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
Introduction

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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-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 comprises 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 Bacillus 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 probiotic 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, strains 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.

For details on the literature search on metabolites and toxins refer to (2015).

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

Report: KII M 5.2/04 - (2015) Statement concerning hazards to man during the use or handling of Bacillus subtilis strain NRRL QST 713 unpublished report, owner: Bayer CropScience AG.
Report-no. n/a
M-582269-01-1

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 one 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 workforce 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: KII M 5.2/05 - (2015) Statement concerning hazards to man during the use or handling of Bacillus subtilis strain NRRL QST713 unpublished report, owner: Bayer

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 - 2015 Statement concerning hazards to man during the use or handling of *Bacillus subtilis* strain NRRL QST713 unpublished report, owner: Bayer CropScience AG.

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 or 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 - 2015 Statement concerning hazards to man during the use or handling of *Bacillus subtilis* strain NRRL QST713 unpublished report, owner: Bayer CropScience AG.

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 or 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 2001.

**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 and (2014) reported the results from a database 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 deemed 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 \textit{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 \textit{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 \textit{B. amylofectaciens} or \textit{B. subtilis} QST 713, or by any other strain used in agriculture, was found.

Cited references (bibliographic data and abstracts):


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, reference reference list of relevant reviews and matched the findings with a list of agents detailed 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 co-exposure to various laboratory animals and association with moderate evidence level (***) was obtained for α-amylase from \textit{Aspergillus oryzae}, various enzymes from \textit{Bacillus subtilis}, papain, bakery flour, amylase, storage mites, western red cedar, latex, psyllium, farming (animals, cereals, 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, toluidine disocyanate and platinum salts.

Conclusions: 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 allergen agent 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) (2010) Extrinsic alveolitis caused by \textit{Bacillus subtilis} in a biological detergent – Diagnosis and follow up Allergologie, 33, 570-572 M-520007-91-1

Abstract: A case report with follow up about an allergic alveolitis due to \textit{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).
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 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 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, vomiting; and 2, diarrhea after fermented soybean ingestion. The interval between fermented soybean ingestion and onset of symptoms was 2 to 14 hours (mean, 9.6 hours). All patients were positive on skin 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 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.
Search number: 2
Date of search: 14.08.2013 and 14.04.2014
Publication dates: 2003-2014

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 genome NOT control NOT efficacy NOT analytic? AND bacillus amyloliquefaciens (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 genome NOT control NOT efficacy NOT analytic? AND bacillus amyloliquefaciens (in title) NOT genome NOT degradation NOT expression AND bacillus subtilis

Search number: 5
Date of search: 22.06.2015
Search terms: Tox? OR infect? OR genotox? AND subtilis

Search number: 6
Date of search: 22.06.2015
Search terms: Control OR synthesis OR efficacy OR antimicrobial AND subtilis

Search number: 7
Date of search: 22.06.2015
Search terms: Search 5 NOT Search 6

Search number: 8
Date of search: 22.06.2015
Search terms: Tox? OR metabolite AND subtilis

Search number: 9
Date of search: 22.06.2015
Search terms: Allergy OR sensiti? AND amyloliquefaciens

Search number: 10
Date of search: 22.06.2015
Search terms: Allergy OR sensiti? AND subtilis

Search number: 11
Date of search: 22.06.2015
Search terms: Search 10 NOT Search 6

Search number: 12
Date of search: 22.06.2015
Search terms: Tox? OR metabolite AND subtilis
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 non-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 clinical cases or adverse events 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 (PYR) 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 swimming motility and siderophore-mediated iron uptake from transferrin through the destruction of transferrin. 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 swimming motility (Ye et al. 2006).

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 patients’ 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 (S.H. 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-trauma endophthalmitis were collected. Bacteria were identified by 16S rRNA sequencing and sequence analysis. *B. subtilis* was identified in 4 cases (S.H. 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 arteriosus. The dog responded to aggressive antibiotic therapy (Sennekamp 2010).

S.H. 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, stuff 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 cell culture test. The examination of the reconstituted milk in the lab showed that both species of *Bacillus* were able to enter, the 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 109 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 Schmehlmann (2015) 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 traditional food in Japan consisting in soybeans fermented by *B. subtilis* natto. Within 5 years, 7 patients were identified which had 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):


**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.


**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, autoimmunne, 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.

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


**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.


**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 medications 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 microbial pathogens and their resistance patterns for better outcome in ocular surgeries.

Report: KIIM 5.2.4/06 – 一旦奄, 一 daughters, 一 ones, 一 in (2014)
Causative organisms of post-traumatic endophthalmitis: a 20-year retrospective study, published report
BMC Ophthalmology, 14, 1-7
M-330509-01-1

Abstract: Background: A wide range of organisms that can 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, 64 (12.1%) 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* (6.8%) 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; or infections caused by *P. aeruginosa*, a combination therapy of ciprofloxacin, doxycycline, and one of the cephalosporins might provide optimal coverage according to data from the second decade.
Specific. The rapid molecular technique was useful in detection 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 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, 16 specimens were positive, illustrating the presence of bacterial infection (56.4%). Twelve species including Escherichia coli, Enterobacter cloacae, Bacillus subtilis, Neisseria gonorrhoea, Streptococcus pneumoniae, Haemophilus influenzae, Chlamydia trachomatis, Staphylococcus aureus, Neisseria meningitidis, 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, infecting endophthalmitis.


M-530032-01-1

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 4-year-old, 12 kg female Border collie and presented with anorexia, weight loss, pyrexia (40.1 °C) and lameness. A continuous murmur with maximal intensity over the left heart base (2/6) was detected on auscultation. Echocardiography revealed a PDA and severe aortic stenosis (AS) caused by aortic valve vegetations. 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.


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 4-year-old, 12 kg female Border collie and presented with anorexia, weight loss, pyrexia (40.1 °C) and lameness. A continuous murmur with maximal intensity over the left heart base (2/6) was detected on auscultation. Echocardiography revealed a PDA and severe aortic stenosis (AS) caused by aortic valve vegetations. 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.


M-192141-01-1

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 – (2014) **Allergens causing occupational asthma: an evidence-based evaluation of the literature**, published report Int Arch Occup Environ Health, 87, 399-633, 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 of various laboratory animals, an association with moderate evidence level (“***”) was obtained for amylase from Apergillus 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, dishmear, norway lobster, polyn, snow crab, seafood, trout and turbot, reactive dyes, toluene disocyanates 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 allergen 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 5.2.4/14 – (2010) **Extrinsic alveolitis caused by Bacillus subtilis in a bio-logical detergent – Diagnosis and follow up** Allergologie, 33, 570 – 572 M-520009-01-1

**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 lactate dehydrogenase (LDH).


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 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 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.

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

Report-no. 6791109-A2-05-01 M-535690-01-1

For details on the literature research, please refer to (Yzqä・Rzて6gac・v, 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 genotoxicity 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 TA98, TA100, TA1535, and TA1537, and Escherichia coli WP2 uvrA/pKM101 and dose ranging from 3.25 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 vivo. 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 twice daily. Mice in the positive control group received cyclophosphamide 40 mg/kg at distilled water through single intraperitoneal 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 an increase in MNPCE, whereas cyclophosphamide significantly increased MNPCE (p < 0.05). Taken together, these findings suggest that surfactin C has no genotoxic potential in vivo or in vitro (Jeong et al. 2008).

Cited references (bibliographic data and abstracts):


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 5.3.5/02 - 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 (z9cDo 7h?.Räた1g2015).

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 amyllosin 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 Jang et al., (2008). In this study, the genotoxicity 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 intraperitoneal 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 (MNPCCs) 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 in MNPC, whereas cyclophosphamide significantly increased MNPC (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 (GDD) 6 through 17 at the doses of 0 (control), 125, 250, and 5000 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 the 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 external 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 alterations in body weight, body weight gain and food consumption, alterations on the hematological parameters, differences in organ weights compared with the control group, or histopathological findings were noted at the lowest dose tested 500 mg/kg per day. The NOAEL was set on 500 mg/kg based on decreased bw, increased levels in serum of alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase, and increased liver weight and hydrophic necrosis on hepatocyte at the highest doses tested (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 by intra-gastric gavage during 28 days. No treatment-related mortality was observed in any dose tested. No clinical signs of toxicity alterations in body weight, body weight gain and food consumption, alterations on the hematological parameters, differences in organ weights compared with the control group, or histopathological findings were noted at the lowest dose tested 500 mg/kg. The NOAEL was set on 500 mg/kg based on decreased bw, increased levels in serum of alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase, and increased liver weight and hydrophic necrosis on hepatocyte 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 insect 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 Bacillus 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 ultra-high-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 cytotoxicity of Bacillus-associated lipopeptides and their tolerance in...
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 exposing concentration of 135 nM and hyperpolarized their cell membrane and depolarized their mitochondria at exposure concentration of 33.67 nM for 10 min. Amylosin was cytotoxic to feline lung cells at concentrations of 270 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 (BLM) with selectivity K(+) over Na(+) at a concentration of 26 nM. The same concentration at which amylosin was toxic to boar sperm cells. The amylosin cation channels were cholesterol- and ATP-dependent and more effective with K(+) than with Na(+). The authors proposed that the toxicity of amylosin may be due to 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* 2564/96 depolarized the mitochondria in intact colon carcinoma cells, used to model the contact with the human digestive 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* (*Kunz et al.* 2009).

In a recent publication (*Mikkola et al.* 2015), the *in vitro* effects of amylosin produced by strains of *B. amyloliquefaciens* isolated from moisture-damaged buildings were assessed in human macrophages, 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 (*Lee 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
et al. (2012) investigated the thrombolytic effects of Douchi fibrinolytic enzyme (DFE) from Bacillus 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) (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 in B. cereus, as well as a heat spore inactivation assay were carried out. Cytotoxic activity was detected in 5 strains of B. amyloliquefaciens (N20.3, S68, S70, S80.2, S85.2, S77.1, S109.3, ATCC 43173) 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 in 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. pumilus and Paenbacillus 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 exist: 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.

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 chromatography-capillary 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 genetic 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 assay and related 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: KIM 5.4/85, Y-H., B-K., J-H., M-S., I-B., S-
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 genotoxic 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.

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 hematological 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. Zona necrosis of hepatocyte around the hepatic vein was observed after administration of the same doses in a dose-dependent manner. On the present study, the no observed adverse-effect level (NOAEL) of surfactin C was 500 mg/kg following oral administration in rats.

J Food Prot, 77, 2039-2140.
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 food product, natto, were isolated in order to clarify their potential to produce the surfact type lipopeptides. The isolated bacilli were characterized as Bacillus subtilis. They were β-hemolytic and have 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 surfactin per single 50-g natto serving apparently without suffering any ill effects, indicating a low human toxicity.

Arch Microbiol, 181, 314-323
M-518859-01-1
Abstract: Fungicidal 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 a chromophore(s) absorbing at 365 nm. In boar sperm and human neural cells (Paju), the compound depolarized the transmembrane potentials of mitochondria (ΔΨm) and the plasma membrane, with a selectivity K(+) > Na(+) 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 - 518861
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 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 concentrations of 33-67 nM for 10 min. In a 30 min 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 triphosphate (ATP)-independent cation influx into isolated rat liver mitochondria (RLM), inducing swelling of the mitochondria at concentrations of 200 nM K(+) > Na(+) medium. In the K(+) 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 selective and ATP-independent and more effective with K(+) than with Na(+). We propose that the toxicity of amylosin may be due to its ionophoric properties, representing the first K(+)Na(+) channel-forming substance reported from Bacillus amyloliquefaciens.

Report: KIIM 5.4/09 - 518861
M-518863-01-1
Abstract:

AIM: To screen and characterize toxic, heat-stable substances produced by food borne strains of 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 potential. 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 11-27 degrees C for B. mojavensis. Both species produced amylosin in air as well as at 7-8% CO2 with 8-9% O2.

Conclusions: Food borne illness related strains of B. subtilis and B. mojavensis, produce the heat-stable toxin amylosin.

SIGNIFICANCE AND IMPACT OF THE STUDY:

This is the first report that suggests a role for the heat-stable non-channel forming toxin amylosin, as a virulence factor in food borne Bacillus.


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 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.


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.


**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 PGA (produced by *B. subtilis* natto ATCC 15245) on the viability of probiotic bacteria during freeze drying and to test the toxicogenic potential of *B. subtilis* natto. 50% γ-PGA was found to protect *Lactobacillus paracasei* significantly better than 50% sucrose, whereas it showed comparable cryoprotective 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 toxicogenic potential of its source, *B. subtilis* natto. Presence of six genes that are known to encode for toxins were investigated: three component hemolysin (hl-D/A), three-component non-haemolytic enterotoxin (nheB), *B. cereus* enterotoxin (*tet*), *B. subtilis* enterotoxin (FM1), sphingomyelinase (sfh) and phosphatidylcholinespecific phospholipase (pip). From our investigations, none of these six genes were present in *B. 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 survivability during the manufacturing process.


**Abstract:** Background: 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, the 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. Thirty mice 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.


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 whether 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 toxigenic 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 N5892), it was low or very low. PCR analyses detected the presence of genes involved in the production of NHE, HBL or Cytotoxins 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 A 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.


Abstract: In this study, production of the excreted toxin, cereulide, in 30 Bacillus strains was analysed by using a cellular cytotoxicity (4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay, polymerase chain reaction (PCR), and micellar electrokinetic chromatography-capillary electrophoresis (MEKC-CE) analysis. The Bacillus cereus emetic strains produced 80 ~ 287 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 excreted and diarrheal toxins. Collectively, these results indicate that several Bacillus species produce toxin(s) with structures and properties similar to those of cereulide.

M-535690-01-1

For details on the literature search on metabolites and toxins please refer to [27] (2015).

**HIM 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.

[28] et al. (2008) reported the *in vitro* and *in vivo* assessment of the safety of *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 10⁸ spores of HU36 or Natto for 30 days. With this continuous dosing regime, there were no adverse effects, neither on the general health status, nor on the 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 × 10¹⁰ CFU spores) of HU36 or Natto spores was administered to Harley Dunkin guinea pigs by oral gavage. There were no noticeable abnormalities or deaths 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 day 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 cell 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 ([29], 2011).

[30] and [31] (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 cells, 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 (inmunized 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):

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 TST29, HE 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 *Bacillus 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.

EFSA Journal, 9(9): 2375-2388
M-2007-001-1

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 clinically 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.

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 – R.C., J.H.; M.T., W.B.; R.C., J.H., R.S., E.G., E.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 (2015) Literature review on effects on human health of Bacillus amyloliquefaciens QST 713 and its metabolites unpublished report, owner: Bayer CropScience AG.

Report-no: 8791109.A2-05-01 M-535698-01-1

For details on the literature search on metabolites and toxins please refer to Doc M, IIM, Sec. 3, P. 5 (2015).

### IIM 5.5 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 genotoxicity 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 intraperitoneal 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 (Ueoh et al. 2008).

Cited references (bibliographic data and abstract):


**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.


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

**IIM 5.5.3 Genotoxicity – in vivo studies in germ cells**

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

**IIM 5.5.6 Summary of mammalian toxicity and overall evaluation**

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

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