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Synergism and distribution of emetic *B. cereus* food-born toxins and development of strategies for preventing toxin production („*Emetic B. cereus toxins*“)

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Research objective:

Whether animal or plant based, whether produced in the food industry or in our own domestic homes: The world wide ubiquitous soil bacterium *Bacillus cereus* is not only present in the soil, but in a wide variety of foods. Even at very strict hygienic standards, in many branches of the food industry it is almost impossible to avoid a bacterial contamination due to the formation of heat and acid stable endospores. Exemplary, dairy products (up to 75%), salads and dried mushrooms (89-90%), and spices (almost 100%) exhibited high contamination rates. The bacteria spores survive even high temperatures, and certain strains can produce the emetic toxin cereulide and its structural homologous isocereulides, which were first described in the IGF project AiF 16845 N. The enormous stability against heat, acid and pressure as well as its small molecular structure (approx. 1.2 kDa) of those dodecadepsipeptides strongly complicate their removal by e.g. heating or filtration processes, rendering it almost impossible. Thus, the emetic toxin can reach the consumer via contaminated foods and cause strong health issues after consumption.

In the past years the reports of cereulide intoxications accumulated: The consumption of contaminated food typically leads to acute emesis, but also can cause severe illness, occasionally including fatalities. As recent product recalls show, *B. cereus* presents a severe safety hazard for small as well as large food industrial companies.

Due to a lack of evidence-based threshold values, a “zero-tolerance” policy is currently employed in the USA, leading to discussing a similar option for the German industry. Considering the permanently increasing gain of sensitivity of analytical procedures, this “zero-tolerance” policy will not be practicable. It confronts producers with massive technological problems and possibly results in the blocking or wastage of whole product batches without contributing to the consumer safety in a significant way. In light of the demands of a future circular economy, new paths are necessary to reduce food waste while ensuring food safety.

Thus, the objectives of this research project were to clarify the biological effects of the newly identified isocereulides compared to cereulide and investigate possible additive or synergistic effects between the single toxins as a basis for possibly evidence-based threshold values. Furthermore, the entry of (iso)cereulide via raw materials as well as their distribution during the food processing, e.g. by sticking to food biopolymers or bacterial spores was examined. The identification of food components and parameters inhibiting bacterial growth or (iso)cereulide production posed an additional research objective, to pave the ground for a guideline to help the food industry in their sampling chain to prevent cereulide based food intoxications. Additionally, in collaboration with the members of the industrial association a checklist should be prepared, which highlights the critical control points for cereulide contaminations.

Research results:

Within this project, additionally to the already described isocereulides A–D and F+G (IGF project AiF 16845 N), seven new isovariants N-H were identified and their structure could be elucidated. The existing UPLC-MS/MS quantitation method for cereulide and isocereulides A–G (IGF project AiF 16845 N), was enlarged to allow quantitation of all isocereulides A–N.

In vitro cytotoxicity assays showed that three of the isovariants are 3-7-fold more toxic than cereulide. Further, additive as well as synergistic interactions could be shown for the single isovariants in combination with cereulide. The strongest synergistic effect was found for the natural ratio of cereulide and isovariants A-N, with a toxicity about 14-fold higher compared to cereulide.

Localization studies of cereulide within milk revealed a shift in toxin distribution from the aqueous to the fatty phase with the increase in milk fat content. These results suggest that special attention should be paid to the analytics of cereulide in foods with high fat content, in the frame of analyses in food production.

Studies to elucidate whether cereulide can be spread by spores in food production processes and in foodstuffs revealed a high affinity of Bacillus spores for cereulide. It was also shown that cereulide can pass over from bound spores into milk, depending on its fat content.

A combinatorial approach, including a bacterial cell culture assay and a fast UPLC-MS/MS method, allowed the parallel investigation of cell viability and quantification of cereulide. This newly developed system was employed for a broad screening of plant natural products and polyphosphates, which allowed to identify natural substances showing a strong inhibitory effect on bacterial growth and/or cereulide production.

Based on the project results, a checklist was developed together with the industrial project partners, allowing to specifically analyze food processing for potential contamination with the toxin cereulide and to define critical control points as well as to prospectively identify sensitive process steps.

Economic relevance:

Although the emetic form of the food intoxications caused by B. cereus is often reported in connection to rice and pasta dishes, recent case reports showed an association of outbreaks in Germany and Switzerland with dairy products. Moreover, emetic B. cereus strains could be isolated from milk, pudding, protein-containing infant food as well as ice cream.

The data collected within this project regarding the entry as well as distribution of the toxins offer an opportunity to the food industry to use the checklist for the identification of contaminants, sampling from the food processing, inhibition and distribution of the toxins and, if necessary, applying for a fast and cost-saving LC-MS analysis. This strategy helps to avoid incident of contamination with an image tarnishing recall and consumer compensation especially jeopardizes SMEs economically, in the worst case bankrupting a company.

Especially new competitors from Asia pose enormous challenges in the context of global trading and can hamper the performance of SMEs. Therefore, the newly developed checklist offers a valuable tool in prevention strategies to ensure the safeguarding industry's competitiveness.

With annual sales of approx. € 28 billion (2019), the German dairy industry is one of the highest-turnover sectors of the food industry. Although no statistical data is available on the financial damage of the food industry in Germany caused by *B. cereus*, the estimation is in the range of several Mio €.

Food additives identified in frame of this project, which are to suppress the formation of the emetic (iso)cereulides of B. cereus without significantly influencing the production conditions, will make an important contribution to minimizing the contamination of food processing and end products with cereulide toxins. Thus, this project provides, particularly for SMEs, new options for taking preventive action to avert economic damage caused by production stops, disposal measures or recalls and claims for compensation. Measures preventing toxin formation not only increase product safety and avoid high follow-up costs, but also make an important contribution to the sustainability of food production. Thus, this project contributes to both, food safety and food security.

Results of this project are of importance for all food producers, especially for the German milk-producing and -processing industry and their suppliers, e.g. manufacturers of spices, cocoa or vegetables and for producer of pet food, food additives, convenience and dietary food products.

Publications (selection):

1. **FEI-final report 2021**
2. Dietrich, RN; Jessberger, M; Ehling-Schulz, M; Märtlbauer, E; Granum, PE (2021): The food poisoning toxins of *Bacillus cereus*. Toxins, 13, 98. https://doi.org/10.3390/toxins13020098
3. Kalbhenn, EM; Bauer, T; Stark, TD; Knüpfer, M; Grass, G; Ehling-Schulz, M (2021): Detection and isolation of emetic *Bacillus cereus* toxin cereulide by reversed phase chromatography. Toxins, 13, 115. doi.org/10.3390/linebreak toxins13020115
4. Kranzler, M; Walser, V; Frenzel, E; Lücking, G; Hofmann, TF; Stark, TD; Ehling-Schulz, M (2021): Impact of phytochemicals on viability and cereulide toxin synthesis in *Bacillus cereus* revealed by a novel high-throughput method, coupling an AlamarBlue-based assay with UPLC-MS/MS. In preparation
5. Rouzeau-Szynalski, K; Stollewerk, K; Messelhäusser, U; Ehling-Schulz, M. (2020): Why be serious about emetic *Bacillus cereus*: Cereulide production and industrial challenges. Food Microbiol. 2020; 85:103279
6. Walser, V; Kranzler, M; Ehling-Schulz, M; Stark, TD; Hofmann, TF (2021): Structure Revision of Isocereulide A, an Isoform of the Food Poisoning Emetic Bacillus cereus Toxin Cereulide. Molecules. 2021; 26(5):1360.
7. Walser, V; Kranzler, M; Dawid, C; Ehling-Schulz, M; Stark, TD; Hofmann, TF (2021): *Bacillus cereus* toxin repertoire: the diversity of (iso)cereulide(s). In preparation

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