirmed GRAS requires a complete genome sequence of the strain to be conducted and a risk assessment then made based on each and every gene present on the genome. This analysis has been conducted for *B. subtilis* RO179 (Lallemand) and *B. subtilis* HU58 (Viridis Pharma PVT) in their self-affirmed GRAS dossiers. Genome sequencing clearly identifies any gene with a potential risk. From this type of analysis it is also apparent that most strains of *B. subtilis* carry multiple hemolysins (e.g., the 168 type strain carries 8 hemolysin genes). The Natto strain carries genes for surfactin biosynthesis and indeed the strain produces large quantities of surfactin yet is eaten daily by large numbers of Japanese. It seems impossible that *Bacillus* researchers working and being exposed to 168 have never suffered adverse effects not Japanese who consume Natto. Having identified putative genes in the genomic risk assessment the applicant can then assess the levels of molecule produced. Having said this it is apparent also that any biochemical or physiological tests to evaluate hemolysis or surfactin activity can be flawed and may not reflect the physiological state when the spores enter the GI-tract or nasopharanyx. A further concern is that physiological tests assess the live vegetative cell and not the spore. The next question is what numbers of spores germinate in the GI-tract? No one knows and it seems unwise to assume that simply because a strain can lyse red blood cells this is of concern. The test by its very nature simply says that the cell or spore is able to disrupt cell membranes at high concentration. This is not surprising when most *B. subtilis* strains carry 8 or more hemolysin genes together with a number of surfactants including surfactin, amylosin and fengycin. I would propose that EFSA adopt the US approach of requiring strains to be evaluated as follows: 1) complete genome sequence and a risk-assessment made on the basis of putative virulence genes 2) in vitro assessment using established methods 3) safety/toxicology in a rodent model. In the end, what matters is that high dose no adverse effects are seen. The way things are moving it seems that there is a need to diminish the relevance of *B. subtilis* and its relatives as food supplements based on often unreliable and outdated tests of little physiological relevance and ignoring that these products are in use worldwide and have a good safety record.