Bacillus amyloliquefaciens QST 713

Microbial pest control agent against plant pathogenic fungi and bacteria

Dossier according to OECD dossier guidance for microbial agents and microbial pest control products – August 2006

Summary documentation, Tier II

Annex IIM, Section 3

Point IIM 5: Toxicological and Exposure Data and Information on the Microbial Pest Control Agent

Date: September 2015

Applicant

Bayer CropScience AG

Table of Contents

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Introduction

The company Bayer CropScience AG is submitting a dossier for the re-approval of the microorganism *Bacillus amyloliquefaciens* QST 713 as an active substance under regulation (EC) 1107/2009, previously designated as *Bacillus subtilis* QST 713. Due to most current information on taxonomy, *B. subtilis* QST 713 is classified as a member of *B. amyloliquefaciens* group. As a consequence, the active substance is now named as *B. amyloliquefaciens* subsp. *plantarum* QST 713, hereinafter named as *B. amyloliquefaciens* QST 713.

The initial evaluation of *Bacillus subtilis* QST 713 was performed under Directive 91/414. Data provided in the initial dossier and in subsequent additional submissions according to the OECD dossier guidance (2006) are submitted as a "Baseline Dossier", separately.

Here we submit all new data and information basing on previous literature searches and studies.

IIM 5 Toxicological and Exposure Data and Information on the Microbial Pest Control Agent

Introduction

IIM 5.1 Summary: Potential of microbial pest control agent to be hazardous to humans with consideration of its pathogenic potential, its ability to infect and pattern of clearance and its toxicological effects

Report: KIIM 5.1/02 - [redacted] (2015) Literature review on effects on human health of Bacillus amyloliquefaciens QST 713 and its metabolites unpublished report, owner: Bayer CropScience AG.
Report-no. 6791109-A2-05-01
M-535690-01-1

For the background information, please refer to the baseline dossier.

A search of the published literature has been conducted using the DIMDI database provided by the German Institute of Medical Documentation and comprised of searches in MEDLINE, BIOSIS, CAB and SCISEARCH databases. The strategy aimed to find all references concerning the occurrence of toxicological adverse effects caused by *B. amyloliquefaciens*, *B. subtilis* or its metabolites. As a result, a large amount of literature was found. However, from the total of publications retrieved, only a few reported on the occurrence of toxicological adverse effects, including infections on patients suffering specific predisposing factors and allergies, that were caused by or related to strains of *B. subtilis* or *B. amyloliquefaciens* different to the strain QST 713, or to any other strain used as a plant protection product. In addition, results from recent reports on toxicity assessment of strains of *B. subtilis* used as probiotics or vaccine adjuvants further support the safety of these species for humans, also demonstrated by the large history of safe exposure to strains of *B. subtilis* and *B. amyloliquefaciens* or its metabolites, used in industrial fermentation, the food industry and agriculture. Moreover, species of *B. subtilis* and *B. amyloliquefaciens* were assigned the Qualified Presumption of Safety (QPS) status for intentional addition to food or feed by the EFSA Panel on Biological Hazards[1].

For details on the literature search on metabolites and toxins please refer to [redacted] (2015).

IIM 5.2 Occupational health surveillance report on workers during production and testing of MCPA

Report: KIIM 5.2/04 - [redacted], M.J. (2015) Statement concerning hazards to man during the use or handling of *Bacillus subtilis* strain NRRL QST713 unpublished report, owner: Bayer CropScience AG.
Report-no. n/a
M-532269-01-1

Summary: It is certified that no adverse effects to man during the use or handling as well as to employees involved in the research for use of the active ingredient *Bacillus subtilis* strain QST 713 have been reported since October 1995.

No effects to man during the use or handling as well as to employees involved in the research for use of the active ingredient *Bacillus subtilis* strain QST 713 have been reported. Since October 1995 investigations in the laboratories of Bayer CropScience, Biologics have been carried out with the strain. Currently the work force consists of 100 employees in the research laboratory. No indications of any toxicological or allergenic effects to the laboratory or production team were observed. Not a single incident, no allergic response, sensitisation or irritation did occur.

Report: KIIM 5.2/05 - [redacted]. (2015) Statement concerning hazards to man during the use or handling of *Bacillus subtilis* strain NRRL QST713 unpublished report, owner: Bayer

[1] EFSA BIOHAZ Panel (EFSA Panel on Biological Hazards), 2013. Scientific Opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed (2013 update). EFSA Journal 2013;11(11):3449

CropScience AG.
Report-no. n/a
M-532275-01-1

Summary: It is certified that no adverse effects to man during the use or handling as well as to employees involved in the research for use of the active ingredient *Bacillus subtilis* strain QST 713 have been reported since March 2001.
The development work in the production plant in México has focused on the strain since March 2001. Currently the work force consists of 115 employees in the production plant. No indications of any toxicological or allergenic effects to the laboratory or production team were observed. Not a single incident, no allergic response, sensitisation or irritation did occur.

IIM 5.2.1 Sensitisation and allergenic response of workers

Report: KIIM 5.2.1/01 - [redacted] (2015) Statement concerning hazards to man during the use or handling of *Bacillus subtilis* strain NRRL QST713 unpublished report, owner: Bayer CropScience AG.
Report-no. n/a
M-532269-01-1

Summary: It is certified that no adverse effects to man during the use or handling as well as to employees involved in the research for use of the active ingredient *Bacillus subtilis* strain QST 713 have been reported since October 1995.

No sensitisation or allergenic responses have occurred of have been reported during the use or handling as well as to employees involved in the research for use of the active ingredient *Bacillus subtilis* strain QST 713 since October 1995.

Report: KIIM 5.2.1/02 - [redacted] (2015) Statement concerning hazards to man during the use or handling of *Bacillus subtilis* strain NRRL QST713 unpublished report, owner: Bayer CropScience AG.
Report-no. n/a
M-532275-01-1

Summary: It is certified that no adverse effects to man during the use or handling as well as to employees involved in the research for use of the active ingredient *Bacillus subtilis* strain QST 713 have been reported since March 2001.

No sensitisation or allergenic responses have occurred of have been reported during the use or handling as well as to employees involved in the research for use of the active ingredient *Bacillus subtilis* strain QST 713 since March 1995.

IIM 5.2.2 Details on any occurrence of hypersensitivity and chronic sensitisation

As part of the search of the published literature conducted using MEDLINE, BIOSIS, CAB and SCISEARCH databases, references concerning the occurrence of toxicological adverse effects, including hypersensitivity and chronic sensitisation, due to *B. amyloliquefaciens*, *B. subtilis* or its metabolites were searched.

Among the publications retrieved, a recent work by [redacted] and [redacted] (2014) reported the results from a data base search with the aim to find agents eliciting occupational asthma due to proven IgE-mediated sensitisation was found. The authors conducted a database search with MEDLINE via PubMed, screening reference lists of relevant reviews and matching the findings with a list of agents denoted as "may cause sensitisation by inhalation" by the phrase H334 (till 2011 R42). The quality of the selected studies was rated with the Scottish Intercollegiate Guideline Network (SIGN) grading system. The evidence level for each causative agent was graded using the modified Royal College of General Practitioners (RCGP) three-star system. A total of 865 relevant papers were identified, which covered 372 different causes of allergic work-related asthma. From these, a total of 327 cases per agent with moderate evidence level was found for various enzymes from *B. subtilis* (alcalase, protease, maxatase, maxapem, esperase, cellulase, α-amylase, lipase, subtilisin).

Similarly, [redacted] and [redacted] (2010) reported a case of allergic alveolitis caused by *B. subtilis*, in which the source of the allergen was identified to be a biological detergent at the working place.

[redacted] et al. (2007) reported a clinical review of allergic reactions to natto, a traditional food in Japan consisting in soybeans fermented by *B. subtilis* natto. Within 5 years, 7 patients were identified which had all experienced anaphylactic reactions within 5-14 hours, without early phase reactions, after ingestion of natto. This clinical review revealed that allergic reactions after ingestion of natto could mostly show a novel clinical course of IgE-mediated, late-onset anaphylaxis without early phase reactions.

No publication reporting on hypersensitivity or chronic sensitisation caused by *B. amyloliquefaciens* or *B. subtilis* QST 713, or by any other strain used in agriculture, was found.

Cited references (bibliographic data and abstracts):

Report: KIIM 5.2.2/01 – [redacted], [redacted] (2014) Allergens causing occupational asthma: an evidence-based evaluation of the literature, published report
Int Arch Occup Environ Health, 87, 339-633.
M-530019-01-1

Abstract:
PURPOSE: The aim of this work is to provide an evidence-based evaluation and overview of causative substances in order to improve disease management.

Methods: A database search with MEDLINE via PubMed, screened reference lists of relevant reviews and matched the findings with a list of agents denoted as "may cause sensitisation by inhalation" by the phrase H334 (till 2011 R42). After exclusion of inappropriate publications, quality of the selected studies was rated with the Scottish Intercollegiate Guideline Network (SIGN) grading system. The evidence level for each causative agent was graded using the modified Royal College of General Practitioners (RCGP) three-star system.

Results: A total of 865 relevant papers were identified, which covered 372 different causes of allergic work-related asthma. The highest level achieved using the SIGN grading system was 2++ indicating a high-quality study with a very low risk of confounding or bias and a high probability of a causal relationship. According to the modified RCGP three-star grading system, the strongest evidence of association with an individual agent, profession or worksite ("***") was found to be the co-exposure to various laboratory animals. An association with moderate evidence level ("**") was obtained for α-amylase from *Aspergillus oryzae*, various enzymes from *Bacillus subtilis*, papain, bakery (flour, amylase, storage mites), western red cedar, latex, psyllium, farming (animals, cereal, hay, straw and storage mites), storage mites, rat, carmine, egg proteins, atlantic salmon, fishmeal, norway lobster, prawn, snow crab, seafood, trout and turbot, reactive dyes, toluene diisocyanates and platinum salts.

Conclusion: This work comprises the largest list of occupational agents and worksites causing allergic asthma. For the first time, these agents are assessed in an evidence-based manner. The identified respiratory allergic agents or worksites with at least moderate evidence for causing work-related asthma may help primary care physicians and occupational physicians in diagnostics and management of cases suffering from work-related asthma. Furthermore, this work may possibly provide a major contribution to prevention and may also initiate more detailed investigations for broadening and updating these evidence-based evaluations.

Report: KIIM 5.2.2/02 – [redacted], [redacted] (2010) Extrinsic alveolitis caused by *Bacillus subtilis* in a biological detergent – Diagnosis and follow up
Allergologie, 33, 570 - 572
M-520007-01-1

Abstract: A case report with follow up about an allergic alveolitis due to *Bacillus subtilis* is presented. As main source of the allergen a bio-logical detergent at the working place could be identified. An amplification by bacterial contamination of a room fontaine at home is very likely. A subclinical reactivation was detected by serial antibody titers and increases of C-reactive protein and lactatdehydrogenase (LDH).

Report: KIIM 5.2.2/03 – [redacted], [redacted], [redacted], [redacted], [redacted], [redacted], [redacted] (2007) Late-onset anaphylaxis after ingestion of *Bacillus subtilis*-fermented soybeans (Natto): clinical review of 7 patients
Allergol Int, 56(3), 257-261
M-520045-01-1

Abstract:
Background: Allergic reactions after ingestion of fermented soybeans have rarely been reported. Fermented soybeans were recently reported to be a causative food of IgE-mediated, late-onset anaphylaxis without early phase responses. The objectives of our study are to clarify the clinical and laboratory features and to characterize the allergens in allergy due to fermented soybeans.

Methods: Seven patients with suspected hypersensitivity to fermented soybeans, from whom informed consent had been obtained, underwent skin prick-prick tests with fermented soybeans and challenge test with fermented soybeans. Additionally, specific IgE against fermented soybeans and the allergens of fermented soybeans were detected by ELISA and IgE-immunoblotting, respectively.

Results: Seven male patients, aged 26 to 42 years (mean age, 33.1 years), participated. All patients reported generalized urticaria and dyspnea; 5, loss of consciousness; 2, collapse; 2, vomiting; and 2, diarrhea after fermented soybean ingestion. The interval between fermented soybean ingestion and onset of symptoms was 5 to 14 hours (mean, 9.6 hours). All patients were positive on skin prick-prick tests with fermented soybeans. In 2 patients, oral challenge with fermented soybeans was positive 5.5 and 13 hours after ingestion. In ELISA, all 5 patients tested showed elevated IgE levels to the fermented soybean extract. Furthermore, IgE-immunoblotting using 5 patients' sera showed six bands, of which three bands at 38, 28, and 26-kd were bound to sera from 4 patients.

Conclusions: Cases with hypersensitivity after ingestion of fermented soybeans most frequently correspond to IgE-mediated, late-onset anaphylactic reactions due to fermented soybeans, and not from *Bacillus subtilis*.

Report: KIIM 5.2.2/04 - [redacted], C. (2015) Literature review on effects on human health of Bacillus amyloliquefaciens QST 713 and its metabolites unpublished report, owner: Bayer CropScience AG.
Report-no. 6791109-A2-05-01
M-535690-01-1

For details on the literature research, please refer to [redacted] (2015).

IIM 5.2.3 Any significant clinical findings related to exposure, with special attention to those whose susceptibility may be affected

For the background information, please refer to the baseline dossier.

IIM 5.2.4 Published reports of adverse effects, especially reports of clinical cases and follow-up studies; list databases and key words used in a literature search

A literature research was conducted on the DIMDI database provided by the German Institute of Medical Documentation and comprised of searches in MEDLINE, BIOSIS, CAB and SCISEARCH databases. Search strategy aimed to find all references that are of toxicological relevance regarding reports of adverse effects, clinical cases and follow-up studies referring to *B. amyloliquefaciens, B. subtilis* or to metabolites or toxins produced by these species.

Search number: 1
Date of search: 14.08.2013 and 14.04.2014
Publication dates: 2003-2014
Search terms: Tox? OR pathogen? OR infectiv? OR allerg? OR genotox? AND bacillus subtilis (in title) NOT control NOT efficacy NOT analytic? AND bacillus subtilis

Search number: 2
Date of search: 14.08.2013 and 14.04.2014
Publication dates: 2003-2014
Search terms: Tox? OR pathogen? OR infectiv? OR allerg? OR genotox? AND bacillus amyloliquefaciens (in title) NOT control NOT efficacy NOT analytic? AND bacillus amyloliquefaciens

Search number: 3
Date of search: 14.08.2013 and 14.04.2014
Publication dates: 2003-2014
Search terms: Toxin OR metabolite AND tox? OR allerg? OR genotox? AND bacillus subtilis (in title) NOT genome NOT degradation NOT expression AND bacillus subtilis

Search number: 4
Date of search: 14.08.2013 and 14.04.2014
Publication dates: 2003-2014
Search terms: Toxin OR metabolite AND tox? OR allerg? OR genotox? AND bacillus amyloliquefaciens (in title) NOT genome NOT degradation NOT expression AND bacillus amyloliquefaciens

Search number: 5
Date of search: 22.06.2015
Publication dates: 2014-2015
Search terms: Tox? OR infect? OR genotox? AND subtilis

Search number: 6
Date of search: 22.06.2015
Publication dates: 2014-2015
Search terms: Control OR synthesis OR efficacy OR antimicrobial AND subtilis

Search number: 7
Date of search: 22.06.2015
Publication dates: 2014-2015
Search terms: Search 5 NOT Search 6

Search number: 8
Date of search: 22.06.2015
Publication dates: 2014-2015
Search terms: Tox? OR infect? OR genotox? AND amyloliquefaciens

Search number: 9
Date of search: 22.06.2015
Publication dates: 2014-2015
Search terms: Allergy OR sensiti? AND amyloliquefaciens

Search number: 10
Date of search: 22.06.2015
Publication dates: 2014-2015
Search terms: Allergy OR sensiti? AND subtilis

Search number: 11
Date of search: 22.06.2015
Publication dates: 2014-2015
Search terms: Search 10 NOT Search 6

Search number: 12
Date of search: 22.06.2015
Publication dates: 2014-2015
Search terms: Tox? OR metabolite AND subtilis

Search number: 13
Date of search: 22.06.2015

Publication dates: 2014-2015
Search terms: Search 12 NOT Search 6

Search number: 14
Date of search: 22.06.2015
Publication dates: 2014-2015
Search terms: Tox? OR metabolite AND amyloliquefaciens

Following the combination of the 14 searches and the elimination of duplicates, the total amount of literature retrieved accounted for 1554 publications. References were assessed by the information contained on titles and abstracts. Of these, 1518 publications were considered to be not relevant based on the title and abstracts. Thirty six references were selected as potentially relevant and subjected to a full text assessment.
Of these, 7 publications were found to report on clinical cases or adverse effects in which different strains of *B. subtilis* were isolated and/or suggested to be the causative agent. One report was found describing an outbreak of food borne intoxication in which *B. subtilis* was identified as part of the source of contamination. Three further publications reported on sensitisation and allergic reaction caused by strains of *B. subtilis*.

None of these publications referred to the strain QST 713 or to any other strain employed as a plant protection product.

A highly serine protease producing *B. subtilis* strain (PRY) was isolated from the wounds, infected with mixed bacteria, on the legs of a traffic accident patient. The serine proteases produced by the PYR strain were found to facilitate swarming motility and siderophore-mediated iron uptake from transferrin through the destruction of transferring. The authors suggested that proteases from *B. subtilis* may play a significant role in the pathogenesis of human infections by facilitating siderophore-mediated iron uptake from transferrin and swarming motility ([redacted] et al. 2006).

[redacted] et al. (2009) reported two cases of severe hepatic injury in patients, one male and one female, following long-term consumption of Herbalife® products. Toxicology screening of the products revealed no relevant contamination with pesticides, heavy metals, antibiotics, alkyl phosphates, and softeners. Immunoallergic activation towards the used Herbalife products was not detectable neither by skin hypersensitivity testing nor by assaying lymphocyte stimulation indicative of drug-induced hypersensitivity. Four samples of Herbalife products, two of the seven ingested by the female patient and the only sample ingested by the male patient as well as one sample of a sealed batch showed growth of Gram positive rods after 48 h of incubation. Bacteria from three out of four were subsequently identified by sequencing the 16S rRNA gene as *Bacillus* spp. (one product sample ingested by the female patient also harboured *Paenibacillus* spp.). *Bacillus* spp. was analysed to the species level by performing *gyr* B gene sequencing and identified as *Bacillus subtilis,* although the strains involved are not indicated. *B. cereus* was also identified in one of the products consumed by the female patient. The bacterial supernatants from cultures of *B. subtilis* showed dose- and time-dependent hepatotoxicity *in vitro* as indicated by the increase of LDH leakage in HepG2 cells. Causality of Herbalife® products as the precipitating factor of liver damage was assessed according to CIOMS and scored "probable" in both cases due to exclusion of other causes as immunoallergic sensitisation or contamination by toxicant chemicals, and due to the immediate resolution of liver damage after de-challenge.

Jeon et al. (2012) reported an unusual bacteraemia and mediastinitis in a patient with an oesophageal perforation probably caused by tablets swallowed to alleviate chest pain. *B. subtilis* and *B. licheniformis* were identified by 16S rRNA sequence analysis of colonies grown in multiple blood cultures, pleural fluid and pus. The condition of the patient improves following treatment with antibiotics and the localization of the oesophageal perforation.

In a study designed to characterize the phenotype and genotype of *Bacillus* spp isolated from diabetic patient's eyes, the eyes of 25 patients with type II diabetes mellitus, with at least 10 years of diabetes history, were analyzed for the presence of *Bacillus* spp. Isolates were identified by morphological, and biochemical tests, and confirmed by the VITEK system. The strain *B. subtilis* PCA 11.2-1 was identified in one of the 28 *Bacillus* spp. isolates. The strain showed resistance to the antibiotic Moxifloxacin (Kivanç et al., 2014).

The spectrum of pathogens and antibiotic susceptibility in post-traumatic endophthalmitis patients was investigated through a retrospective study of 912 patients. Among the culture proven microorganism identified, *Bacillus subtilis* was isolated from 31 cases (8.7%). *B. subtilis* showed susceptibility (100%) to ciprofloxacin, gentamicin, ofloxacin, cefuroxime, and ceftazidime ([redacted] et al. 2014).

With the aim of identifying the causative organisms of endophthalmitis using a procedure based on polymerase chain reaction, ocular specimens from patients with clinical diagnosis of post-operative endophthalmitis were collected. Bacteria were identified by 16S rRNA sequencing and sequence analysis. *B. subtilis* was identified in 4 cases ([redacted] et al. 2015), although the strains identified were not indicated.

B. subtilis together with *Corynebacterium* spp. were isolated from blood cultures from a dog suffering infective endocarditis of the aortic valve with patent ductus arteriosis. The dog responded to aggressive antibiotic therapy ([redacted] et al. 2015).

[redacted] et al. (2005) reported of an outbreak of food borne intoxication in a kindergarten. Twelve out of the 25 children exposed to breakfast had symptoms of food poisoning such as nausea, headache and vomiting, in the afternoon. The analysis of the food, staff involved in food preparation and contaminated children showed the presence of heat stable toxin producing *B. subtilis* and *B. licheniformis* in the power milk, as the source of contamination. Contamination of milk powder with toxin producing *B. licheniformis* and *B. subtilis* was proved by vacuolation assay and MTT cell culture test. The examination of the reconstituted milk in the lab showed that both species of *Bacillus* were able to enter log phase of growth within 2 hours of storage at room temperature. Thus it was concluded that the main error in milk powder handling was to prepare the milk and cocoa beverage 2 hours prior to consumption without boiling.

In a study conducted with the aim to find agents eliciting occupational asthma due to proven IgE-mediated sensitisation, Baur and Bakehe (2014) conducted a database search with MEDLINE via PubMed, screening reference lists of relevant reviews and matching the findings with a list of agents denoted as "may cause sensitisation by inhalation" by the phrase H334 (till 2011 R42). The quality of the selected studies was rated with the Scottish Intercollegiate Guideline Network (SIGN) grading system. The evidence level for each causative agent was graded using the modified Royal College of General Practitioners (RCGP) three-star system. A total of 865 relevant papers were identified, which covered 372 different causes of allergic work-related asthma. From these, a total of 327 cases per agent with moderate evidence level was found for various enzymes from *B. subtilis* (alcalase, protease, maxatase, maxapem, esperase, cellulase, α-amylase, lipase, subtilisin).

Schulte and Sennekamp (2010) reported a case of allergic alveolitis caused by *B. subtilis* in which the source of the allergen was identified to be a biological detergent at the working place.

Inomata et al. (2011) reported a clinical review of allergic reactions to natto, a tradictional food in Japan consisting in soybeans fermented by *B. subtilis* natto. Within 5 years, 7 patients were identified which had all experienced anaphylactic reactions within 5-14 hours, without early phase reactions, after ingestion of natto. This clinical review revealed that allergic reactions after ingestion of natto could mostly show a novel clinical course of IgE-mediated, late-onset anaphylaxis without early phase reactions.

Cited references (bibliographic data and abstracts):

Report: KIIM 5.2.4/02– [redacted] Y., [redacted] Y., [redacted] H., [redacted] H., [redacted] Y., [redacted], S.H. (2006) Proteases of a *Bacillus subtilis* clinical isolate facilitate swarming and siderophore-mediated iron uptake via proteolytic cleavage of transferrin, published report
Biol Pharm Bull, 29, 850-853.
M-519890-01-1

Abstract: A highly serine protease-producing Bacillus subtilis strain (PRY) was isolated from a clinical sample and identified it through biochemical testing and ribosomal DNA sequencing. The PRY strain exhibited a robust swarming behavior and was able to digest human transferrin efficiently, concomitantly with the production of catechol-siderophore in the exponential growth

phase. The growth of PRY was in proportion to increased iron availability resulting from transferrin destruction. These results suggest that proteases of the B. subtilis PRY strain may play a significant role in the pathogenesis of human infections by facilitating siderophore-mediated iron uptake from transferrin and swarming motility.

Report: KIIM 5.2.4/03 – [redacted], [redacted], [redacted], [redacted]., [redacted]., [redacted], S.L. (2009) Severe hepatotoxicity following ingestion of Herbalife nutritional supplements contaminated with *Bacillus subtilis*, published report
J Hepatol, 50, 111-117
M-520048-01-1

Abstract:
Background/AIMS: Nutritional supplements are widely used. Recently, liver injury after consumption of Herbalife preparations was reported but the underlying pathogenesis remained cryptic.

Methods: Two patients presented with cholestatic hepatitis and pruritus, and cirrhosis, respectively. Viral, alcoholic, metabolic, autoimmune, neoplastic, vascular liver diseases and synthetic drugs as the precipitating causes of liver injury were excluded. However, both patients reported long-term consumption of Herbalife products. All Herbalife products were tested for contamination with drugs, pesticides, heavy metals, and softeners, and examined for microbial contamination according to standard laboratory procedures. Bacteria isolated from the samples were identified as Bacillus subtilis by sequencing the 16S rRNA and gyrB genes.

Results: Causality between consumption of Herbalife products and disease according to CIOMS was scored "probable" in both cases. Histology showed cholestatic and lobular/portal hepatitis with cirrhosis in one patient, and biliary fibrosis with ductopenia in the other. No contamination with chemicals or heavy metals was detected, and immunological testing showed no drug hypersensitivity. However, samples of Herbalife products ingested by both patients showed growth of *Bacillus subtilis* of which culture supernatants showed dose- and time-dependent hepatotoxicity.

Conclusions: Two novel incidents of severe hepatic injury following intake of Herbalife products contaminated with *Bacillus subtilis* emphasize its potential hepatotoxicity.

Report: KIIM 5.2.4/04 – [redacted]., [redacted]J., [redacted]J., [redacted], [redacted]., [redacted], [redacted], J.T., [redacted], [redacted]., [redacted]C., [redacted] (2012) Combined *Bacillus licheniformis* and *Bacillus subtilis* infection in a patient with esophageal perforation, published report
J Med Microbiol, 61, 1766-1769
M-518668-01-1

Abstract: Species of the genus *Bacillus* are a common laboratory contaminant, therefore, isolation of these organisms from blood cultures does not always indicate infection. In fact, except for *Bacillus anthracis* and *Bacillus cereus*, most species of the genus *Bacillus* are not considered human pathogens, especially in immunocompetent individuals. Here, we report an unusual presentation of bacteraemia and mediastinitis due to co-infection with *Bacillus subtilis* and *Bacillus licheniformis*, which were identified by 16S RNA gene sequencing, in a patient with an oesophageal perforation.

Report: KIIM 5.2.4/05 – Kıvanç, S.A., Kıvanç, M., Güllülü, G. (2014) Automated ribotyping and antibiotic resistance determining *of Bacillus* spp from conjunctiva of diabetic patients, published report
Iran J Basic Med Sci, 17, 138-144.
M-530307-01-1

Abstract:
Objective(s): Characterization of the phenotype and genotype of *Bacillus* spp isolated from diabetic patients' eyes, by studying the drug sensitivity patterns with a disc-diffusion method.

Materials and Methods: Fifty eyes of 25 patients with type II diabetes mellitus, with at least 10

years of diabetes history, were included in the study. The eyes were analyzed for the presence of *Bacillus* spp.; presumptive isolates were identified by morphological, and biochemical tests, and confirmed by the VITEK system. Automated EcoRI ribotyping was performed with a RiboPrinter(®) Microbial Characterization System. The antibiotic resistance of the isolates was determined by the Kirby-Bauer disc diffusion test.

Results: Seven out of 25 patients were on insulin treatment; 7 on oral anti-diabetic medication; and 11 on combination therapy of insulin and oral medications. Among the 28 *Bacillus* spp isolates, 14 were *B. cereus*, 11 were *B. pumilus*, 2 were *B. mojavensis* and 1 was *B. subtilis*. Almost all the strains were either resistant or multiresistant, particularly towards cefuroxime, methicillin, and ceftazidime.

Conclusion: Diabetic patients seem to be more prone to *B. cereus* infections than healthy individuals. It would be greatly beneficial to understand and recognize the prevalence of microorganisms and their resistance patterns for better outcome in ocular surgeries.

Report: KIIM 5.2.4/06 – [redacted], [redacted], [redacted], [redacted], [redacted], [redacted] (2014)
Causative organisms of post-traumatic endophthalmitis: a 20-year retrospective study, published report
BMC Ophthalmology, 14, 1-7
M-530509-01-1

Abstract: Background: A wide range of organisms that enter the eye following ocular trauma can cause endophthalmitis. This study was to investigate the spectrum of pathogens and antibiotic susceptibility of bacterial isolates from a large cohort of post-traumatic endophthalmitis cases.
Methods: A retrospective study of 912 post-traumatic endophthalmitis patients treated at a tertiary eye-care center in China was performed. The associations between risk factors and the most common isolated organisms were investigated by Chi square Test. The percent susceptibilities for the first 10 years (1990–1999) and the second 10 years (2000–2009) were compared by Chi square test. $p < 0.05$ was considered statistically significant.

Results: Three-hundred-forty-seven (38.1%) cases of endophthalmitis were culture-positive, and 11 (3.2%) showed mixed infections (Gram-negative bacilli and fungi), yielding a total of 358 microbial pathogens. Culture proven organisms included 150 (41.9%) Gram-positive cocci, 104 (29.1%) Gram-negative bacilli, 44 (12.3%) Gram-positive bacilli, and 60 (16.8%) fungi. The coagulase-negative staphylococcal (CNS) species S. epidermidis (21.8%) and *S. saprophyticus* (12.0%) were the predominant pathogens, followed by *Bacillus subtilis* (8.7%), *Pseudomonas aeruginosa* (7.8%), and *Escherichia coli* (6.4%). Delayed repair over 24 h ($p < 0.001$) and metallic injury ($p < 0.01$) were significantly associated with positive culture of CNS. The most frequent fungal species were *Aspergillus* (26/60), followed by yeast-like fungi (18/60). *P. aeruginosa* was relatively sensitive to ciprofloxacin (83.3%), cefoperazone (75%), tobramycin (75%), cefuroxime (75%), and ceftazidime (75%) during the second decade. Multi-drug resistance was observed in the predominant Gram-negative bacteria.

Conclusion: Identification of a broad spectrum of microbes causing post-traumatic endophthalmitis, with Gram-positive cocci the most frequently identified causative organism, followed by *Bacillus* species, fungi, and mixed infections. CNS infection was statistically associated with delayed repair and metallic injury. Variation in antibiotic susceptibility was observed among isolated bacteria and between different periods. Ciprofloxacin and ceftazidime in the first and second decades of the study, respectively, showed the highest activity against bacterial post-traumatic endophthalmitis. For infections caused by *P. aeruginosa*, a combination therapy of ciprofloxacin, tobramycin, and one of the cephalosporins might provide optimal coverage according to data from the second decade.

Report: KIIM 5.2.4/07 – [redacted] (2015) PCR detection and identification of bacterial contaminants in ocular samples from post-operative endophthalmitis, published report
J Clin Diagn Res, 9, 1-3.
M-530032-01-1

Abstract: Background: Bacterial endophthalmitis is a sight-threatening complication of ocular surgery which requires urgent medical consideration including comprehensive diagnosis. Polymerase chain reaction (PCR) as a sensitive molecular method has been extensively used for detection of microbial species in clinical specimens.

AIM: The aim of this study was to identify the causative organisms of endophthalmitis in our patient population using a procedure based on PCR.

Materials and Methods: Vitreous samples from 32 patients with post-operative endophthalmitis were collected. Total vitreous DNA was extracted and then assessed by agarose gel electrophoresis. Bacterial 16S rRNA gene was amplified from genomic DNA using PCR with a pair of HAD2 universal primers. Library of PCR products from 16S rRNA, cloned into the pTZ57R/T vector. The ligated products were then transformed into E. coli DH5α strain and grown in the LB-ampicillin/X-Gal/IPTG plate.

Results: From the total of 32 vitreous samples, 18 specimens were positive, illustrating the presence of bacterial infection (56.4 %). Twelve species including *Escherichia coli*, *Enterobacter cloacae*, *Bacillus subtilis*, *Neisseria gonorrhoeae*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Chlamydia trachomatis*, *Staphylococcus aureus*, *Neisseria meningitides*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa* and *Bacillus cereus* were identified using BLAST for known 16S rRNA sequences.

Conclusion: Polymerase chain reaction (PCR) accompanied with cloning and sequencing approved to be sensitive and specific. The rapid molecular technique was useful in detection of 12 major microbial species, in infectious endophthalmitis.

Report: KIIM 5.2.4/08 – [redacted] (2015) Infective endocarditis of the aortic valve in a Border collie dog with patent ductus arteriosus, published report
J Vet Med Sci, 77, 331-336
M-530031-01-1

Abstract: Infective endocarditis (IE) in dogs with cardiac shunts has not been reported previously. However, we encountered a dog with concurrent patent ductus arteriosus (PDA) and IE. The dog was a 1-year-old, 13.9-kg female Border collie and presented with anorexia, weight loss, pyrexia (40.4 °C) and lameness. A continuous murmur with maximal intensity over the left heart base (Levine 5/6) was detected on auscultation. Echocardiography revealed a PDA and severe aortic stenosis (AS) caused by aortic-valve vegetative lesions. *Corynebacterium* spp. and *Bacillus subtilis* were isolated from blood cultures. The dog responded to aggressive antibiotic therapy, and the PDA was subsequently surgically corrected. After a series of treatments, the dog showed long-term improvement in clinical status

Report: KIIM 5.2.4/09 – [redacted] I., [redacted] (2005) An outbreak of food poisoning in a kindergarten caused by milk powder containing toxigenic *Bacillus subtilis* and *Bacillus licheniformis*, published report
Archiv für Lebensmittelhygiene, 56, 20-22
M-519210-01-1

Abstract: In February 2000 an outbreak of food borne intoxication was registered in a small kindergarten in the town of Split (Croatia). Out of 25 exposed children, 12 exhibited symptoms of nausea, headache and vomiting 5-8 hours after eating breakfast which consisted of sandwiches made of sliced bread and rolled ham together with milk and cocoa reconstituted from milk and cocoa powder. Food analysis regarding contamination with potentially toxigenic food borne

bacteria which was performed according to ISO Standards revealed no pathogenic microflora, including rotaviruses and adenoviruses. The same applies to the swabs of hands, noses, throats of the kitchen staff and from working surfaces and utensils from the kindergarten. Analysis of stool samples taken from staff as well as infected children also revealed no pathogenic microorganisms. The samples of milk and cocoa powder were examined in parallel using nonselective media. Contamination of milk powder with toxin producing *B. licheniformis* and *B. subtilis* was proved by vacuolation assay and MTT cell culture test. The examination of reconstituted milk performed in our lab showed that both species of *Bacillus* were able to enter the log phase of growth within 2 hours storage of the reconstituted milk at room temperature. Therefore, it was concluded that the main error in milk powder handling was to prepare the milk and cocoa beverage 2 hours prior to consumption without boiling instead of preparing it immediately before consumption taking care to boil the beverage. The application of the main HACCP principles is therefore recommended in order to avoid such incidents in small kindergartens.

Report: KIIM 5.2.4/10 – [redacted], [redacted] (2014) Allergens causing occupational asthma: an evidence-based evaluation of the literature, published report
Int Arch Occup Environ Health, 87, 339-633.
M-530019-01-1

Abstract: Purpose: The aim of this work is to provide an evidence-based evaluation and overview of causative substances in order to improve disease management.

Methods: A database search with MEDLINE via PubMed, screened reference lists of relevant reviews and matched our findings with a list of agents denoted as "may cause sensitisation by inhalation" by the phrase H334 (till 2011 R42). After exclusion of inappropriate publications, quality of the selected studies was rated with the Scottish Intercollegiate Guideline Network (SIGN) grading system. The evidence level for each causative agent was graded using the modified Royal College of General Practitioners (RCGP) three-star system.

Results: A total of 865 relevant papers were identified, which covered 372 different causes of allergic work-related asthma. The highest level achieved using the SIGN grading system was 2++ indicating a high-quality study with a very low risk of confounding or bias and a high probability of a causal relationship. According to the modified RCGP three-star grading system, the strongest evidence of association with an individual agent, profession or worksite ("***") was found to be the co-exposure to various laboratory animals. An association with moderate evidence level ("**") was obtained for α-amylase from *Aspergillus oryzae*, various enzymes from *Bacillus subtilis*, papain, bakery (flour, amylase, storage mites), western red cedar, latex, psyllium, farming (animals, cereal, hay, straw and storage mites), storage mites, rat, carmine, egg proteins, atlantic salmon, fishmeal, norway lobster, prawn, snow crab, seafood, trout and turbot, reactive dyes, toluene diisocyanates and platinum salts.

Conclusion: This work comprises the largest list of occupational agents and worksites causing allergic asthma. For the first time, these agents are assessed in an evidence-based manner. The identified respiratory allergic agents or worksites with at least moderate evidence for causing work-related asthma may help primary care physicians and occupational physicians in diagnostics and management of cases suffering from work-related asthma. Furthermore, this work may possibly provide a major contribution to prevention and may also initiate more detailed investigations for broadening and updating these evidence-based evaluations.

Report: KIIM 5.2.4/11 – [redacted], [redacted] (2010) Extrinsic alveolitis caused by *Bacillus subtilis* in a biological detergent – Diagnosis and follow up
Allergologie, 33, 570 – 572
M-520007-01-1

Abstract: A case report is presented herein with follow up about an allergic alveolitis due to *Bacillus subtilis*. As main source of the allergen a bio-logical detergent at the working place could be identified. An amplification by bacterial contamination of a room fontaine at home is very likely. A subclinical reactivation was detected by serial antibody titers and increases of C-reactive

protein and lactatdehydrogenase (LDH).

Report: KIIM 5.2.4/12 – [redacted] (2007) Late-onset anaphylaxis after ingestion of *Bacillus subtilis*-fermented soybeans (Natto): clinical review of 7 patients
Allergol Int, 56(3), 257-261
M-520045-01-1

Abstract: Background: Allergic reactions after ingestion of fermented soybeans have rarely been reported. Fermented soybeans were recently reported to be a causative food of IgE-mediated, late-onset anaphylaxis without early phase responses. The objectives of our study are to clarify the clinical and laboratory features and to characterize the allergens in allergy due to fermented soybeans.

Methods: Seven patients with suspected hypersensitivity to fermented soybeans, from whom informed consent had been obtained, underwent skin prick-prick tests with fermented soybeans and challenge test with fermented soybeans. Additionally, specific IgE against fermented soybeans and the allergens of fermented soybeans were detected by ELISA and IgE-immunoblotting, respectively.

Results: Seven male patients, aged 26 to 42 years (mean age, 33.1 years), participated. All patients reported generalized urticaria and dyspnea; 5, loss of consciousness; 2, collapse; 2, vomiting; and 2, diarrhea after fermented soybean ingestion. The interval between fermented soybean ingestion and onset of symptoms was 5 to 14 hours (mean, 9.6 hours). All patients were positive on skin prick-prick tests with fermented soybeans. In 2 patients, oral challenge with fermented soybeans was positive 5.5 and 13 hours after ingestion. In ELISA, all 5 patients tested showed elevated IgE levels to the fermented soybean extract. Furthermore, IgE-immunoblotting using 5 patients' sera showed six bands, of which three bands at 38, 28, and 26 kd were bound to sera from 4 patients.

Conclusions: Cases with hypersensitivity after ingestion of fermented soybeans most frequently correspond to IgE-mediated, late-onset anaphylactic reactions due to fermented soybeans.

Report: KIIM 5.2.4/13 [redacted], C. (2015) Literature review on effects on human health of Bacillus amyloliquefaciens QST 713 and its metabolites unpublished report, owner: Bayer CropScience AG.
Report-no. 6791109-A2-05-01
M-535690-01-1

For details on the literature research, please refer to ([redacted], 2015).

IIM 5.2.5 Proposed first aid measures and medical treatment

For the background information, please refer to the baseline dossier.

IIM 5.3 Basic studies

IIM 5.3.1 Sensitisation properties

For the background information, please refer to the baseline dossier.

According to COMMISSION REGULATION (EU) No 283/2013, the available methods for testing dermal sensitisation are not suitable for testing micro-organisms. Therefore, no study with *B. amyloliquefaciens* QST 713 is presented. However, the following labelling phrase is proposed: "Contains *Bacillus amyloliquefaciens* QST 713. Micro-organisms may have the potential to provoke sensitising reactions."

IIM 5.3.2 Acute oral infectivity, toxicity and pathogenicity

For the background information, please refer to the baseline dossier.

IIM 5.3.3 Acute intratracheal/inhalation infectivity, toxicity and pathogenicity

For the background information, please refer to the baseline dossier.

IIM 5.3.4 Acute intravenous/intraperitoneal infectivity

For the background information, please refer to the baseline dossier.

IIM 5.3.5 Genotoxic potential, especially for fungi and actinomycetes: a discussion of the potential for genotoxin production based on the relationship of the microorganism to a genus/species known to produce genotoxins. If a related fungus/ actinomycete produces a genotoxin, either an appropriate and sensitive analytical test (e.g. HPLC) must be done to detect its presence in the MPCA (for Canada), or genotoxicity testing is required (for EC).

For the background information, please refer to the baseline dossier.

As part of the literature research submitted with this dossier, which comprises searches in MEDLINE, BIOSIS, CAB and SCISEARCH databases, references regarding the production of toxins or metabolites of toxicological concern by *Bacillus amyloliquefaciens* or *B. subtilis* have been searched. One of the references retrieved reported the investigation of the genotoxic potential *in vitro* and *in vivo* of surfactin C from *B. subtilis* BC1212, the most prominent surfactant produced by strains of *B. amyloliquefaciens* or *B. subtilis*.

The genotoxicty of surfactin C was investigated *in vitro* in a reverse mutation assay and *in vivo* in the bone marrow micronucleus assay. The *in vitro* test was performed using 4 strains of *Salmonella typhimurium* including TA 98, TA 100, TA 1535, and TA 1537, and *Escherichia coli* WP2 uvrA/pkM 101 and doses ranging from 312.5 to 5000 µg surfactin C/plate, with a common ratio of 2 in at least two independent experiments using three plates per dose in the presence or absence of the S9 metabolic activation system. No mutagenic toxicity was observed at all doses tested. The positive controls induced significant increases in the mutant frequencies, verifying the sensitivity of the strains used ($p < 0.05$). The numbers of revertants caused by exposure to surfactin C were close to those of negative control. Surfactin C had no mutagenic effect both in the presence and absence of metabolic activation *in vitro*. To conduct the bone marrow micronucleus assay, 8-week-old male ICR mice were administered distilled water (negative control) or surfactin C at 2000, 3000 or 4000 mg/kg body weight, given by gavage in twice daily. Mice in the positive control group received cyclophosphamide 40 mg/kg in distilled water through single intraperitonial injection. Five animals each from the vehicle control, positive control, and three surfactin C-treated groups were sacrificed by cervical dislocation 24 hr after dosing. Bone marrow smears from the treated animals were stained in 5% (v/v) Giemsa solution and observed for the frequency of cells with micronuclei using light microscopy. The incidence of micronucleated cells (MNPCEs) per 2000 polychromatic erythrocytes (PCEs) per animal was measured. The proportion of polychromatic erythrocytes was assessed by examination of a total of 200 erythrocytes per animal. No significant increase in the incidence of PCEs in the surfactin C treated groups, compared with that of negative control, was observed. Surfactin C did not cause increases of MNPCE, whereas cyclophosphamide significantly increased MNPCE ($p < 0.05$). Taken together, these findings suggest that surfactin C has no genotoxic potential *in vitro* or *in vivo* ([redacted] et al. 2008).

Cited references (bibliographic data and abstracts):

Report: KIIM 5.3.5/01 – [redacted], Y-H., [redacted], B-K., [redacted], J-H., [redacted], M-S., [redacted], I-B., [redacted], S-C., [redacted], H-I. (2008) Evaluation of genetic and developmental toxicity of surfactin C from *Bacillus subtilis* BC1212, published report
Journal of Health Science, 54, 101-106.
M-520043-01-1

Abstract: Surfactin C is a biosurfactant produced by *Bacillus subtilis* from Korean soybean paste.

Surfactin C is known to have several therapeutic effects including anti-inflammatory, fibrinolytic, and thrombolytic activities. However, there is little information concerning its safety. In this study, we evaluated the genetic and developmental toxicity of surfactin C. Bacterial reverse mutation and rodent micronucleus assays were performed to determine its genotoxic potentials. Surfactin C at 0, 125, 250, and 500 mg/kg of body weight/day was administered orally to pregnant ICR mice during the period of major organogenesis. There was no genetic toxicity related to surfactin C treatment in *in vitro* and *in vivo* systems. In the developmental study, surfactin C did not demonstrate maternal toxicity, fetotoxicity, and teratogenicity, and hence the no observed5.4.8 effect level was concluded 500 mg/kg per day in ICR mice.

Report: KIIM 5.3.5/02 - [redacted] (2015) Literature review on effects on human health of Bacillus amyloliquefaciens QST 713 and its metabolites unpublished report, owner: Bayer CropScience AG.
Report-no. 6791109-A2-05-01
M-535690-01-1

For details on the literature research, please refer to ([redacted] 2015).

IIM 5.3.6 Cell culture study, for viruses and viroids or specific bacteria and protozoa with intracellular replication

For the background information, please refer to the baseline dossier.

IIM 5.3.7 Short-term toxicity (including inhalatory short-term toxicity), pathogenicity, infectivity

For the background information, please refer to the baseline dossier.

IIM 5.3.7.1 Short-term toxicity, pathogenicity, infectivity (28-day minimum)

For the background information, please refer to the baseline dossier.

IIM 5.3.7.2 Inhalatory short-term toxicity

For the background information, please refer to the baseline dossier.

IIM 5.4 Toxicity studies on metabolites (especially toxins)

For the background information, please refer to the baseline dossier.

As part of the literature research submitted with this dossier, which comprised searches in MEDLINE, BIOSIS, CAB and SCISEARCH databases, references regarding the production of toxins or metabolites of toxicological concern by *B. amyloliquefaciens or B. subtilis* have been searched.

The references found, considered of relevance, mainly report on toxicity studies with amylosin produced by *B. amyloliquefaciens* strains isolated from moisture-damaged buildings or surfactin isolated from *B. subtilis* natto.

The genetic and developmental toxicity of surfactin C from *B. subtilis* BC1212 have been reported by Hwang et al., (2008). In this study, the genotoxicty of surfactin C was investigated *in vitro* in a reverse mutation assay and *in vivo* in the bone marrow micronucleus assay. The *in vitro* test was performed using 4 strains of *Salmonella typhimurium* including TA 98, TA 100, TA 1535, and TA 1537, and *Escherichia coli* WP2 uvrA/pkM 101 and doses ranging from 312.5 to 5000 µg surfactin C/plate, with a common ratio of 2 in at least two independent experiments using three plates per dose in the presence or absence of the S9 metabolic activation system. No mutagenic toxicity was observed at all doses tested. The positive controls induced significant increases in the mutant frequencies, verifying the sensitivity of the strains used ($p < 0.05$). The numbers of revertants caused

by exposure to surfactin C were close to those of negative control. Surfactin C had no mutagenic effect both in the presence and absence of metabolic activation *in vitro*. To conduct the bone marrow micronucleus assay, 8-week-old male ICR mice were administered distilled water (negative control) or surfactin C at 2000, 3000 or 4000 mg/kg body weight, given by gavage in twice daily. Mice in the positive control group received cyclophosphamide 40 mg/kg in distilled water through single intraperitonial injection. Five animals each from the vehicle control, positive control, and three surfactin C-treated groups were sacrificed by cervical dislocation 24 hr after dosing. Bone marrow smears from the treated animals were stained in 5% (v/v) Giemsa solution and observed for the frequency of cells with micronuclei using light microscopy. The incidence of micronucleated cells (MNPCEs) per 2000 polychromatic erythrocytes (PCEs) per animal was measured. The proportion of polychromatic erythrocytes was assessed by examination of a total of 200 erythrocytes per animal. No significant increase in the incidence of PCEs in the surfactin C treated groups, compared with that of negative control, was observed. Surfactin C did not cause increases of MNPCE, whereas cyclophosphamide significantly increased MNPCE ($p < 0.05$). Taken together, these findings suggest that surfactin C has no genotoxic potential *in vitro* or *in vivo*.

To evaluate the developmental toxicity of surfactin C, groups of inseminated mice (3 treatment groups of 14-15 animals each) were given surfactin C by gavage daily over gestational day (GD) 6 through 17 at the doses of 0 (control), 125, 250, and 500 mg/kg per day. The control received deionized water at 10 ml/kg per day. Animals were observed for their daily signs of toxicity throughout the experimental period. Body weight and feed consumption were recorded. On GD 18, tested animals were sacrificed by carbon dioxide. Maternal necropsy was performed and their organ weights were measured. Uteri of tested animals were exposed and determined for the presence and position of resorption sites, survival of fetuses (dead or alive), and the number of implantation sites. The live fetuses were weighed and examined for external and visceral malformations. There were no deaths or abortions during the experimental period. No clinical signs in maternal animals were observed. No significant difference in maternal body weight was observed among the dose groups. Feed and water consumption among the experimental groups was not statistically different. There were no significant alterations in the relative organ weights.
Surfactin C treatment showed no differences in death, early and late resorptions, sex ratio, and body weight of fetuses among the treatment groups. There were no observed external and skeletal abnormalities of fetuses. Based on these results, the no observed effects level of surfactin C is suggested as the highest dose tested of 500 mg/kg per day. Taken together the results from this study, the authors concluded that surfactin C from *Bacillus subtilis* BC1212 showed no genetic and developmental toxicity in *in vitro* and *in vivo* systems (Hwang et al., 2008).

The subchronic (28 day) oral toxicity of surfactin C has been studied in Sprague-Dawley rats. Surfactin C was administered to female and males rats (3 groups of 15 animals/sex) at doses of 500, 1000 or 2000 mg/kg bw by intra-gastric gavage during 28 days. No treatment-related mortality was observed at any dose tested. No clinical signs of toxicity, alterations in body weight, body weight gain and food consumption, alterations on the hemathological parameters, differences in organ weights compared with the control group, or histopathological findings were noted at the lowest dose tested (500 mg/kg bw). The NOAEL was set on 500 mg/kg bw based on decreased bw, increased levels in serum of alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase, and increased liver weight and hydrophic necrosis on hepathocyte at the highest doses tested (Hwang et al., 2009).

In a study conducted with the aim to get information on surfactin levels in actual human foods, the potential to produce lipopeptides and the presence of lipopeptides in final products was assessed using three different intact commercial samples of the Japanese traditional bean product natto, which is prepared from steamed soybeans using starters based on specific *bacilli*. Bacteria isolated from all natto samples were identified as *B. subtilis* by 16S rDNA sequencing and they all were β-hemolytic and gave a positive signal in the polymerase chain reaction screen for genes associated with surfactin production. Their culture extracts were cytotoxic to boar sperm cells. Organic extracts of both *Bacillus* cultures and the natto samples, were analyzed for their surfactin content using ultrahigh-performance liquid chromatography with high-resolution mass spectrometry. All the strains proved to be surfactin producers (15 to 39 µg/ml culture medium) and the natto samples contained as much as 2.2 mg/g of surfactins. This means that consumers can ingest at least approximately 80 to 100 mg of surfactins per single 50-g natto serving apparently without suffering any ill effects, which indicates a very low human toxicity. These results also suggest a lack of correlation between the *in vitro* cytotoxicty of *Bacillus*-associated lipopeptides and their tolerance *in*

vivo, and confirm the low oral toxicity of these compounds as revealed in rodent feeding trials ([redacted] et al. 2014).

[redacted] et al. (2004) reported the isolation of strains of *B. amyloliquefaciens* from moisture-damaged buildings, which produce a cation- specific forming channels heat-stable toxin of 1197 Da and surfactin. Both toxins inhibited motility of boar spermatozoa within 15 min of exposure and killed feline lung cells in high dilution in 1 day. In boar sperm and human neural cells (Paju), the 1197 Da toxin depolarized the transmembrane potentials of mitochondria and the plasma membrane after a 20-min exposure and formed cation selective channels in lipid membranes. According to the authors, the *in vitro* observed simultaneous collapse of both cytosolic and mitochondrial ATP in the affected mammalian cell, induced by the 1197-Da cation channel, suggests potential health risks for occupants of buildings contaminated with such toxins.

In a subsequent paper, Mikkola et al. (2007) identified the 1197 toxin produced by strains of *B. amyloliquefaciens* from moisture-damaged buildings as amylosin. Purified amylosin inhibited motility of boar sperm cells at an exposure concentration of 135 nM and hyperpolarized their cell membrane and depolarized their mitochondria at exposure to concentration of 33-67 nM for 10 min. Amylosin was cytotoxic to feline lung cells at concentrations of <170 nM. Purified amylosin provoked ATP-independent cation influx into isolated rat liver mitochondria (RLM), inducing swelling of the mitochondria at concentrations of 200 nM K(+) or >250 nM Na(+) medium. In the K(+)- or Na(+)-containing medium, amylosin uncoupled RLM, causing oxidation of pyridine nucleotides, loss of the mitochondrial membrane potential, and suppressed ATP synthesis. Purified amylosin produced cation channels in black-lipid membranes (BLMs) with a selectivity K(+)>Na(+) at a concentration of 26 nM, i.e. the same concentration at which amylosin was toxic to boar sperm cells. The amylosin cation channels were cholesterol- and ATP-independent and more effective with K(+) than with Na(+). The authors proposed that the toxicity of amylosin may be due its ionophoric properties, representing the first K(+)/Na(+) channel-forming substance reported from *B. amyloliquefaciens.*

The heat-stable toxin amylosin was also identified in six isolates originating from two food poisoning outbreaks through screening of *Bacillus* sp, other than *B. cereus*, associated with food borne and using the boar sperm motility inhibition assay. The toxic isolates were identified as *B. subtilis* and *B. mojavensis*. The extract of *B. subtilis* F 2564/96 depolarized the mitochondria in intact colon carcinoma cells, used to model the contact with the human gigestive tract, similarly as in sperm cells. Amylosin was identified as the substance responsible for these effects. It was suggested that amylosin could play a role as a virulence factor in foodborne *Bacillus* ([redacted]-Constantin et al., 2009).

In a recent publication ([redacted] et al. 2015), the *in vitro* effects of amylosin produced by strains of *B. amyloliquefaciens* isolated from moisture-damaged buildings were assessed in human macrophagues, peripheral blood mononuclear cells (PBMC), human keratinocytes (HaCaT) and porcine spermatozoa. Amylosin was shown to stimulate the secretion of cytokines IL-1β and IL-18 in human macrophages, to cause dose-dependent potassium efflux from PBMC, HaCaT and porcine spermatozoa cells, and to depolarize human and porcine membrane potentials and to impair motility of porcine spermatozoa. In addition, amylosin-producing *B. amyloliquefaciens* strains and amylosin inhibited microbial growth. According to the authors, results from this study indicate that exposure to amylosin activates the innate immune system which could be related with the inflammatory symptoms experienced by occupants of moisture-damaged buildings.

As part of the investigation on the effects of Poly-γ-glutamic acid (γ-PGA), produced by *Bacillus subtilis* (chungkookjang) on corneal wound healing, the eye irritation potential of γ-PGA was assessed in New Zealand White rabbits. The study is indicated to have been conducted according to OECD guideline 405. No eye irritation of the cornea, iris or conjunctivae was observed in any of the 3 animals at 1, 24, 48, 72 or 96 h after administration of 0.1 mL of γ-PGA solution. The authors concluded that γ-PGA did not induce eye irritation in rabbits ([redacted] et al. 2010).

In a study to test the effect of γ-PGA (produced by *B. subtilis* natto ATCC 15245) on the viability of probiotic bacteria during freeze drying, the toxigenic potential of *B. subtilis* natto was assessed. The presence of six genes that are known to encode four toxins, including three component hemolysin (hbl D/A), three component non-haemolytic enterotoxin (nheB), *B. cereus* enterotoxin T (bceT), enterotoxin FM (entFM), sphingomyelinase (sph) and phosphatidylcholine-specific phospholipase

(piplc), were investigated in *B. subtilis* natto by polymerase chain reaction. None of these six genes were present in *B. subtilis* natto. Haemolytic and lecithinase activities *in vitro* were found to be absent ([redacted] et al. 2013).

[redacted] et al. (2012) investigated the thrombolityc effects of Douchi fibrinolytic enzyme (DFE) from *B. subtilis* LD-8547 *in vitro* and *in vivo*. As part of this study, the acute oral toxicity of DFE was assessed in Kunming female and male mice. Groups of 5 male and 5 female mice were given 691.586 U DFE/kg by the oral route. Animals were observed for clinical signs of toxicity and mortality during 2 weeks following treatment. According to the authors, DFE showed no obvious acute toxicity to mice. Morphology of viscera from treated mice was nearly the same as the control. There were no abnormal changes of the pathologic section in the hearts, livers, spleens, lungs, kidneys, stomachs and intestines of all mice. No obvious differences were found in the body weight and viscera (P >0.05) ([redacted] et al. 2012). The study was not conducted according to guidelines.

Fifty-four spore-forming bacterial strains isolated from bread ingredients and bread, mainly belonging to the genus *Bacillus* (including strains of *B. cereus*, *B. amyloliquefaciens* and *B. subtilis*), together with 11 reference strains were investigated to evaluate their cytotoxic potential and heat survival in order to ascertain if they could represent a risk for consumer health. Screening test of cytotoxic activity on human intestinal HT-29 cells using bacterial culture filtrates were conducted. Moreover, immunoassays and polymerase chain reaction (PCR) analyses, specifically targeting already known toxins and related genes of *B. cereus*, as well as a heat spore inactivation assay were carried out. Cytotoxic activity was detected in 8 strains of *B. amyloliquefaciens* (N20.3, S68, S70, S80.2, S85.2, S77.1, S109.3, ATCC 8473) and in one strain of *B. subtilis* (DSM10) although it was low or very low in comparison with strains of *B. cereus*. Genes responsible for cereulide production were not detected in any of the tested strains. Production of NHE and HBL toxins was confirmed by specific immunoassays only for *B. cereus* strains, even if PCR analyses revealed the presence of related toxin genes also in some strains of *B. amyloliquefaciens* or *B. subtilis*. Viable spore count was ascertained after a heat treatment simulating the bread cooking process. Results indicated that *B. amyloliquefaciens* strains almost completely survived the heat treatment showing less than 2 log-cycle reductions similarly to two strains of *B. cereus* group III and single strains belonging to *B. subtilis*, *B. mojavensis* and *Paenibacillus* spp. It was concluded that spore-forming bacteria contaminating bread ingredients and bread could represent a risk for consumer health if specific characteristics of the strains coexist: specifically the ability to produce toxic substances and a thermal resistance enough to survive the bread cooking conditions. In this regard, some strains of the *B cereus* group III are source of concern and single strains of other species should be also considered ([redacted] et al. 2015).

[redacted] et al. (2014) reported the analysis of the production of the emetic toxin cereulide in 30 *Bacillus* strains (including *B. subtilis* and *B. amyloliquefaciens*) by using the cellular cytotoxicity MTT assay, polymerase chain reaction (PCR), and micellar electrokinetic chromatographycapillary electrophoresis (MEKC-CE) analysis. MEKC-CE results showed that some strains of *Bacillus* species other than *B. cereus*, including *B. subtilis*, produced putative cereulide at levels similar to those produced by emetic *B. cereus*. *B. amyloliquefaciens* strains produced putative cereulide, but at lower levels. However, only *B. cereus* emetic strains were found to be toxic to bovine fibroblast cells in the MTT assays and resulted positive in the analysis of the emetic toxin gene by PCR.

None of these publications reported on toxicity data of metabolites produced by the strain QST 713 or by any other strain used as a biopesticide.

Cited references (bibliographic data and abstracts):

Report: KIIM 5.4/05 – [redacted], Y-H., [redacted], B-K., [redacted], J-H., [redacted], M-S., [redacted], I-B., [redacted], S-[redacted]. (2008) Evaluation of genetic and developmental toxicity of surfactin C from *Bacillus subtilis* BC1212, published report
Journal of Health Science, 54, 101-106.
M-520043-01-1

Abstract: Surfactin C is a biosurfactant produced by *Bacillus subtilis* from Korean soybean paste. Surfactin C is known to have several therapeutic effects including anti-inflammatory, fibrinolytic,

and thrombolytic activities. However, there is little information concerning its safety. In this study, we evaluated the genetic and developmental toxicity of surfactin C. Bacterial reverse mutation and rodent micronucleus assays were performed to determine its genotoxic potentials. Surfactin C at 0, 125, 250, and 500 mg/kg of body weight/day was administered orally to pregnant ICR mice during the period of major organogenesis. There was no genetic toxicity related to surfactin C treatment in *in vitro* and *in vivo* systems. In the developmental study, surfactin C did not demonstrate maternal toxicity, fetotoxicity, and teratogenicity, and hence the no observed effect level was concluded 500 mg/kg per day in ICR mice.

Report: KIIM 5.4/06– [redacted], Y-H., [redacted], M-S., [redacted], I-B., [redacted], B-K., [redacted], J-H., [redacted], S-[redacted]. (2009) Subacute (28 day) toxicity of surfactin C, a lipopeptide produced by *Bacillus subtilis*, in rats, published report
Journal of Health Science, 55, 351-355.
M-519895-01-1

Abstract: Surfactin C, produced by *Bacillus subtilis* isolated from Korean soybean paste, was given to Sprague-Dawley rats of both sexes at dose of 500, 1000 or 2000 mg/kg for 28 days. There were no surfactin C-related toxicities in survival, clinical signs, and haematological parameters in the experimental period. The highest dose of surfactin C showed the decrease in body weight gain despite normal food and water consumptions and the increase in relative liver weight. Alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) levels were increased in animals administered with surfactin C of 1000 or 2000 mg/kg. Zonal necrosis of hepatocyte around the hepatic vein was observed after administration of the same doses in a dose-dependent manner. In the present study, the no-observed-adverse-effect level (NOAEL) of surfactin C was 500 mg/kg following oral administration in rats.

Report: KIIM 5.4/07– [redacted], [redacted], [redacted], K.P., [redacted] (2014) Surfactins in natto: the surfactin production capacity of the starter strains and the actual surfactin contents in the products, published report
J Food Prot, 77, 2139-2143
M-530101-01-1

Abstract: Surfactin-type lipopeptides are suspected of being implicated in the rare food poisonings caused by Bacillus species outside the *Bacillus cereus* cluster. In order to get information on surfactin levels in actual human foods, bacilli from three commercial samples of a Japanese traditional bean product, natto, were isolated in order to clarify their potential to produce the suspect lipopeptides. The isolated bacilli were characterized as *Bacillus subtilis*. They were β-hemolytic and gave a positive signal in the PCR screen for genes associated with surfactin production, and their culture extracts were cytotoxic to boar sperm cells. Organic extracts of both Bacillus cultures and the natto samples were analyzed for their surfactin content using ultrahigh-performance liquid chromatography with high-resolution mass spectrometry. All the strains proved to be surfactin producers (15 to 39 µg/mL culture medium); the natto samples contained as much as 2.2 mg g^{-1} of surfactins. This means that consumers can ingest at least approximately 80 to 100 mg of surfactins per single 50-g natto serving apparently without suffering any ill effects, indicating a very low human toxicity.

Report: KIIM 5.4/08 – [redacted], [redacted], M.A., [redacted], [redacted]V., [redacted], N.E., [redacted], F.A., [redacted]S (2004) *Bacillus amyloliquefaciens* strains isolated from moisture-damaged buildings produced surfactin and a substance toxic to mammalian cells, published report
Arch Microbiol, 181, 314-323
M-518859-01-1

Abstract: Fungicidic *Bacillus amyloliquefaciens* strains isolated from the indoor environment of moisture-damaged buildings contained heat-stable, methanol-soluble substances that inhibited motility of boar spermatozoa within 15 min of exposure and killed feline lung cells in high dilution in 1 day. Boar sperm cells lost motility, cellular ATP, and NADH upon contact to the bacterial extract (0.2 microg dry wt/mL). Two bioactive substances were purified from biomass of the fungicidal isolates. One partially characterized substance, 1,197 Da, was moderately hydrophobic and contained leucine, proline, serine, aspartic acid, glutamic acid and tyrosine, in addition to chromophore(s) absorbing at 365 nm. In boar sperm and human neural cells (Paju), the compound depolarized the transmembrane potentials of mitochondria (Delta Psi(m)) and the plasma membrane (Delta Psi(p)) after a 20-min exposure and formed cation-selective channels in lipid membranes, with a selectivity K(+):Na(+):Ca(2+) of 26:15:3.5. The other substance was identified as a plasma-membrane-damaging lipopeptide surfactin. Plate-grown biomass of indoor *Bacillus amyloliquefaciens* contained ca. 7% of dry weight of the two substances, 1,197 Da and surfactin, in a ratio of 1:6 (w:w). The in vitro observed simultaneous collapse of both cytosolic and mitochondrial ATP in the affected mammalian cell, induced by the 1,197-Da cation channel, suggests potential health risks for occupants of buildings contaminated with such toxins.

Report: KIIM 5.4/09 – [redacted], [redacted], M.A., [redacted], [redacted], [redacted], [redacted], [redacted], [redacted], [redacted] N.E., [redacted], [redacted] S. (2007) Amylosin from *Bacillus amyloliquefaciens*, a K^+ and Na^+ channel-forming toxic peptide containing a polyene structure, published report
Toxicon, 49, 1158-1171
M-518861-01-1

Abstract: *Bacillus amyloliquefaciens* strains isolated from the indoor environment of moisture-damaged buildings produce a 1197 Da toxin, named amylosin. Nuclear magnetic resonance (NMR) data showed that amylosin contains a chromophoric polyene structure and the amino acids leucine/isoleucine, proline, aspartic acid/asparagine, glutamic acid/glutamine and tyrosine. A quantitation method for amylosin was developed using commercially available amphotericin B as a reference compound and a known concentration of amylosin determined by NMR with the electronic reference to access in vivo concentration (ERETIC) method. Purified amylosin inhibited motility of boar sperm cells at an exposure concentration of 135 nM and hyperpolarized their cell membrane and depolarized their mitochondria at exposure to concentration of 33-67 nM for 10 min. In a 3-d exposure time only 27 nM of amylosin was needed to provoke the same toxicity functions. Amylosin was cytotoxic to feline lung cells at concentrations of <170 nM. Purified amylosin provoked adenosine 5'-triphosphate (ATP)-independent cation influx into isolated rat liver mitochondria (RLM), inducing swelling of the mitochondria at concentrations of 200 nM K(+) or >250 nM Na(+) medium. In the K(+)- or Na(+)-containing medium, amylosin uncoupled RLM, causing oxidation of pyridine nucleotides (PN), loss of the mitochondrial membrane potential, and suppressed ATP synthesis. Purified amylosin produced cation channels in black-lipid membranes (BLMs) with a selectivity K(+)>Na(+) at a concentration of 26 nM, i.e. the same concentration at which amylosin was toxic to boar sperm cells. The amylosin cation channels were cholesterol- and ATP-independent and more effective with K(+) than with Na(+). We propose that the toxicity of amylosin may be due its ionophoric properties, representing the first K(+)/Na(+) channel-forming substance reported from *B. amyloliquefaciens*.

Report: KIIM 5.4/010 – [redacted], [redacted], [redacted], M.A., [redacted], [redacted], [redacted], [redacted] (2009) *Bacillus subtilis* and *B. mojavensis* strains connected to food poisoning produce the heat stable toxin amylosin, published report
J Appl Microbiol 106, 1976-1985
M-518663-01-1

Abstract:
AIM: To screen and characterize toxic, heat-stable substances produced by food borne strains from Bacillus subtilis group.
Methods and Results: Using the boar sperm motility inhibition assay, six isolates from two outbreaks, out of the 94 isolates from 26 foods, were found to produce ethanol-soluble heat-stable substances that were toxic to sperm cells by depleting the mitochondrial membrane potentials. The toxic isolates were identified as *Bacillus subtilis* and *B. mojavensis*. Colon carcinoma cells (Caco-2) were used to model the contact with the human digestive tract. The extract of B. subtilis F 2564/96 depolarized the mitochondria in intact Caco-2 cells similarly as in sperm cells. The substance responsible for these effects was purified using HPLC and identified by electron spray ionization ion trap mass spectrometry analysis as amylosin. The temperature requirement for amylosin production was 21-37 degrees C for *B. subtilis* and 11-21 degrees C for *B. mojavensis*. Both species produced amylosin in air as well as in 7-8% CO_2 with 8-9% O_2.
Conclusions: Food borne illness related strains of *B. subtilis* and *B. mojavensis*, produced the heat-stable toxin amylosin.

SIGNIFICANCE AND IMPACT OF THE STUDY:
This is the first report that suggests a role for the heat-stable, ion-channel forming toxin amylosin, as a virulence factor in food borne *Bacillus*.

Report: KIIM 5.4/11 – [redacted] S, [redacted] VV, [redacted] MA, [redacted] R, [redacted] P, [redacted] S, [redacted] CG, [redacted] LC, [redacted] M. (2015) The peptide toxin amylosin of *Bacillus amyloliquefaciens* from moisture-damaged buildings is immunotoxic, induces potassium efflux from mammalian cells, and has antimicrobial activity., published report
Appl Environ Microbiol 81, 2939-2949
M-530348-01-1

Abstract: Amylosin, a heat-stable channel-forming non-ribosomally synthesized peptide toxin produced by strains of *Bacillus amyloliquefaciens* isolated from moisture-damaged buildings, is shown in this paper to have immunotoxic and cytotoxic effects on human cells as well as antagonistic effects on microbes. Human macrophages exposed to 50 ng of amylosin ml(-1) secreted high levels of cytokines interleukin-1β (IL-1β) and IL-18 within 2 h, indicating activation of the NLRP3 inflammasome, an integral part of the innate immune system. At the same exposure level, expression of IL-1β and IL-18 mRNA increased. Amylosin caused dose-dependent potassium ion efflux from all tested mammalian cells (human monocytes and keratinocytes and porcine sperm cells) at 1 to 2 μM exposure. Amylosin also inhibited the motility of porcine sperm cells and depolarized the mitochondria of human keratinocytes. Amylosin may thus trigger the activation of the NLRP3 inflammasome and subsequently cytokine release by causing potassium efflux from exposed cells. The results of this study indicate that exposure to amylosin activates the innate immune system, which could offer an explanation for the inflammatory symptoms experienced by occupants of moisture-damaged buildings. In addition, the amylosin-producing *B. amyloliquefaciens* inhibited the growth of both prokaryotic and eukaryotic indoor microbes, and purified amylosin also had an antimicrobial effect. These antimicrobial effects could make amylosin producers dominant and therefore significant causal agents of health problems in some moisture-damaged sites.

Report: KIIM 5.4/12 – [redacted], S.R., [redacted], [redacted], J.C., [redacted], [redacted], C.J., [redacted], M.H. (2010) Effects of ultra high molecular weight poly-gamma-glutamic acid from *Bacillus subtilis* (chungkookjang) on corneal wound healing, published report
J Microbiol Biotechnol, 20, 803-808.
M-520052-01-1

Abstract: Poly-gamma-glutamic acid (gamma-PGA) is a natural, edible polypeptide in which glutamate is polymerized via gamma-amide linkages. First, the eye irritancy potential of gamma PGA in rabbits was assessed. Additionally, we studied the effects of gamma-PGA on corneal wound healing, due to the anti-inflammatory properties and water retaining abilities of gamma-PGA. In this study, the effects of gamma-PGA on corneal wound healing after an alkali burn were evaluated. Thirty eyes wounded by alkali burning in 30 white rabbits were divided into three

groups: group A was treated with 0.1% 5000 kDa gamma-PGA for 2 days, group B was treated with 0.1% hyaluronic acid, and group C was not treated, as a control. The area of corneal epithelial defect was examined at 12, 24, 30, 36, 42, and 48 h after corneal alkali wounding to determine initial wound healing. It was determined that gamma-PGA promoted corneal wound healing, compared with controls, and showed similar effects to hyaluronic acid. These results indicate that gamma-PGA stimulates corneal wound healing by an anti-inflammatory effect and enhancing cell migration and cell proliferation. gamma-PGA is a promising biomaterial that may be a substitute for hyaluronic acid in corneal wound healing treatment.

Report: KIIM 5.4/13 – [redacted], A.R., [redacted], V.U., [redacted], [redacted], [redacted], [redacted], M.R., [redacted], [redacted] (2013) *Bacillus subtilis* natto: a non-toxic source of poly-γ-glutamic acid that could be used as a cryoprotectant for probiotic bacteria, published report
AMB Express, 3(1), 36-44.
M-518931-01-1

Abstract: It is common practice to freeze dry probiotic bacteria to improve their shelf life. However, the freeze drying process itself can be detrimental to their viability. The viability of probiotics could be maintained if they are administered within a microbially produced biodegradable polymer - poly-γ-glutamic acid (γ-PGA) - matrix. Although the antifreeze activity of γ-PGA is well known, it has not been used for maintaining the viability of probiotic bacteria during freeze drying. The aim of this study was to test the effect of γ-PGA (produced by *B. subtilis* natto ATCC 15245) on the viability of probiotic bacteria during freeze drying and to test the toxigenic potential of B. subtilis natto. 10% γ-PGA was found to protect *Lactobacillus paracasei* significantly better than 10% sucrose, whereas it showed comparable cryoprotectant activity to sucrose when it was used to protect *Bifidobacterium breve* and *Bifidobacterium longum*. Although γ-PGA is known to be non-toxic, it is crucial to ascertain the toxigenic potential of its source, *B. subtilis* natto. Presence of six genes that are known to encode for toxins were investigated: three component hemolysin (hbl D/A), three component non-haemolytic enterotoxin (nheB), *B. cereus* enterotoxin T (bceT), enterotoxin FM (entFM), sphingomyelinase (sph) and phosphatidylcholine-specific phospholipase (piplc). From our investigations, none of these six genes were present in *B. subtilis* natto. Moreover, haemolytic and lecithinase activities were found to be absent. This work contributes a biodegradable polymer from a non-toxic source for the cryoprotection of probiotic bacteria, thus improving their survival during the manufacturing process.

Report: KIIM 5.4/14 – [redacted], [redacted], [redacted], [redacted], [redacted], [redacted] (2012) Thrombolytic effects of Douchi fibrinolytic enzyme from *Bacillus subtilis* LD-8547 *in vitro* and *in vivo*, published report
BMC Biotechnol, 12: 36-44
M-520056-01-1

Abstract:
Background: Today, thrombosis is one of the most widely occurring diseases in modern life. Drugs with thrombolytic functions are the most effective methods in the treatment of thrombosis. Among them, Douchi fibrinolytic enzyme (DFE) is a promising agent. DFE was isolated from Douchi, a typical and popular soybean-fermented food in China, and it can dissolve fibrin directly and efficiently. A strain, *Bacillus subtilis* LD-8547 produced DFE with high fibrinolytic activity has been isolated in our lab previously.

Results: In the study, thrombolytic effect of DFE from *Bacillus subtilis* LD-8547 was studied *in vitro* and *in vivo* systematically. The results showed that DFE played a significant role in thrombolysis and anticoagulation *in vitro*. And the thrombolytic effects correlated with DFE in a dose-dependent manner. *In vivo*, the acute toxicity assay showed that DFE had no obvious acute toxicity to mice. Test of carrageenan-induced thrombosis in mice indicated that the DFE significantly prevented tail thrombosis, and arterial thrombosis model test indicated that Douchi fibrinolytic enzyme DFE had thrombolytic effect on carotid thrombosis of rabbits in vivo. Other results in vivo indicated that DFE could increase bleeding and clotting time obviously.

Conclusions: The DFE isolated from *Bacillus subtilis* LD-8547 has obvious thrombolytic effects *in vitro* and *in vivo*. This function demonstrates that this enzyme can be a useful tool for preventing

and treating clinical thrombus.

Report: KIIM 5.4/15 – [redacted], [redacted], [redacted], [redacted], [redacted], [redacted] (2015) Toxigenic potential and heat survival of spore-forming bacteria isolated from bread and ingredients, published report
Int J Food Microbiol, 197, 30-39
M-530102-01-1

Abstract: Fifty-four spore-forming bacterial strains isolated from bread ingredients and bread, mainly belonging to the genus *Bacillus* (including *Bacillus cereus*), together with 11 reference strains were investigated to evaluate their cytotoxic potential and heat survival in order to ascertain if they could represent a risk for consumer health. Therefore, we performed a screening test of cytotoxic activity on HT-29 cells using bacterial culture filtrates after growing bacterial cells in Brain Heart Infusion medium and in the bread-based medium Bread Extract Broth (BEB). Moreover, immunoassays and PCR analyses, specifically targeting already known toxins and related genes of *B. cereus*, as well as a heat spore inactivation assay were carried out. Despite of strain variability, the results clearly demonstrated a high cytotoxic activity of *B. cereus* strains, even if for most of them it was significantly lower in BEB medium. Cytotoxic activity was also detected in 30% of strains belonging to species different from *B. cereus*, although, with a few exceptions (e.g. *Bacillus simplex* N58.2), it was low or very low. PCR analyses detected the presence of genes involved in the production of NHE, HBL or CytK toxins in *B. cereus* strains, while genes responsible for cereulide production were not detected. Production of NHE and HBL toxins was also confirmed by specific immunoassays only for *B. cereus* strains even if PCR analyses revealed the presence of related toxin genes also in some strains of other species. Viable spore count was ascertained after a heat treatment simulating the bread cooking process. Results indicated that *B. amyloliquefaciens* strains almost completely survived the heat treatment showing less than 2 log-cycle reductions similarly to two strains of *B. cereus* group III and single strains belonging to *Bacillus subtilis*, *Bacillus mojavensis* and *Paenibacillus* spp. Importantly, spores from strains of the *B. cereus* group IV exhibited a thermal resistance markedly lower than *B. cereus* group III with high values of log-cycle reductions. In conclusion, our results indicate that spore-forming bacteria contaminating bread ingredients and bread could represent a source of concern for consumer health related to the presence of strains, such as strains of *B. cereus* group III and single strains of other species, showing the ability to produce toxic substances associated to a thermal resistance enough to survive the bread cooking conditions.

Report: KIIM 5.4/16 – U7k, J.G., [redacted], J-H., [redacted], S-Y., [redacted], J-Y., [redacted], S-W., [redacted], B-Y., [redacted], M-H. (2014) Analysis of emetic toxin production by *Bacillus* species using cellular cytotoxicity, molecular, and chromatographic assays, published report
Biotechnology and Bioprocess Engineering, 19: 978-983.
M-530473-01-1

Abstract: In this study production of the emetic toxin, cereulide, in 30 *Bacillus* strains was analysed by using a cellular cytotoxicity (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; MTT) assay, polymerase chain reaction (PCR), and micellar electrokinetic chromatographycapillary electrophoresis (MEKC-CE) analysis. The *Bacillus cereus* emetic strains produced 60 ~ 227 µg/mL of cereulide when analyzed using MEKC-CE. Some other *Bacillus* species, including *B. subtilis*, *B. pumilus*, and *B. megaterium*, produced putative cereulide at levels similar to those produced by emetic *B. cereus*, whereas *B. mycoides* and *B. thuringiensis* did not produce the putative toxin or produced it at a concentration less than 2 µg/mL. Only *B. cereus* emetic strains were found to be toxic to bovine fibroblast cells, with the exception of one *Bacillus* diarrheal strain. The PCR results correlated with the MTT assay results, except in the case of one *B. cereus* diarrheal strain. This strain may produce either unusually heat-stable enterotoxins or is a co-producer of emetic and diarrheal toxins. Collectively, these results indicate that several *Bacillus* species produce toxin(s) with structures and properties similar to those of cereulide.

Report: KIIM 5.4/17 - [redacted] (2015) Literature review on effects on human health of Bacillus amyloliquefaciens QST 713 and its metabolites unpublished report, owner: Bayer CropScience AG.
Report-no. 6791109-A2-05-01

M-535690-01-1

For details on the literature search on metabolites and toxins please refer to [redacted] (2015).

IIM 5.5 Other/special studies

As part of the literature research conducted during the preparation of this dossier, the following reports on toxicity assessment of strains of *B. subtilis* used as probiotics or vaccine adjuvants were found.

[redacted] et al. (2008) reported the i*n vitro* and *in vivo* assessment of the safety of *B. subtilis* PY79, *B. subtilis* var. natto and *B. indicus* HU36 as food probiotics. Cultured cell lines were used to evaluate adhesion, invasion and cytotoxicity. The natto strain was shown to be able to invade and lyse cells and to form biofilms. Neither species was able to adhere significantly to any cell line. No strain produced any of the known *Bacillus* enterotoxins as shown by polymerase chain reaction analysis. Disc-diffusion assays using a panel of antibiotics listed by the EFSA showed that only *B. indicus* carried resistance to clindamycin at a level above the minimum inhibitory concentration breakpoints set by the EFSA. In a short-term continuous-exposure study, New Zealand White rabbits received daily oral doses of 10^9 spores of HU36 or Natto for 30 days. With this continuous dosing regime, there were no adverse effects, neither on the general health status of the animals nor their feed intake. No changes in selected visceral organs and tissues (liver, kidneys, spleens, small intestines, and mesenteric lymph nodes) were observed. No significant differences in the haematological indexes were observed in blood from control and treated rabbits. In an acute oral toxicity study, a single oral dose (1×10^{12} CFU spores) of HU36 or Natto spores was administered to Harley Dunkin guinea pigs by oral gavage. There were no noticeable abnormalities 17 days after the administration of spores in their feed intake. No significant differences were seen in weight gains between male or female animals receiving Natto and HU36 spores. Comparison of treated animals and those of the control group did reveal some differences at a significant level. These were at day 7 in the female groups receiving HU36 spores at days 7, 14 and 17 and in the female group at day 14 for those receiving Natto spores. Histological analysis of organs and tissues revealed no signs of inflammation or pathological changes and no differences in the haematological indexes measured in blood from control and treated animals. In general, no toxicity was observed in animals following *in vivo* assessments of acute and chronic dosing in guinea pigs and rabbits.

The EFSA Panel on Additives and Products or Substances used in Animal Feed reported the assessment of the toxigenic potential and resistance to relevant antibiotics of Animavit®, a feed additive based on viable cells of *B. subtilis* CBS 117162, as part of the authorization procedure.
It was concluded that Animavit® is safe for the target animals, consumers and the environment based on the lack of toxigenic potential, established on the basis of the full genome sequence analysis and a series of cytotoxicity assays, and considering that the minimum inhibitory concentrations of the antibiotics tested all fell below the defined breakpoints. Animavit® is not irritant to skin and eyes, but is a skin sensitiser and should be labelled accordingly ([redacted], 2011).

[redacted] and [redacted] (2014) evaluated spores of *B. subtilis* PXN21 as a potential probiotic treatment against *Clostridium difficile* infection (CDI). Using a murine model of infection, it was shown that oral administration of *B. subtilis* spores can attenuate the symptoms of infection. Suppression of symptoms was better if spores were administered post infection. Results from this study indicate that germination of the spore to a vegetative cell may be an integral part of how CDI is suppressed and highlight the potential of this bacterium as a probiotic treatment for CDI.

To evaluate the safety of *B. subtilis* spores as vaccine adjuvant, the hematological profile (white blood, monocytes, lymphocytes, neutrophils and eosinophils) of mice immunized with three doses of live or heat-killed spores was examined. No evidence of hematologic disturbances was observed after the subcutaneous administration of the spores. No differences were found between the control group (immunized subcutaneously with PBS) and the group immunized with spores regarding physical changes (hair loss and local inflammation), histologic alterations of the inguinal lymph nodes, and non-specific biochemical markers of inflammatory reactions. These data indicate that *B. subtilis* spores, in the complete immunization regimen via the subcutaneous route, are safe for use as a promising adjuvant (de Souza et al. 2014).

Cited references (bibliographic data and abstracts):

Report: KIIM 5.5/01 – [redacted], H.A., [redacted] M., [redacted], [redacted], L.V., [redacted], M.C., [redacted], S.M. (2008) The safety of *Bacillus subtilis* and *Bacillus indicus* as food probiotics, published report
J Appl Microbiol, 105, 510-520.
M-519896-01-1

Abstract:
AIMS: To conduct *in vitro* and *in vivo* assessments of the safety of two species of *Bacillus*, one of which, *Bacillus subtilis*, is in current use as a food supplement.

Methods and Results: Cultured cell lines, Caco-2, HEp-2 and the mucus-producing HT29-10E cell line, were used to evaluate adhesion, invasion and cytotoxicity. The Natto strain of *B. subtilis* was shown to be able to invade and lyse cells. Neither species was able to adhere significantly to any cell line. The Natto strain was also shown to form biofilms. No strain produced any of the known *Bacillus* enterotoxins. Disc-diffusion assays using a panel of antibiotics listed by the European Food Safety Authority (EFSA) showed that only *Bacillus indicus* carried resistance to clindamycin at a level above the minimum inhibitory concentration breakpoints set by the EFSA. In vivo assessments of acute and chronic dosing in guinea pigs and rabbits were made. No toxicity was observed in animals under these conditions.

Conclusions: *Bacillus indicus* and *B. subtilis* should be considered safe for oral use although the resistance of *B. indicus* to clindamycin requires further study.

Significance and impact of the study:
The results support the use of *B. subtilis* and *B. indicus* strains as food supplements.

Report: KIIM 5.5/02 – [redacted]; [redacted]; [redacted]; [redacted]; [redacted]; [redacted] (2010) Scientific Opinion on Animavit® (*Bacillus subtilis* CBS 117162) as feed additive for piglets and pigs for fattening, published report
EFSA Journal, 9(9): 2375-2388
M-520072-01-1

Abstract: Animavit® is the trade name for a feed additive based on viable cells of a strain of *Bacillus subtilis*. It is intended for use with piglets and pigs for fattening at a minimum dose of 2 x 10^9 and a maximum dose of 1 x 10^{10} CFU/kg complete feed. The product has not been previously authorised in the European Union. *B. subtilis* is a species which EFSA recognises as being suitable for the QPS approach to the assessment of safety. This requires an assessment of toxigenic potential and resistance to relevant antibiotics. The lack of toxigenic potential was established on the basis of the full genome sequence analysis and a series of cytotoxicity assays. The minimum inhibitory concentrations of the antibiotics tested all fell below the defined breakpoints. Consequently, the FEEDAP Panel concludes that Animavit® is safe for the target animals, consumers and the environment. Animavit® is not irritant to skin and eyes. However, the product is a skin sensitiser and should be labelled accordingly. Given the nature of the product and the evidence of skin sensitisation, Animavit® should be considered as also having the potential to cause sensitisation via the respiratory route. On the basis of the in vivo studies provided, Animavit® was shown to have the potential to improve the daily weight gain of weaned piglets in three studies at the dose of 2 x 10^9 CFU/kg feed. A further three studies with pigs for fattening showed the same effects at the same dose.

Report: KIIM 5.5/03 – [redacted], [redacted], S.M. (2014) Use of *Bacillus subtilis* PXN21 spores for suppression of Clostridium difficile infection symptoms in a murine model, published report
FEMS Microbiol Lett, 358, 154-161
M-530028-01-1

Abstract: *Clostridium difficile* is the primary cause of nosocomial diarrhoea in healthcare centres of the developed world. Only a few antibiotics are available for treatment, and relapses are common in patients undergoing antibiotic therapy. New approaches are required to reduce reliance on antibiotics, the use of which represents a primary risk factor for development of *C. difficile* infections. Supplementation of the gut flora with probiotics represents a key area for producing more successful treatment options for *C. difficile* infection (CDI). In this study, spores of *B. subtilis* have been evaluated as a potential probiotic treatment against CDI. Using a murine model of infection oral administration of *B. subtilis* spores can attenuate the symptoms of infection. Furthermore the data demonstrate (1) suppression of symptoms was better if spores were administered post infection, and (2) germination of the spore to a vegetative cell may be an integral part of how CDI is suppressed. The results of this study highlight the potential of this bacterium as a probiotic treatment for CDI.

Report: KIIM 5.5/04 – [redacted], J.H.; [redacted]., [redacted], M.T., [redacted], W.B., [redacted], R.C., [redacted], J.H., [redacted], R.S., [redacted], E.G., [redacted], L.C., Bacillus subtilis spores as vaccine adjuvants: further insights into the mechanisms of action published report
PLoS One, 9, 1-10
M-530018-01-1

Abstract: *Bacillus subtilis* spores have received growing attention regarding potential biotechnological applications, including the use as probiotics and in vaccine formulations. *B. subtilis* spores have also been shown to behave as particulate vaccine adjuvants, promoting the increase of antibody responses after co-administration with antigens either admixed or adsorbed on the spore surface. In this study, the immune modulatory properties of *B. subtilis* spores using a recombinant HIV gag p24 protein as a model antigen is evaluated. The adjuvant effects of *B. subtilis* spores were not affected by the genetic background of the mouse lineage and did not induce significant inflammatory or deleterious effects after parenteral administration. The results demonstrated that co-administration, but not adsorption to the spore surface, enhanced the immunogenicity of that target antigen after subcutaneous administration to BALB/c and C57BL/6 mice. Spores promoted activation of antigen presenting cells as demonstrated by the upregulation of MHC and CD40 molecules and enhanced secretion of pro-inflammatory cytokines by murine dendritic cells. In addition, in vivo studies indicated a direct role of the innate immunity on the immunomodulatory properties of *B. subtilis* spores, as demonstrated by the lack of adjuvant effects on MyD88 and TLR2 knockout mouse strains.

Report: KIIM 5.5/05 – [redacted]. (2015) Literature review on effects on human health of Bacillus amyloliquefaciens QST 713 and its metabolites unpublished report, owner: Bayer CropScience AG.
Report-no. 6791109-A2-05-01
M-535690-01-1

For details on the literature search on metabolites and toxins please refer to [redacted] (2015).

IIM 5.5.1 Specific toxicity, pathogenicity and infectiveness studies

For the background information, please refer to the baseline dossier.

IIM 5.5.2 Genotoxicity- in vivo studies in somatic cells

For the background information, please refer to the baseline dossier.

As part of the literature research submitted with this dossier, a publication reporting the investigation of the genotoxic potential *in vitro* and *in vivo* of surfactin C from *B. subtilis* BC1212, the most prominent surfactant produced by strains of *B. amyloliquefaciens* or *B. subtilis* was found. In the study, the genotoxicty of surfactin C was investigated *in vitro* in a reverse mutation assay and *in vivo* in the bone marrow micronucleus assay. To conduct the bone marrow micronucleus assay, 8-week-old male ICR mice were administered distilled water (negative control) or surfactin C at 2000,

3000 or 4000 mg/kg body weight, given by gavage in twice daily. Mice in the positive control group received cyclophosphamide 40 mg/kg in distilled water through single intraperitonial injection. Five animals each from the vehicle control, positive control, and three surfactin C-treated groups were sacrificed by cervical dislocation 24 hr after dosing. Bone marrow smears from the treated animals were stained in 5% (v/v) Giemsa solution and observed for the frequency of cells with micronuclei using light microscopy. The incidence of micronucleated cells (MNPCEs) per 2000 polychromatic erythrocytes (PCEs) per animal was measured. The proportion of polychromatic erythrocytes was assessed by examination of a total of 200 erythrocytes per animal. No significant increase in the incidence of PCEs in the surfactin C treated groups, compared with that of negative control, was observed. Surfactin C did not cause increases of MNPCE, whereas cyclophosphamide significantly increased MNPCE ($p < 0.05$). Taken together, these findings suggest that surfactin C has no genotoxic potential *in vitro* or *in vivo* ([redacted] et al., 2008).

Cited references (bibliographic data and abstract):

Report: KIIM 5.5.2/07 – [redacted], Y-H. [redacted], B-K., [redacted], J-H. [redacted], M-S., [redacted], I-B. [redacted], S. [redacted]. (2008) Evaluation of genetic and developmental toxicity of surfactin C from *Bacillus subtilis* BC1212, published report
Journal of Health Science, 54, 101-106.
M-520043-01-1

Abstract: Surfactin C is a biosurfactant produced by *Bacillus subtilis* from Korean soybean paste. Surfactin C is known to have several therapeutic effects including anti-inflammatory, fibrinolytic, and thrombolytic activities. However, there is little information concerning its safety. In this study, we evaluated the genetic and developmental toxicity of surfactin C. Bacterial reverse mutation and rodent micronucleus assays were performed to determine its genotoxic potentials. Surfactin C at 0, 125, 250, and 500 mg/kg of body weight/day was administered orally to pregnant ICR mice during the period of major organogenesis. There was no genetic toxicity related to surfactin C treatment in *in vitro* and *in vivo* systems. In the developmental study, surfactin C did not demonstrate maternal toxicity, fetotoxicity, and teratogenicity, and hence the no observed effect level was concluded 500 mg/kg per day in ICR mice.

Report: KIIM 5.5.2/08 - [redacted]. (2015) Literature review on effects on human health of Bacillus amyloliquefaciens QST 713 and its metabolites unpublished report, owner: Bayer CropScience AG.
Report-no. 6791109-A2-05-01
M-535690-01-1

For details on the literature research, please refer to ([redacted], 2015).

IIM 5.5.3 Genotoxicity – in vivo studies in germ cells

For the background information, please refer to the baseline dossier.

IIM 5.6 Summary of mammalian toxicity and overall evaluation

For the background information, please refer to the baseline dossier.