SECTION: BACKGROUND AS PROVIDED BY EFSA (lines 43-76)

- 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 from Natto products was clearly hemolytic on sheep blood agar after 2 days and toxic in a boar sperm motility test.

Additional internal tests on 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.
SECTION: 1. INTRODUCTION (lines 81-101)

- 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.

SECTION: 3. SAFETY CONCERNS CAUSED BY BACILLUS SPECIES (lines 109-186)

3.2. Assessment of Bacillus species other than the Bacillus cereus group (lines 121-146)

- 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. 

Ref.:


- **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., 20137; Plaza et al., 20068, Walter et al., 20109). 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., 20109). According to Madslien et al 2013 75% of the 52 Bacillus strains tested showed β hemolysis. Proteas 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.

Ref.:


- Lines 137-140: Hong et al.\textsuperscript{10} have isolated \textit{Bacillus subtilis} strains from the gastro-intestinal tract of healthy humans. They found that all isolates were hemolytic. As such, hemolytic \textit{B. subtilis} strains are naturally present in the gastro-intestinal tract without causing adverse events (\textit{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 \textit{B. subtilis} is not recommended and safety should be investigated \textit{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\textsuperscript{11} 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.

Ref.: 
\textsuperscript{10}Hong et al. (2009) \textit{Bacillus subtilis} isolated from the human gastrointestinal tract. Research in Microbiology. 160. 134-143.


- 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.
3.3. Assessment of species belonging to the Bacillus cereus (lines 147-186)

- **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.

**SECTION: Appendix (Lines 287-344)**

- AMFEP & FEFANA 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 fluorescens 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 14C-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.