Bacillus amyloliquefaciens QST 713

Microbial Pest Control Agent against plant pathogenic fungi and bacteria

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

Summary documentation, Tier II

Annex IIM, Section 6

Point IIM 8: Effects on Non-Target Organisms

Date: September 2015

Applicant

Bayer CropScience AG

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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 8 Effects on non-target organisms

The literature research was conducted on the DIMDI database provided by the German Institute of Medical Documentation and comprised searches in MEDLINE; BIOSIS, CAB Abstracts and SCISEARCH databases in order to identify scientific peer-reviewed open literature on the active substance *Bacillus amyloliquefaciens* QST 713 which may affect the assessment of *B. amyloliquefaciens* and *B. subtilis* on non-target organisms ([redacted], 2015). In total, 1473 articles were evaluated based on their titles or abstracts. After rapid assessment for relevance, 59 documents were assessed in detail. Main relevant findings are summarised below, as well as in the literature review report:

Report: IIM 8/01 – [redacted](2015), Literature review on *Bacillus amyloliquefaciens* QST 713: Section 6: Effects on non-target organisms
Unpublished report.
Owner: Bayer CropScience AG
M-535712-01-1

Abstract: This report presents a detailed literature research review on the influence of *Bacillus amyloliquefaciens* QST 713 on non-target organisms, using DIMDI engine from German Institute of Medical Documentation and comprised of searches in MEDLINE, BIOSIS, CAB Abstracts and SCISEARCH databases. Based on the evaluation of 59 references, 41 were considered as relevant for the assessment of the effect of *B. amyloliquefaciens* QST 713 on non-target organisms.

IIM 8.1 Effects on birds

Bacillus sp. are not described to have pathogenic effects on birds. Moreover, some *Bacillus* sp. strains are used as probiotics in birds' diet. Please refer to the baseline dossier for the background information.

A literature search was conducted to identify publications on toxic or pathogenic effects of *B. amyloliquefaciens* QST 713 on birds by combination of the keywords "*Bacillus subtilis*" or "*Bacillus amyloliquefaciens*" and "bird" (please refer to Point IIM 8). After removal of doubles, 28 articles were recorded. After rapid assessment of titles or abstracts, 8 articles were evaluated by their full text, and 6 articles were identified as relevant However, all relevant articles deal with the probiotic effect of *Bacillus* sp. No articles were identified, describing toxicity or pathogenicity of *B. subtilis* or *B. amyloliquefaciens* on birds.

[redacted] (2013) studied the effect of probiotics on the behaviour of turkey poults by feeding Ecobiol® probiotic (*B. amyloliquefaciens* spores) at a dose rate of 0.01 g/day (1×10^{10} CFU/g feed). Visual observations were performed, to study the behaviour of the birds. Control and treated group consist of 350 birds, each. Probiotic administration had variable effects on turkey poults. No effects were observed in their maintenance behaviour (ingestive behaviour, sleeping and resting times). Also, probiotics did not affect kinesis (walking, running) and comfort behaviour. On the other hand, social and agonistic behaviour were influenced significantly by probiotic administration: Distress calls, fighting and biting activities were significant reduced at Turkey poults fed with *B. amyloliquefaciens* spores.

A study on the effect of *B. subtilis* probiotic Enviva Pro^{TM} 202 GT at a level of 7.5×10^4 CFU/g feed on broiler performance and intestinal mucosa-associated avian pathogenic *E. coli* was evaluated ([redacted] et al., 2013). Therefore, seven pens of 50 male broilers were fed with either *B. subtilis* spores, zinc bacitracin or the unsupplemented control diet for 42 days. However detailed information on intake of *B. subtilis* cells per kg body weight and per day were not provided. Nevertheless, probiotic diet was observed to influence positively the birds' performance on day 35 in comparison to both, the control and the antibiotic supplemented diet. Thus, probiotic diet was recommended for an alternative use to antibiotic growth promotors. However, no effects on the number of intestinal mucosa-associated avian pathogenic *E. coli* were detected.

[redacted] et al. (2012) confirmed these results by a similar study of *B. subtilis* treatment on chickens challenged with *Eimeria tenella* (2×10^4 sporulated oocyst/bird), the causative agent of avian coccidiosis. 300 day-old chickens were fed for 6 weeks with various probiotic microorganisms or an anticoccidial lasalocid. Regarding *B. subtilis* 588, feed was supplemented 5×10^8 CFU/kg feed. To assess the effect of the probiotics, body weight, feed intake per pan, feed conversion ratio,

as well as extent of bloody diarrhea, excreta oocyst numbers and mortality were recorded weekly. No significant effects on body weight values were detected between the treated (*B. subtilis* spores) and the untreated controls (challenged or non-challenged with the pathogen). Whereas, *B. subtilis* supplementation reduced oocyst numbers and mortality of birds (challenged with *E. tenella*) significantly.

Influence of *B. subtilis* probiotic was also examined on 60 one day old mail broilers challenged with *Salmonella minnesota* (Carmrago [redacted] et al., 2012). Chickens were fed with a diet containing *B. subtilis* DSM17299 2.13 × 10^6 CFU/g feed) for 35 days. At 14 days of age, chickens were challenged with *S. minnesota* solution at a population of 1 × 10^8 CFU/mL. However, no information on the detailed intake of cells probiotics and pathogens per gram body weight is provided. Anyway, *B. subtilis* intake reduced significantly *Salmonella* spp. counts in chickens and increased number of CD4+ cells (immune cells in the ileum und cecum mucosa). These results were confirmed by a study of [redacted] et al. (2012). Thereby, 600 day-old broilers were fed with solid-state fermented cottonseed meal, whereas fermentation was performed with *B. subtilis* BJ-1 1.4 × 10^8 CFU/mL. However total rate of CFU intake was not described. Nevertheless, growth performance and immunity was shown to be improved by *B. subtilis* treated feed.

[redacted] and [redacted] (2014) studied recently the efficacy of probiotics feed basing on *Enterococcus faecium*, *B. subtilis* ATCC PTA-6737 spores or *Saccharomyces cerevisiae* on Japanese quails. The diet was supplemented with 1 × 10^7 CFU/kg feed *B. subtilis* spores. Feed conversion ratio (FCR) was significantly improved in the birds with both probiotic feed additives. However no significant differences of body weight or weight gains were measured between *B. subtilis* supplementation and the untreated control. Nevertheless, no pathogenic or toxic effects of *B. subtilis* spores on Japanese quails were reported.

In conclusion, there are no reports on pathogenic effects of *B. subtilis* or *B. amyloliquefaciens* on birds. Moreover, supplementation of feed with *B. amyloliquefaciens* or *B. subtilis* was shown to improve birds' behaviour and/or health when treated or not with birds pathogens. Thus, they are recommended for probiotic use in birds' nutrition.

Cited references (abstracts):

Report: IIM 8.1/02 – [redacted]. (2013) Do probiotics affect the behaviour of turkey poults?
Published report.
J Vet Med Animal Health 5, 144-148
M-518928-01-1

Abstract: With the concept that measuring behavior is often the first step to take when studying how the brain operates, this study was conducted to investigate the effect of probiotic on turkey poult's behavior which will confirm the new concept that gut microbes can influence the brain. Ecobiol® probiotic, spores of *Bacillus amyloliquefaciens* and a carrier as serum of milk with a minimum guaranteed 1×10^{10} CFU/g was given with a dose of 0.01 g/day for each bird in the drinking water to group (P; n=350) and the other group (C; n=350) were kept as controls. Behavioral observations were carried out by direct personal observation without bird disturbance from outside the pen with a good view over the whole pen. Maintenance, comfort behavior, kinesis and agonistic behaviors were recorded. The obtained results indicated that probiotics increased the feeding frequency and duration and decreased distress call and aggressive behaviors in turkey poults.

Report: IIM 8.1/03 – [redacted]., [redacted], P.W., [redacted], [redacted] (2013), Effect of feeding diets containing a probiotic or antibiotic on broiler performance, intestinal mucosa-associated avian pathogenic *E. coli* and litter water-soluble phosphorus,
Published report.

Journal of Applied Animal Nutrition, 1, 1-7
M-530479-01-1

Abstract: The aim of the present experiment was to examine the influence of feeding diets containing a probiotic containing three *Bacillus subtilis* strains or zinc bacitracin (BMD) on bird performance, intestinal mucosa-associated avian pathogenic *Escherichia coli* (APEC), intestinal morphology and litter water-soluble phosphorus (WSP) of broilers fed corn-based diets. Three treatments were employed, either a control diet; the control diet supplemented with probiotic at 7.5 × 10^4 colony forming units (cfu) /g of feed or the supplemented with BMD (50g/tonne feed). Broiler starter and finisher diets, based on corn and soybean meal, were formulated and fed *ad libitum* to seven pens of 50 male broilers per treatment from days 1 to 42. During the 1-35 d periods, feed intake and weight gain were not influenced (P > 0.05) by dietary treatments, but probiotic supplementation improved (P < 0.05) 35-d FCR compared to the control and BMD-supplemented diets (1.395, 1.447 and 1.484, respectively). During the 1-42d period, feed intake and weight gain were not influenced (P > 0.05) by dietary treatments. However, probiotic improved (P = 0.05) and BMD tended (P = 0.07) to improve FCR compared to control diet. Villus height and crypt depth in the duodenum and jejunum were not influenced (P > 0.05) by dietary treatment. The number of mucosa-associated APEC was not influenced (P > 0.05) by dietary treatments. Probiotic and BMD supplementation had no effect (P > 0.05) on litter total phosphorus. However, BMD reduced (P < 0.05) litter WSP compared to control and probiotic supplemented diet. In conclusion, probiotic supplementation had no effect on intestinal morphology or WSP but improved broiler performance and can be used as an alternative to antibiotic growth promoters.

Report: IIM 8.1/04 – [redacted], [redacted], [redacted], [redacted], [redacted] [redacted], [redacted], [redacted] (2012), Assessment of dietary supplementation with probiotics on performance, intestinal morphology and microflora of chickens infected with *Eimeria tenella,* published report
Vet Parasitol, 188, 31-40
M-518923-01-1

Abstract: The effect of dietary supplementation with different preparations of probiotics on the performance of broiler chickens was evaluated. Broiler chickens were experimentally infected with 2 × 10^4 sporulated oocysts of *Eimeria tenella* at 14 days of age. Three hundred, day-old, Cobb-500 chicks, as hatched, were separated into 10 equal groups with three replicates. Two of the groups, one challenged with *E. tenella* oocysts and the other not, were given a basal diet and served as controls without medication. The other challenged groups were given the anticoccidial lasalocid (60 mg/kg) or *Enterococcus faecium* (5 × 10^8 or 5 × 10^9 cfu/kg feed), Bifidobacterium animalis (5 × 10^8 cfu/kg feed), *Lactobacillus reuteri* (5 × 10^8 cfu/kg feed), *Bacillus subtilis* (5 × 10^8 cfu/kg feed), or a multi-species probiotic mix at 5 × 10^8 or 5 × 10^9 cfu/kg feed, respectively. The trial lasted 6 weeks. Individual body weight, feed intake per pen and feed conversion ratio values were recorded weekly, along with the extent of bloody diarrhea, excreta oocyst numbers and bird mortality. Caecal lesions were assessed and intestinal samples were taken for histopathological and bacteriological evaluation from ileum and caecum. Overall growth performance of chickens fed the multi-species probiotic mix at both levels was higher (P<0.05) compared to the infected control. Overall oocyst shedding was lowest (P<0.05) in the lasalocid supplemented group. Villous height was higher (P<0.05) in *Bacillus* supplemented groups compared to infected controls. The *Lactobacillus* supplemented group had the highest (P<0.05) numbers of both *Lactobacillus* and *Bifidobacterium* in ileum and caecum. In conclusion, dietary probiotics are promising for further investigation on improving intestinal health and growth performance of broiler chickens experimentally challenged with *E. tenella.*

Report: IIM 8.1/05 – [redacted]., [redacted], L.N., [redacted], [redacted], [redacted], [redacted] (2012), Effects of *Bacillus subtilis* in the dynamics of infiltration of immunological cells in the intestinal mucosa of chickens challenged with *Salmonella* Minnesota, Published report.
International Journal of Poultry Science, 11, 630-634
M-518904-01-1

Abstract: The use of *Bacillus subtilis* (BS) as a probiotic in bird feed was studied through the evaluation of its effect on the infiltration of immune cells in the ileum and cecum mucosa of chickens challenged with *Salmonella* Minnesota (SM). The birds were divided into three treatment groups; Negative control, containing unchallenged birds; Positive control, with SM challenged birds; and Probiotic, with SM challenged birds fed with a diet containing BS (DSM 17299 2.13 x 10^6 cfu/g). The birds fed BS showed increased goblet and CD4+ cell counts in the ileum and cecum before being challenged with SM in comparison to the birds not fed BS. After the SM challenge, the birds fed BS showed a reduction in the *Salmonella* counts at 48 Post Inoculation (PI) in the cloaca and cecum swabs and in litter samples and furthermore a reduction in CD8+ cells in the cecum compared to the challenged birds. Based on the results, it is concluded that feeding BS as a probiotic to broilers reduced the *Salmonella* spp. counts and thus, affected the mobilization of CD4+ and CD8+ cells in the ileum and cecum mucosa.

Report: IIM 8.1/06 – [redacted], [redacted], X.H., [redacted], Y.F., [redacted], [redacted], J (2012), Effects of Replacement of Soybean Meal by Fermented Cottonseed Meal on Growth Performance, Serum Biochemical Parameters and Immune Function of Yellow-feathered Broilers,
published report.
Asian-Aust. J. Anim. Sci.,25, 393-400
M-519808-01-1

Abstract: The study was conducted to examine the effects of partially replacing soybean meal (SBM) by solid-state fermented cottonseed meal (FCSM) on growth performance, serum biochemical parameters and immune function of broilers. After inoculated with *Bacillus subtilis* BJ-1 for 48 h, the content of free gossypol in cottonseed meal was decreased from 0.82 to 0.21 g/kg. A total of 600, day-old male yellow-feathered broilers were randomly divided into four groups with three replicates of 50 chicks each. A corn-SBM based control diet was formulated and the experimental diets included 4, 8 or 12% FCSM, replacing SBM. Throughout the experiment, broilers fed 8% FCSM had higher ($p<0.05$) body weight gain than those fed 0, 4 and 12% FCSM. The feed intake in 8% FCSM group was superior ($p<0.05$) to other treatments from d 21 to 42. On d 21, the concentration of serum immunoglobin M in the 4% and 8% FCSM groups, as well as the content of complements (C3, C4) in 8% FCSM group were greater ($p<0.05$) than those in the SBM group. Besides, birds fed 8% FCSM had increased ($p<0.05$) serum immunoglobin M, immunoglobulin G and complement C4 levels on d 42 compared with bird fed control diet. No differences ($p>0.05$) were found between treatments regarding the serum biochemical parameters and the relative weights of immune organs. In conclusion, FCSM can be used in broiler diets at up to 12% of the total diet and an appropriate replacement of SBM with FCSM may improve growth performance and immunity in broilers.

Report: IIM 8.1/07 – [redacted], [redacted] (2014), Efficacy of a feed probiotic bacteria (*Enterococcus faecium* NCIMB 10415), spore (*Bacillus subtilis* ATCC PTA-6737) and yeast (*Saccharomyces cerevisiae*) in Japanese quails,
Published report
Animal Science and Biotechnologies, 71, 63-70
M-530525-01-1

Abstract: Efficacy of a bacteria (*enterococcus faecium* NCIMB 10415), spore (*bacillus subtilis ATCC PTA-6737*) and yeast (*saccharomyces cerevisiae*) probiotic was tested in quails from day 2 to day 35. A control diet (CON) was supplemented at the recommended dosages to obtain three diets containing a bacteria (D-bacteria), a spore (D-spore) and a yeast (D-yeast). The results indicated that the birds fed on D-yeast significantly ($P<0.05$) consumed more feed at the age of 16, 30 and 37 days than the birds fed on CON, D-bacteria and D-spore, whose food intake (FI) were almost similar throughout the fattening period. Body weights (BW) and weight gains (WG) of birds fed on probiotic diets were higher than the birds fed on CON diet, especially BW and GW of the birds fed on D-yeast were significantly ($P<0.05$) greater than birds on CON at the age of 9, 16 and 30 days, and than birds on D-spore at the age of 30 days. Feed conversion ratio (FCR) was ($P<0.05$) improved in the birds of all probiotic diets, compared to the control bird group at the age of 9 and 16 days. As the birds get older the differences in FCR between control group and probiotic groups were not significant; all FCR were almost similar. Carcass yield was significantly ($P<0.05$)

high in the birds of D-bacteria. A significant increase in the weight and length of digestive tract was seen with the birds of D-spore group. The results indicated that the use of selected probiotics enhanced bird performance, and the effect of D-yeast probiotic was better.

IIM 8.2 Effects on fish

Studies on acute toxicity and/or pathogenicity and infectivity to fish revealed that *B. amyloliquefaciens* QST 713 is not toxic to fish. Please refer to the baseline dossier for the background information. No pathogenic effects of *B. subtilis* or *B. amyloliquefaciens* on fish were described. Thus, the conclusions from the baseline dossier was that the overall risk *B. amyloliquefaciens* QST 713 to fish is considered to be acceptable.

A literature search was conducted to identify publications on the toxic or pathogenic effects of *B. amyloliquefaciens* QST 713 on aquatic organisms by combination of the keywords "*Bacillus subtilis*" and "*Bacillus amyloliquefaciens*" and concerned aquatic organisms (fish, daphnia, algae, please refer to Point IIM 8). After rapid assessment of titles and abstracts, four articles were evaluated by their full text, dealing with fish or other aquatic organisms. Regarding information on fish toxicity, 3 publications were identified as relevant, among which two dealt with the probiotic effect of *Bacillus* sp. No articles were identified that described toxicity or pathogenicity of *B. subtilis* or *B. amyloliquefaciens* to fish. A discussion of these article is provided below.

B. amyloliquefaciens or *B. subtilis* can produce cyclic lipopeptides which are recommended for safe and effective use against mosquitos (please refer to Point IIM 8.8). Therefore, aquatic organisms can be exposed to these cyclic lipopeptides where mosquito control programms are implemented. In the study from [redacted] & [redacted] (2006), crude cyclic lipopeptides from two *B. subtilis* strains (DM-03 and DM-4) were examined for their potential risk to fish. Effects were assessed in the Indian mayor carp, *Labio rohita* (35 g/fish) exposed to at 120 mg/L and 300 mg/L. It was shown that crude lipopeptides did not influence the fish physiology as well as serum biochemical parameters in this concentration range. No adverse effect on fish was identified. Thus, crude cyclic lipopeptides from *B. subtilis* were recommended as safe biopesticide for effective mosquito control by the authors.

Moreover, *B. subtilis* or substances produced by these were reported for the safe use as probiotics in fish diet. [redacted] et al. (2005) studied the immune modulatory effects caused by dietary administration of living *B. subtilis* CECT 287 cells alone (10^7 CFU/g) or in conjunction with *Lactobacillus delbrüeckii* spp. *lactis* (0.5×10^7 CFU/g each) to the gilthead seabream. Measured cellular immune parameters were leucocyte peroxidase content, phagocytosis, respiratory burst activity and cytotoxicity. Fish were fed for 1, 2 or 3 weeks with the supplemented feed and for one more week with the control diet before examination. After two weeks phagocytic ability rose significantly in fish treated with *B. subtilis*, but decreased significantly at the end of the experiments. Leucocyte cellular peroxidase content was significantly lower in the fish fed with *B. subtilis*. The other parameters studied were not significantly affected. In conclusion, both bacterial strains produced similar stimulating effects on the host cellular immune parameters. However, effects were restricted to the experimental period, indicating a low persistence in the seabream gut.

Probiotic effects of *B. amyloliquefaciens* spores in combination with *Saccharomyces cerevisiae* culture or live cells were tested on juvenile common carp (*Cyprinus carpio*) ([redacted] et al., 2015). The influence of supplemented diet on growth performance, gut mucosal morphology, general welfare and disease resistance against *Aeromona hydrophila* were evaluated. Supplemented diets containing *B. amyloliquefaciens* were (a) *S. cerevisiae* culture product containing 10^7 CFU *B. amyloliquefaciens* spores /kg feed and (b) the commercial product Changlijian containing living *S. cerevisiae* cells and *B. amyloliquefaciens* spores at a concentration of 2×10^6 CFU/g and 2.4×10^6 CFU/g, respectively. Neither effects on growth performance nor on the intestinal index of weight (WII [%] = 100 × intestinal weight × body weight-1) were detected. On the other hand, fish exposed to. *hydrophila* had higher survival rates when treated with *B. amyloliquefaciens* in combination with *S. cerevisiae*. Furthermore, treatment (a) significantly increased posterior intestinal microvillus length. In general, this indicates more mature epithelia and enhanced absorptive function. However, opposite effects were shown for treatment (b). In conclusion, no fish mortality or toxic effect was detected due to application of *B. amyloliquefaciens* cells in combination with *S. cerevisiae*. Moreover, administration of these microorganisms may have positive effects on fish health.

A new *B. amyloliquefaciens* strain (ZJHD3-06) was isolated from marine fish *Epinephelus areolatus*, which was shown to produce a novel bacteriocin named CAMT2 ([redacted] et al., 2015). This

20.0 kDa bacteriocin inhibits important food spoilage and food-borne pathogens (e.g. *Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli* and *Vibrio parahaemolyticus*). It was resistant for up to 100 °C and pH ranging 2-10. Activity loss was shown to occur when treated with protease K. Hence, CAMT2 was suggested to have potential for use as food biopreservative. This novel bacteriocin might participate in the mode of action of *B. amyloliquefaciens* QST 713. However, it cannot be easily produced as pure substance and is not intended to be applied alone.

In conclusion, no evidence for toxicity or pathogenicity of *B. subtilis* and *B. amyloliquefaciens* to fish was found in the open literature. Moreover, some studies determined positive probiotic effects of *B. subtilis* and *B. amyloliquefaciens*.

<u>Cited references (abstracts):</u>

Report: IIM 8.2/05 – [redacted], [redacted] (2006), Assessment of mosquito larvicidal potency of cyclic lipopeptides produced by *Bacillus subtilis* strains.
Published report
Acta Trop, 97, 168-173
M-530022-01-1

Abstract: In this study, mosquito larvicidal potency of cyclic lipopeptides (CLPs) secreted by two *Bacillus subtilis* strains were determined. LC_{50} of the crude CLPs secreted by *B. subtilis* DM-03 and DM-04 strains against third instar larvae of *Culex quinquefasciatus* was 120.0+/-5.0 and 300.0+/-8.0 mg/L respectively post 24 h of treatment. Physico-chemical factors such as pH of water, incubation temperature, heating and exposure to sunlight hardly influenced the larvicidal potency of these CLPs. Present study provided the evidence that *B. subtilis* lipopeptides were safe to Indian major carp *Labeo rohita*, a non-target aquatic organism. These properties of *B. subtilis* CLPs can be exploited for the formulation of a safer, novel biopesticide for effective control of mosquito larvae.

Report: IIM 8.2/06 – [redacted], [redacted], [redacted], M.A., [redacted] (2005), Dietary administration of *Lactobacillus delbrüeckii* and *Bacillus subtilis*, single or combined, on gilthead seabream cellular innate immune responses.
Published report
Fish Shelfish Immunol, 19, 67-77
M-530499-01-1

Abstract: The effects of oral administration of *Lactobacillus delbrüeckii* ssp. *lactis* and *Bacillus subtilis*, single or combined, on gilthead seabream cellular innate immune responses were investigated. Fish were fed four different diets: control (non-supplemented); or diet supplemented with 10^7 cfu g^{-1} *L. delbrüeckii* ssp. *lactis*; 10^7 cfu g(-1)*B. subtilis*; or with 0.5 × 10^7 cfu g^{-1} *L. delbrüeckii* ssp. *lactis* and 0.5 × 10^7 cfu g^{-1} *B. subtilis*. This feeding regime lasted for 3 weeks, and all experimental groups were then fed the control commercial diet for another week. Six fish were sampled at weeks 1, 2, 3 and 4. Head-kidney leucocytes were isolated and the main cellular innate immune parameters (leucocyte peroxidase content, phagocytosis, respiratory burst activity and cytotoxicity) were evaluated. Leucocyte peroxidase content was lower in all groups at week 3 but the levels tended to recover during the last week of the experiment. Respiratory burst activity was not affected at any time of the experiment in any of the experimental groups. However, phagocytic activity increased after 2 weeks of feeding the single bacteria-supplemented diets, whereas the combination of the two caused an increment which persisted for as long as the bacteria were being administered. Cytotoxic activity was also significantly increased after 3 weeks of feeding the mixture of the two bacteria. After 1 week back on the control diet, the parameters in the experimental groups had recovered or even dropped below those recorded in the control group, suggesting that the bacteria did not persist in the seabream gut.

Report: IIM 8.2/7 – [redacted] (2015), Effects of dietary Saccharomyces cerevisiae culture or live cells with *Bacillus amyloliquefaciens* spores on growth performance, gut mucosal morphology, hsp70 gene expression, and disease resistance of juvenile common carp (*Cyprinus carpio*).
Published report
Aquaculture, 438, 33-38
M-530114-01-1

Abstract: A feeding experiment was conducted to examine the effects of dietary administration of brewer's yeast *Saccharomyces cerevisiae* culture or live cells with *Bacillus amyloliquefaciens* spores on the growth performance, gut mucosal morphology, general welfare, and disease resistance of juvenile common carp (*Cyprinus carpio*). Four practical diets were formulated, i.e. control diet, diet Y1 (supplemented with 1 g/kg Saccharoculture, a Korean-made *S. cerevisiae* culture product containing 10^7 CFU/g *B. amyloliquefaciens* spores), diet Y2 (supplemented with 2 g/kg *S. cerevisiae* culture, DVAQUA®, produced by Diamond V Mills Inc., IA, USA, as the positive control) and diet Y3 (supplemented with Changlijian, a local product with each gram containing 10^{10} CFU live *S. cerevisiae* cells and 1.2×10^{10} CFU *B. amyloliquefaciens* spores, at the recommended dose of 0.2 g product per kg feed). Each diet was randomly assigned to triplicate groups of 12 fish. The experiment was conducted in aquaria for 8 weeks. Results showed that the growth performances were not significantly improved in all treatment groups. Intestinal mucosal morphology analysis indicated that the posterior intestinal microvillus length in treatments Y1 and Y2 was significantly increased compared to control ($P < 0.05$), while significantly reduced posterior intestinal microvillus density was observed in Y3 ($P < 0.05$). The expression of the gene involved in animal welfare (70 kDa-Heat Shock Protein gene) was analyzed. No significant difference in *hsp70* gene expression was observed in the intestine for different dietary groups. In the liver, the expression was decreased in Y1 and Y2, while in Y3 the expression was significantly increased ($P < 0.05$). However, the expression was significantly up-regulated in the kidney for Y2. Lastly, all treatment groups showed some trend of protection against *A. hydrophila* infection in common carp. In conclusion, lower level of *S. cerevisiae* culture with *B. amyloliquefaciens* spores (1 g/kg *Saccharoculture*) may confer some beneficial effect to common carp, but the supplementation of live *S. cerevisiae* cells with *B. amyloliquefaciens* spores didn't provide any improvements under the present experimental conditions.

Report: IIM 8.2/08 – [redacted] (2015), Purification and characterization of a novel bacteriocin CAMT2 produced by *Bacillus amyloliquefaciens* isolated from marine fish *Epinephelus areolatus*.
Published report
Food Control, 51, 278-282
M-530030-01-1

Abstract: A novel bacteriocin named CAMT2 was produced by strain ZJHD3-06 which was isolated from the marine fish *Epinephelus areolatus* and identified as *Bacillus amyloliquefaciens*. Bacteriocin CAMT2 inhibits important food spoilage and food-borne pathogens such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli* and *Vibrio parahaemolyticus*. Bacteriocin CAMT2 was purified by ammonium sulfate precipitation, gel filtration chromatography on Sephadex G-50 and reversed phase chromatography on a C_{18} column. The molecular mass of the purified bacteriocin CAMT2 was about 20.0 kDa and N-terminal sequencing of the peptides revealed low similarity with existing antimicrobial peptides, as determined by an LC–MS/MS system. Bacteriocin CAMT2 was resistant for up to 100C and pH ranging 2–10, but lost its activity when treated with protease K. The bacteriocin also showed significant antimicrobial activity against *L. monocytogenes* in a meat model system. These obtained results suggest that bacteriocin CAMT2 has potential for use as a food biopreservative.

IIM 8.3 Effects on aquatic invertebrates

B. amyloliquefaciens QST 713 is not known to cause toxic effects to aquatic invertebrates. To evaluate effects of *B. amyloliquefaciens* QST 713 (previously designated as *B. subtilis*), toxicity studies on *Daphnia magna* and shrimps (*Palaemonetes pugio*) were performed. For the background

information, please refer to the baseline dossier. The conclusions from the baseline dossier was that the overall risk *B. amyloliquefaciens* QST 713 to aquatic invertebrates is considered to be acceptable.

An extensive literature study has been additionally conducted using DIMDI database provided by the German Institute of Medical Documentation and comprised of searches in MEDLINE, BIOSIS, CAB Abstracts and SCISEARCH databases to identify relevant literature on effects of *B. subtilis* or *B. amyloliquefaciens* on aquatic organisms (please refer to Point IIM 8). Regarding effects on aquatic invertebrates, two reports were identified as relevant, among which one studied the probiotic effects of *B. subtilis* on shrimps.

The probiotic effect of a mixture of two *B. subtilis* strains (L10 and G1) on the white shrimp *Litopenaeus vannamei* was evaluated ([redacted] et al., 2012). Examined factors were growth performance, digestive enzyme activity, immune gene expression and disease resistance. Supplementation with *B. subtilis* was performed with two application dosages; BM5, at a final concentration of 10^5 CFU/g and BM8 at a final concentration of 10^8 CFU/g. Shrimp growth and disease resistance were shown to be improved by *B. subtilis* supplementation.

Some substances produced by *B. subtilis* could be used alternative biocontrol agents. Therefore, the toxicity of these pure substances to aquatic invertebrates has been studied by some authors. [redacted] et al. (2014) recently studied the use of surfactin and mycosubtilin as eco-friendly alternative to control *Bremia lactucae*, the causal agent of downy mildew on lettuce. Effects of mycosubtilin and surfactin on *Daphnia magna* revealed LC_{50} for immobilisation of 6 mg/L and 25 mg/L, respectively. In conclusion, both compounds were found to be efficient against lettuce downy mildew with low toxicity to invertebrates compared to chemical pesticides. These two substances might participate in the mode of action of *B. amyloliquefaciens* QST 713. However, they cannot be easily produced as pure substances and are not intended to be applied alone.

In conclusion, no evidence for toxicity or pathogenicity of *B. subtilis* and *B. amyloliquefaciens* to invertebrates was found in the open literature. Moreover, some studies determined positive probiotic effects of *B. subtilis* and *B. amyloliquefaciens*.

Cited references (abstracts):

Report: IIM 8.3/06 – [redacted], [redacted], [redacted], C.R., [redacted], M.S., [redacted], [redacted], [redacted], N (2012). Effects of *Bacillus subtilis* on the growth performance, digestive enzymes, immune gene expression and disease resistance of white shrimp, *Litopenaeus vannamei.*
Published report
Fish & Shellfish Immunology, 33, 683 – 689
M-530520-01-1

Abstract: Study of the effect of two probiotic *Bacillus subtilis* strains on the growth performance, digestive enzyme activity, immune gene expression and disease resistance of juvenile white shrimp (*Litopenaeus vannamei*). A mixture of two probiotic strains, L10 and G1 in equal proportions, was administered at two different doses 10(5) (BM5) and 10(8) (BM8) CFU g(-1) feed to shrimp for eight weeks. In comparison to untreated control group, final weight, weight gain and digestive enzyme activity were significantly greater in shrimp fed BM5 and BM8 diets. Significant differences for specific growth rate (SGR) and survival were recorded in shrimp fed BM8 diet as compared with the control; however, no significant differences were recorded for food conversion ratio (FCR) among all the experimental groups. Eight weeks after the start of the feeding period, shrimp were challenged with *Vibrio harveyi*. Statistical analysis revealed significant differences in shrimp survival between probiotic and control groups. Cumulative mortality of the control group was 63.3%, whereas cumulative mortality of the shrimp that had been given probiotics was 20.0% with BM8 and 33.3% with BM5. Subsequently, real-time PCR was employed to determine the mRNA levels of prophenoloxidase (proPO), peroxinectin (PE), lipopolysaccharide- and β-1,3-glucan-binding protein (LGBP) and serine protein (SP). The expression of all immune-related genes studied was significantly up-regulated ($P < 0.05$) in the shrimp fed BM5 and BM8 diets compared to the control group. These findings demonstrate that administration of *B. subtilis* strains, L10 and G1, can improve growth performance and disease resistance through an enhanced immune

response in shrimp.

Report: IIM 8.3/07 – [redacted], [redacted], [redacted], [redacted], [redacted]., [redacted], [redacted]., [redacted] (2014), Mycosubtilin and surfactin are efficient, low ecotoxicity molecules for the biocontrol of lettuce downy mildew
Published report
Appl Microbiol Biotechnol, 98, 6255-6264
M-530112-01-1

Abstract: The use of surfactin and mycosubtilin as an eco-friendly alternative to control lettuce downy mildew caused by the obligate pathogen *Bremia lactucae* was investigated. Preliminary ecotoxicity evaluations obtained from three different tests revealed the rather low toxicity of these lipopeptides separately or in combination. The EC50 (concentration estimated to cause a 50 % response by the exposed test organisms) was about 100 mg L(-1) in Microtox assays and 6 mg L(-1) in *Daphnia magna* immobilization tests for mycosubtilin and 125 mg L(-1) and 25 mg L(-1) for surfactin, respectively. The toxicity of the mixture mycosubtilin/surfactin (1:1, w/w) was close to that obtained with mycosubtilin alone. In addition, the very low phytotoxic effect of these lipopeptides has been observed on germination and root growth of garden cress *Lepidium sativum* L. While a surfactin treatment did not influence the development of *B. lactucae* on lettuce plantlets, treatment with 100 mg L(-1) of mycosubtilin produced about seven times more healthy plantlets than the control samples, indicating that mycosubtilin strongly reduced the development of *B. lactucae*. The mixture mycosubtilin/surfactin (50:50 mg L(-1)) gave the same result on *B. lactucae* development as 100 mg L(-1) of mycosubtilin. The results of ecotoxicity as well as those obtained in biocontrol experiments indicated that the presence of surfactin enhances the biological activities of mycosubtilin. Mycosubtilin and surfactin were thus found to be efficient compounds against lettuce downy mildew, with low toxicity compared to the toxicity values of chemical pesticides. This is the first time that *Bacillus* lipopeptides have been tested in vivo against an obligate pathogen and that ecotoxic values have been given for surfactin and mycosubtilin.

IIM 8.4 Effects on algal growth and growth rate

To evaluate the effects of *B. amyloliquefaciens* QST 713 on algal growth, an Alga growth inhibition test was conducted. No adverse effects were observed. For more information, please refer to the baseline dossier. The conclusions from the baseline dossier was that the overall risk *B. amyloliquefaciens* QST 713 to aquatic algae is considered to be acceptable.

To identify relevant literature on algal pathogenicity, a literature research was performed (please refer to Point IIM 8). No articles were identified as relevant.

IIM 8.5 Effects on aquatic plants

No data on the toxicity of *B. amyloliquefaciens* QST 713 to aquatic plants are availalble from the baseline dossier. A literature search was conducted to identify possible toxic or pathogenic effects of *B. subtilis* or *B. amyloliquefaciens* on aquatic plants. Using DIMDI database provided by the German Institute of Medical Documentation and comprised of searches in MEDLINE, BIOSIS, CAB Abstracts and SCISEARCH databases, search resulted in no relevant publications (please refer to Point IIM 8). Therefore, there is currently no evidence for toxicity of *B. amyloliquefaciens* QST 713 to aquatic plants.

IIM 8.6 Effects on terrestrial plants

No data on the toxicity of *B. amyloliquefaciens* QST 713 to terrestrial plants are availalble from the baseline dossier. *B. amyloliquefaciens* and closely related species are known to positively effect plants, accordingly, many of them belong to the plant growth promoting rhizobacteria (PGPR). PGPR are ubiquitous bacteria in rhizosphere, often associated to plant roots. *B. amyloliquefaciens* is able to improve plant health by direct effects on plant pathogens as well as by induction of plant resistance mechanisms and increased stress tolerance (please refer to Annex II, Doc M, Point IIM 2.3.2).

To identify publications presenting data on plant pathogenicity of *B. subtilis* or *B. amyloliquefaciens*, a literature search was conducted on the DIMDI database provided by the German Institute of Medical Documentation and comprised of searches in MEDLINE, BIOSIS, CAB and SCISEARCH databases, using terms "phytotoxic AND *Bacillus amyloliquefaciens*" (please refer to Point IIM 8). The search identified no relevant publications, dealing with adverse effects of *B. subtilis* on plants. It can therefore be concluded that no negative adverse effects of *B. amyloliquefaciens* have been reported in the literature to date.

IIM 8.7 Effects on bees

Several studies were conducted on bees. It was concluded that Serenade poses no significant risk to honeybees when applied at appropriate field rates. Please refer to the baseline dossier for the background information.

On bumblebees (*Bombus terrestris*), a study with the product Serenade MAX, containing *B. amyloliquefaciens* QST 713 was conducted ([redacted], 2006) on tomato-treated plants. Treatments with Serenade MAX (300 g/hL) were applied in greenhouses cultivated tomato either while bumblebees were actively foraging on flowers, to estimate direct exposure or (treatment A) or during the evening when bumblebee were in closed hives, to assess indirect exposure (treatment B). As a toxic standard, the reference Bioroten (250 g/hL) was applied. Thus, direct and indirect exposure (bumblebees exposed to the remains of the produt on the vegetation) were considered. Each treatment was applied three times at 7-day interval. Assessments were performed on days 0, 1, 2 and 4 after the spray application. Parameter used for assessment were mortality and foraging activity of bumblebees, as well as activity at the hives (no. of bumblebees enterin/exiting the hives).

Mortality was almost zero percent in all treated greenhouses throughout the entire study period. Also foraging activity was high in treated greenhouses in comparison to the untreated control. Moreover, activity at the hives was not influenced by direct or indirect treatment. Thus, B. amyloliquefaciens QST 713 containing product Serenade MAX was evaluated to be safe for bumblebees.

A literature search was conducted on the DIMDI database provided by the German Institute of Medical Documentation and comprised of searches in MEDLINE, BIOSIS, CAB and SCISEARCH databases. The search terms "bee" or "honeybee" and "*Bacillus amyloliquefaciens*" did reveal in 6 relevant hits. For more details on the literature search, please refer to [redacted] (2015), submitted in Point IIM 8.

A study was published by [redacted] et al. (2009) when evaluating side effects of microbiological control agents on *Bombus terrestris*. Adult workers were exposed via three different routes: contact application (50 µL, 7.5 × 10^9 CFU/L; 3.75 × 10^5 CFU/bee), orally via sugar water (continuously exposed to 500 mL, 7.5 × 10^9 CFU/L) and orally via treated pollen. Additionally, application of a dilution series (1/2, 1/5, 1/10) was tested for products exceeding lethal effects.

None of the tested microbiological control agents were shown to cause lethal effects on bumble bees in the fist 72 hours. After 11 weeks, high mortality was detected for Serenade® exposure via contact application (88%) or via drinking treated sugar water (100%). No toxicity was detected when Serenade was ingested through treated pollen. Reduced Serenade concentration (1/2) led to a strong reduction of mortality when applied topically (no significant mortality, 20%). However, mortality remains high with the orally application (1/10: 79%). Sublethal effects on drone reproduction was also evaluated. Contact application at concentrations of 1/2, 1/5, 1/10 detrimental effects were induced by Serenade®, with drone production of 29%, 36% and 58% of control nests (34 ± 5.6 drones). Oral application of Serenade® led to low reduction of reproduction rate when applied at 1/1, 1/2, 1/5 and 1/10: 0%, 0%, 4% and 5%, respectively. However; when fed through pollen patties no effects on mortality were shown. The authors also report that tests with dry exposure did not result in toxicity (data was not shown in the paper). Consequently the paper shows that through wet routes of exposure some degree of toxicity can be seen and indicates the potential intrinsic worse case toxicity *of B. subtilis* QST 713 on bumble bees under exaggerated exposure test conditions.

Unfortunately, no results on mortality were reported for the previous weeks, although examination was performed weekly. Moreover, data of the negative control is not presented clearly, however it is stated that it should be zero. Nevertheless, possible toxicity of *B. subtilis* QST 713 on bumble bee was clearly presented, although methods were not performed under good laboratory practice (GLP). Moreover, it has to be considered that agricultural use of *B. subtilis* QST 713 does not imply a

continuous wet administration over such a long time as carried out by [redacted] et al. (2009). The authors themselves stated that the assessment did not reflect the realistic conditions in field. During product use the effect of overspray and drinking water will be minimal for bumble bees (which do not drink water) and the major route of exposure will be through residues in pollen or dried product on plant surfaces. Consequently although the paper shows the potential for toxicity the routes of exposure are not relevant for the condition of use.

These findings were in contrast to a study by [redacted] et al. (2004). The authors examined the ability of Serenade® (*B. subtilis* QRD132) to control the fungal pathogen *Monilinia vacciniicorymbosi* when transmitted on blueberry flowers by honey bees. Honey bees carried about 5.1-6.4 × 10^5 CFU *B. subtilis* per bee. It was shown, that transportation of Serenade® by honey bees significantly reduced mummy berry disease. Although dose rate was relatively similar to the one tested by [redacted] et al. (2009), no toxicity of Serenade® on bees was not reported.

[redacted] et al. (2009) did not report bee toxicity when evaluating the use of bees as carriers of biocontrol agent, as well. Tested formulation was Biopro®, containing 2 × 10^{11} *B. subtilis* BD170 spores/g against *Erwinia amylovora*, the causal agent of fire blight. Dispersal activity was tested for two bee species: *Apis mellifera* and *Osmia cornuta*. Both bee species were shown to transport *B. subtilis* spores onto apple flowers, whereas toxicity on bees was not reported.

Examinations on toxic and hormonal responses of *Bombus impatiens* to *B. subtilis* QST 713 formulation (Serenade® Max) were later repeated by [redacted] & [redacted] (2012). Application of the product was performed at the recommended field concentration (RFC, 1×) as well in a dilution series (0.1×, 0.2×, 0.5×, and 2×). For the topical exposure, 50 µl of the solution were applied to the dorsal thorax of each bee, similar to the application performed to [redacted] et al. (2009). Bees were checked each day for 60 days on mortality, drone production, number of days to oviposition and number of days to drone emergence. Oral application was also performed with the recommended field rate, as well as with a dilution series (0.5×, 2×). Results totally differed from those gained by [redacted] et al. (2009). Topical application with *B. subtilis* did not cause mortality. Moreover, significantly higher drone production was shown when applied at 0.2× and 0.5× RFC. Oral exposure of Serenade® Max did not affect bee survival, as well. However, drone production was reduced with higher concentrations.

Another aspect is the probiotic effect of *B. subtilis* on bees. [redacted] et al. (2012) studied the beneficial effects of *B. subtilis* subsp. *subtilis* Mori2 on the performance of honeybees. Over 8 month, bees were administered ones a month to 1 L of sugar-syrup suspension supplemented with *B. subtilis* spores at a final concentration of 10^5 spores/mL and compared to a non-supplemented sugar-syrup suspension. Observed parameters were the number of bees in each frame, open and operculated brood areas and quantity of honey. Furthermore, infestation with bee pathogens *Nosema* sp. and *Varroa* (V. *destructor*) were assessed to determine bee health. It was shown that *B. subtilis* spores positively affect bee behaviour and health. The total number of bees was 26% higher when treated with bacteria in comparison to the control, since egg laying was stimulated. In follows, honey storage was higher (17%), too. Moreover, infestation with bee pathogens was lower in comparison to untreated control. This finding was confirmed by [redacted] et al. (2014) when testing the antimicrobial activity of *B. subtilis* strains against *Ascosphaera apis*, the causal agent of chalkbrood disease. *B. subtilis* were isolated from healthy worker honeybees and tested on their antimicrobial activities. *B. subtilis* B2 showed the highest antagonistic activity and offers potential for biological control of this serious bee pathogen.

Taken together, toxicity of *B. subtilis* QST 713 has been reported by [redacted] et al. (2009). However, these findings were not confirmed by other authors.

<u>Cited references (abstracts):</u>

Report: IIIM 8.7/20 – [redacted]. (2006), Side effects of the biofungicide *Bacillus subtilis* QST 713 on bumblebees (*Bombus terrestris*) on protected tomato in eastern Sicily. Unpublished Report No. E 06PH011, 27.01.2006
M-473493-01-1

Guideline: EPPO guideline PP 1/170(3)

GLP: Yes

Materials and Methods: The study was conducted during the period 30.11.2005 to 22.12.2005, at the farm holding [redacted]), Sicily. The test material used was Serenade MAX containing *Bacillus subtilis* QST 713. Four *Bombus terrestric* mini-hives were placed within each greenhouse cultivated with protected tomato. The substance Serenade MAX was tested at a content of 300 g/hL corresponding to 3 kg/ha at a spraying volume of 1000L/ha. Bumblebees were directly and indirectly (12 hours after spraying) exposed for a period of 21 days. In total three successive applications with 7-days intervals were conducted. As toxic standard "Bioroten" (Rotenone) was used (250 g/hL = 2.5 kg/ha). Bumblebee mortality, foraging activity (percentage of flowers with bite marks on 200 randomly selected flowers per treatment) and activity at the hives (number of bumblebees entering/exiting mini-hives per time unit) were assessed twice a day at day 0, 1, 2 and 4 after each application. Throughout the study period, the crop was examined for the presence of phytotoxic effects of the tested products.

Findings: Bumblebee mortality was almost 0 % in all treated greenhouses throughout the entire study period. Only two dead bumblebees were detected at day 1 after the first application of Serenade MAX (direct exposure). Foraging activity in the treated greenhouses was always very high and comparable to that of the untreated greenhouse. The percentages of flowers with bit marks ranged from 86 to 99 % during direct exposure and from 83 to 100 % when bumblebees were exposed to residues of *B. subtilis* QST 713 on the crops, respectively. Also bumblebee activity at the hives was not influenced by direct and indirect exposure to *B. subtilis* QST 713, whereas slight negative effects, lasting for a few hours, were observed after application of Bioroton. Phytotoxic effects at the tomato crops did not occur throughout the study period

Conclusions: Serenade MAX can be considered as safe for bumblebees

Report: IIM 8.7/21 – [redacted], [redacted], [redacted], [redacted] (2009) A laboratory evaluation to determine the compatibility of microbiological control agents with the pollinator *Bombus terrestris*, published report.

Pest Management Sci 65:949-955

M-530333-01-1

Abstract:

Background: This study was undertaken to identify any potential adverse side effects of the use of seven microbiological control agents (MCAs) on the bumblebee, *Bombus terrestris* L., in the context of combined use in integrated pest management (IPM).AQ10 (*Ampelomycesquisqualis*), Binab-T-vector (*Hypocreaparapilulifera* + *T. atroviride*;1/1), Prestop-Mix (*Gliocladium catenulatum* J1446), Serenade (*Bacillus subtilis* QST713), Trianum-P (*Trichoderma harzianum* T22), Botanigard (*Beauveria bassiana GHA*) and Granupom (*Cydia pomonella* granulovirus), comprising five biofungicides and two bioinsecticides, were investigated. Bumblebee workers were exposed under laboratory conditions to each MCA at its maximum field recommended concentration (MFRC) via three different routes of exposure: dermal contact and orally via either treated sugar water or pollen.

Results: The tested MCAs were found to be safe for workers of *B. terrestris*, with the exception of Botanigard and Serenade. Exposure to Botanigard via contact at its MFRC caused 92% mortality after 11 weeks, while the 1/10 MFRC killed 46% of exposed workers. For Serenade, topical contact and oral delivery via sugar water resulted in 88 and 100% worker mortality respectively. With lower concentrations (1/2, 1/5 and 1/10 MFRC) the toxicity decreased, but the effect depended on the route of exposure. When fed through pollen patties no effects on mortality were shown. The authors also report that tests with dry exposure did not result in toxicity (data was not shown in the paper). In addition to lethal effects, nests were also evaluated for sublethal effects after treatment with the seven MCAs at their respective MFRCs over 11 weeks. In these bioassays, only Botanigard and Serenade gave rise to a significant (P < 0.05) decrease in drone production. Sublethal effects on foraging behavior were also evaluated, and only Botanigard at its MFRC delivered via treated sugar water induced negative effects.

Conclusion: The results demonstrated that most of the MCAs tested can be considered safe for use in combination with *B. terrestris*, based on the International Organization for Biological Control of Noxious Animals and Plants (IOBC) classification. However, some can be potentially harmful, such as the biofungicide Serenade and the bioinsecticide Botanigard. Therefore, it is recommended that all should be tested before use in combination with pollinators. In this context, it is also

advisable that these MCAs be evaluated in more realistic field situations for the assessment of potentially deleterious effects on foraging behaviour.

Notifiers comment: The findings from paper show that through wet routes of exposure some degree of toxicity can be seen and indicates the potential intrinsic worse case toxicity *of B. subtilis* QST 713 on bumble bees under exaggerated exposure test conditions. During product use the effect of overspray and drinking water will be minimal for bumble bees (which do not drink water) and the major route of exposure will be through residues in pollen or dried product on plant surfaces. Consequently although the paper shows the potential for toxicity the routes of exposure are not relevant for the condition of use.

Report: IIM 8.7/22 – [redacted], [redacted], K.S., [redacted], H. (2004), Effectiveness of honey bees in delivering the biocontrol agent *Bacillus subtilis* to blueberry flowers to suppress mummy berry disease
Published report
Biological control, 31, 422-427
M-518909-01-1

Abstract: Honey bees are important pollinators of commercial blueberries in the southeastern United States, and blueberry producers often use supplemental bees to achieve adequate fruit set. However, honey bees also vector the plant pathogenic fungus *Monilinia vaccinii-corymbosi* which infects open blueberry flowers through the gynoecial pathway causing mummy berry disease. Here, we report the results of a 3-year field study to test the hypothesis that using bee hives equipped with dispensers containing the biocontrol product Serenade, a commercial formulation of the bacterium *Bacillus subtilis* which has shown activity against flower infection by *M. vaccinii-corymbosi* in laboratory experiments, can reduce mummy berry disease incidence when honey bees are used as pollinators in blueberries. Individual honey bees carried 5.1–6.4 × 10^5 colony-forming units (CFU) of *B. subtilis* when exiting hive-mounted dispensers with Serenade. On caged rabbiteye blueberry bushes in the field, population densities of *B. subtilis* vectored by honey bees reached a carrying capacity of $<10^3$ CFU per flower stigma within 2 days of exposure, and there was a highly significant non-linear relationship between *B. subtilis* populations per stigma and bee activity, expressed as number of legitimate flower visits per time interval per cage ($R = 0.6928$, $P < 0.0001$, $n = 32$). Honey bee density (1600 or 6400 individuals per 5.8-m^3 cage) and Serenade treatment (presence or absence of the product in hive-mounted dispensers) significantly ($P < 0.05$) affected the incidence of fruit mummification on caged bushes, whereby increasing bee density increased disease incidence and application of Serenade reduced disease levels. Taken together, results of this study suggest that use of a hive-dispersed biocontrol product such as Serenade as a supplement during pollination can reduce the risk of mummy berry disease. This may be a prudent practice that optimizes the benefits to pollination of high bee densities while reducing the associated disease-vectoring risk.

Report: IIM 8.7/23 – Maccagnani, M., [redacted], [redacted], [redacted], [redacted], [redacted] (2009), *Apis mellifera* and *Osmia cornuta* as carriers for the secondary spread of *Bacillus subtilis* on apple flowers
Published report
BioControl, 54, 123-133
M-530515-01-1

Abstract: The efficiency of two pollinators, *Apis mellifera* L. (Hymenoptera: Apidae) and the mason bee *Osmia cornuta* (Latreille) (Hymenoptera: Megachilidae), as carriers of biocontrol agents (BCA) from flower to flower (secondary colonisation) was investigated on apple cv 'Golden Delicious'. The BCA tested was *Bacillus subtilis*, strain BD170 (Biopro®) developed for the control of the 'fire blight' caused by *Erwinia amylovora* (Burril) Winslow et al. The two insect species were studied as secondary BCA carriers on apple plants in pots under net screened tunnels. Their behaviour and capacity to deposit the BCA in the most receptive flower parts were compared both by washing, diluting and plating the flower organs on a recovery medium and by means of PCR analyses based on a molecular marker. *O. cornuta* showed better performances with respect to *A. mellifera*. For the field trials, pollinators were introduced in four apple orchards. During apple's flowering, the BD170 (100 g hl^{-1}) was sprayed once in two fields, and twice in the others. The

pollinators' efficacy in carrying the BCA from sprayed flowers to the stigmas of newly opened ones at different times after the spray treatment was evaluated. The detection of the BCA was performed by PCR analysis. The percentages of positive PCR flower samples were higher in the internal treated areas of the fields with respect to the external untreated ones, but the high colonisation level found in the latter and in the flowers opened in both areas several days after the treatment(s) demonstrated that pollinators can play an important role as secondary carriers.

Report: IIM 8.7/24 – [redacted], [redacted](2012), Different toxic and hormetic responses of *Bombus impatiens* to *Beauveria bassiana*, *Bacillus subtilis* and spirotetramat

Published report

Pest Manag Sci, 69, 949-954

M-530341-01-1

Abstract:

Background: Pollinator exposure to pesticides is a concern in agricultural systems that depend on pollinators for crop production. However, not all pesticides elicit toxic effects, and response to a pesticide will vary depending on dose and exposure route. The effects of biopesticide formulations of *Bacillus subtilis* and *Beauveria bassiana* and of the tetramic acid insecticide spirotetramat on the common eastern bumblebee, *Bombus impatiens*, were evaluated. Microcolonies of bees were exposed to field-rate or lower concentrations, and data were collected over 60 days.

Results: When ingested, field rates of spirotetramat caused high mortality after 10 days, and *B. subtilis* significantly reduced drone production, number of days to oviposition and number of days to drone emergence. Converse to effects observed following ingestion, topical applications of *B. subtilis* at concentrations less than the recommended field rate resulted in a hormetic response, with significantly increased drone production. Topical application of spirotetramat and oral or topical application of *B. bassiana* had no effects on bees.

Conclusions: Spirotetramat and *B. subtilis* can induce adverse effects on B. impatiens, but hormetic effects following *B. subtilis* treatment can also occur, depending on exposure route. Additional experiments are required to determine whether similar toxic or hormetic effects occur under more realistic field conditions.

Report: IIM 8.7/25 – [redacted]., [redacted], M.S., [redacted]., [redacted], M.C. (2012), Beneficial effects of *Bacillus subtilis* subsp. *subtilis* Mori2, a honey-associated strain, on honeybee colony performance.

Published report

Probiotics and Antimicrobial Proteins, 4, 39-46

M-530529-01-1

Abstract: A *Bacillus* spp. strain isolated from a honey sample in [redacted] (Salta, Argentina) was phylogenetically characterized as *B. subtilis* subsp. *subtilis* Mori2. The strain was administered to bee colonies as a monoculture in one litre of sugarcane syrup (125 g/L) at a final concentration of 10^5 spores/mL to evaluate the bee colony performance. The treated colony was monitored, and any changes were compared with the control hives. All conditions were identical (weather, nourishment and supervision), except for the *Bacillus* spore supplement. The new nourishment, which was administered monthly from May to December 2010, was accepted by the bees and consumed within ca. 24–48 h. Photograph records and statistic analyses revealed significant differences in the open and operculated brood areas between the treated and control groups. The status of the colony improved after the second administration of the *Bacillus* spores until the end of the experiment. A higher number of bees were counted in the treated groups (26% more than the control) with respect to the initial number. Furthermore, at the time of harvest, honey storage in the treated hives was 17% higher than in the control hives. In addition, spore counts of both *Nosema* sp. and *Varroa* sp. foretica in treated hives were lower than in the control hives. These results with experimental hives would indicate that *B. subtilis* subsp. *subtilis* Mori2 favoured the performance of bees; firstly, because the micro-organism stimulated the queen's egg laying, translating into a higher number of bees and consequently more honey. Secondly, because it reduced the prevalence of two important bee diseases worldwide: nosemosis and varroosis.

Report: IIM 8.7/26 – [redacted]., [redacted], A.M., [redacted], M.J., [redacted]., [redacted], B.F., [redacted], [redacted] (2014), Antagonistic effect of gut bacteria in the hybrid Carniolan honey bee, *Apis mellifera carnica*, against *Ascosphaera apis*, the causal organism of chalkbrood disease
Published report
Journal of Apicultural Science, 58, 17-27
M-530344-01-1

Abstract: The objective of this study was to isolate and characterize bacterial strains associated with the gut of the hybrid Carniolan honey bee, *Apis mellifera carnica*, and to determine their in vitro and in vivo potential against *Ascosphaera apis*, the causal organism of chalkbrood disease, with the purpose of exploring feasible biological control. Six bacterial strains were isolated from healthy worker honey bees by culture-dependent methods. Six fungal strains (A3, A4, A7, A8, A9, and A15) of *A. apis* were isolated from larvae suffering from chalkbrood disease on Yeast–Glucose–Starch agar (YGPSA) medium. All bacteria were identified by a combination of morphology, Gram stain, and 16S rRNA sequence analysis, and fungal strains were identified by morphology and 5.8S rRNA. In vitro and in vivo inhibition assays were carried out to determine the ability of bacterial isolates to inhibit *A. apis*, the causal agent of chalkbrood disease. The analysis of 16S rRNA sequences revealed that four bacterial strains (B2, B4, B10, and B100) belong to *Bacillus subtilis* species, and two strains (P1 and P5) belong to *Pseudomonas fluorescence*. Significant differences in antagonistic activity of all bacterial strains were observed. *B. subtilis* isolate B2 showed the highest antagonistic activity, as measured by the inhibition zone against *A. apis*, followed by the P1 strain of *P. fluorescence*. SEM analysis also supports the antagonistic activity of these bacteria against *A. apis*. This study provides a theoretical basis for biological control of honey bee chalkbrood disease.

IIM 8.8 Effects on terrestrial arthropods other than bees

For the evaluation of the effects of *B. amyloliquefaciens* QST 713 on non-target arthropods, several studies were performed:
Dietary pathogenicity and toxicity studies had been performed on the ladybird beetle *Hippodamia convergens* (1998, no adverse effects), green lacewing larvae *Chrysoperla carnea* (1998, no adverse effects), parasitic *Hymenoptera Nasonia vitripennis* and Chrysoperla carnea (1998, no adverse effects are anticipated). Acute toxicity studies had been performed on *Aphidius rhopalosiphi* (2000, no significant effects) and toxicity study to predatory mite *Typhlodromus pyri* (2000, no effects on fertility, low effects on mortality). For more information, please refer to the baseline dossier.

For confirmation, a literature search was conducted on the DIMDI database provided by the German Institute of Medical Documentation and comprised of searches in MEDLINE, BIOSIS, CAB and SCISEARCH databases to identify the potential of environmental risk of *B. amyloliquefaciens* QST 713 on terrestrial arthropods by combination of the search terms “*Bacillus subtilis*” or “*Bacillus amyloliquefaciens*” and “arthropod” or “insect”. Regarding B. amyloliquefaciens QST 713, no effects on non-target arthropods other than bees were identified. Since several studies had been carried out to identify biological control agents against various pest species, also Bacillus strains were identified, which were active against pathogen insects. However, host specifity is strain specific. Thus, it can be concluded that some *B. amyloliquefaciens* strains may have effects on other (pathogenic) insect species, although this is not consequently transferrable to *B. amyloliquefaciens* strain QST 713. For more details on the literature search, please refer to [redacted] (2015), submitted in Point IIM 8.

B. subtilis and *B. amyloliquefaciens* are ubiquitous bacteria with a worldwide distribution, and were isolated from a large variety of plants, environment (e.g. soil, rhizosphere, maritime ecosystems), animals or insects. Several of these isolated bacteria are described for their antimicrobial activity or their potential for use in plant protection against pests or plant pathogens. Some of them are already registered as active substances and have a long history of save use. Thus, bacterial species as *Bacillus* sp. are often screened for their activity against pests or diseases, were alternative control strategies are needed. In some cases, these strains were isolated from the direct environment where the pest occurred. Other tested strains used in screenings, are already registered as active substances for plant protection, as *B. amyloliquefaciens* QST 713 (formerly designated as *B. subtilis* QST 713).

Thus, several studies were identified which evaluated the efficacy of *B. amyloliquefaciens* QST 713 on other pests or diseases, or to identify toxicity to non-target (beneficial) species.

In the course of an evaluation of alternative fungicides against Apple Scab, the efficacy of *Bacillus subtilis* QST 713 (Serenade MAX) was compared with a standard organic sulphur/lime sulphur fungicide program and a non-treated control ([redacted] et al., 2011). Therefore, the maximum labelled application rate of 3.4 kg Serenade MAX/ha was applied. No toxicity effects on the pest species European red mites, two-spotted spider mites, white apple leafhopper and potato leafhopper were detected. Serenade however appeared to have indirect negative impacts on the control of damaging insects when compared to the non-fungicide-treated controls.

B. subtilis formulation Serenade was also studied for its potential use in strawberry production against powdery mildew ([redacted] et al., 2008). Within the same study a monitoring of potential side effects on beneficial predatory mite *Amblyseius andersoni* and efficacy against the pest *Tetranychus urticae* were evaluated. It was shown, that Serenade neither increased populations of phytophagous mites, nor influenced beneficial predatory mite populations. Serenade therefore was regarded safe for the ecological balance of arthropods in strawberry greenhouses.

The beneficial mite species *Amblyseius andersoni* and the parasite mite species *Tetranychus urticae* were also tested with Serenade ([redacted] et al., 2004). When applied at the recommended field concentration (RFC) no effects were observed for this *B. amyloliquefaciens* formulation.

B. subtilis QST 713 formulation Rhapsody AS was tested against adults and larvae of the beneficial coleopteran species *Halyziine coccinellids* ([redacted] et al., 2010). *Halyziine coccinellids* are known to reduce powdery mildew by consumption. The *B. subtilis* formulation was applied directly to the dorsum (0.5 mL) of each individual at a concentration of 135 mg/L *B. subtilis* and the mortality assessed. No significant differences when compared to the control were detected, indicating that the risk for this coleopteran species is very low.

Non-registered *Bacillus* sp. had been isolated form Tunesian olive tree habitats and tested on their larvicidal activity on olive pests *Prays oleae*, *Palpita unionalis*, *Hylesinus oleiperda* and *Phloeotribus scarabaeoides* ([redacted] et al., 2012). *Bacillus* sp. were prevalent in 81% of the samples taken randomly from olive tree habitats, confirming the natural occurence of *Bacillus* sp. The most occuring species were *Paenibacillus polymyxa* and *Bacillus braevis*. Entomopathogenicity on insect larvae was only shown for *Bacillus thuringiensis* supsp. *kurstakii*, *Bacillus licheniformis*, *P. polymyxa* and *B. brevis* but not for *B. subtilis*.

B. subtilis was one of the bacterial species isolated from the pine processionary moth *Thaumetopoea pityocampa* when evaluation potential biocontrol agents against this pest ([redacted] et al., 2008). However, no insecticidal effects on *T. pityocampa* were shown.

To identify microorganisms with insecticidal potential against the olive pest *Bactrocera oleae*, a total of 115 bacterial strains from various ecological niches were tested ([redacted] et al., 2012). Bioassays with bacterial strains and *B. oleae* larvae identified one *B. subtilis* strain (Abs3b), showing 99% larvae mortality. From this strain the crude insecticidal toxins were extracted and tested on their larvicidal activity. The highest mortality was observed at pH7 (LC_{50} = 25.8 µL/mL).

B. subtilis EPC8 was also tested on its efficacy against the 3rd-instar larval stage of the fruit borer (*Helocoverpa armigera*) on tomato ([redacted] et al., 2014). Seeds and roots were soaked in a talc-based fromulation containing 2.5-3 × 10^8 CFU/g. 46 days after planting, leaves were fed to the larvae. Morover, a field trial on tomato plants was conducted. It was shown, that *B. subtilis* effectively controled the fruitborer in field (55%) and larval mortality of 53% was recordet. However, both efficacy and mortality were much lower than with the pesticide control (carbendazim + quinalphos) and with *Bauveria bassiana* application or in combination with *B. bassiana*.

Against cotton leafworm Spodoptera *littoralis, B. subtilis* NRC313, isolated from soil of cotton fields was tested (El-Salam et al., 2011). 3rd-instar larvae were fed with leaves dipped for 2 minutes in cell suspensions of three *B. subtilis* concentrations: 10 × 10^8, 5 × 10^8 and 2.5 ×10^8 CFU/mL. Pupae were fed for a 2 day period with treated leaves. Mortality was about 100%, 79% and 21% for the above mentioned concentrations. LC_{50} was calculated to be 3.3 × 10^8 CFU/mL. Hoever, total amount of bacterial intake was not estimated and trials were not conducted under good experimental praxis (GEP).

Since members of *Bacillus* sp. are known to produce metabolites with insecticidal activity, some of these analysed or non-analysed substances were already tested against a wide range of pest insects to

identify alternative substances for pest control. [redacted] et al. (2012a) studied the insecticidal activity of a lipopeptide biosurfactant from *B. subtilis* SPB1 against the Egyptian cotton leaf worm *Spodoptera littoralis*, which causes considerable damage to many crops. The authors showed toxicity with an LC_{50} of 551 ng/cm² of the studied biosurfactant against neonate larvae of *S. littoralis*. However, the biosurfactant was not further analysed. *B. subtilis* SPB1 biosurfactant was also tested on its histopathological effects in the midgut of the Mediterranean flour moth *Ephestia kuehniella* ([redacted] et al., 2012b). It was shown that the most frequently effects were cell vacuolisation, microvilli damage and epithelium cell contents passing into the midgut lumen. Nevertheless it has to be considered, that effects can not be transferred to *B. amyloliquefaciens* QST 713 due to the species difference.

A crude mosquitocidal toxin (CMT) produced by *B. subtilis* subsp. *subtilis* B-471 was tested on mosquito larvae ([redacted] & Mannomani, 2008). This substance was obtained from the cell free broth by acid precipitation. It was tested against III instar larvae and freshly moulted pupae of the mosquito species *Anopheles stephensi*, *Culex quinqefasciatus* and *Aedes aegyptii*. It was shown, that the CMT was active against all tested species, but LC_{50} differed between developmental stage and species. However, the substance was not further analysed. Toxicity of *B. subtilis* on mosquito larvae was also confirmed by [redacted] & [redacted] (2013) when testing bacterial cells against *Anopheles arabiensis*. Adulticidal activity of a biosurfactant from *B. subtilis* subsp. *subtilis*, containing surfactin as main component was tested on mosquito *Anopheles stephensi* ([redacted] et al., 2012). For this surfactant a LD_{50} of 16.13 mg/m³ on adult mosquitos was detected.

On the other hand, *B. subtilis* was shown to occur naturally in mosquitos. [redacted] et al. (2007) isolated *B. subtilis* from *Aedes aegyptii* and detected their prevalence in the diverticulum of the mosquito gut by PCR analysis.

Since *Bacillus* subtilis are ubiquitous bacteria and worldwide distributed, numerous studies report the prevalence of *Bacillus* species in insects, without causing toxic effects. Soil invertebrates are described to harbour complex microbial communities with a total number of 10^{11} mL/L microbes in the hindgut ([redacted] (2005). For example, *B. subtilis* had been detected in termite guts where *Bacillus* sp. can reach titres of 10^7 mL/L in the gut. They are essential for the digestion of polysaccharides and aromatic compounds.

[redacted] et al. (2012) studied the microbial colonization of five sand fly species and reported the isolation of 5 *B. subtilis* strains from midgut but not from cuticle. [redacted] et al. (2009) isolated *B. subtilis* from the gut of the velvetbean canterpillar (*Anticarsia gemmatalis*), a key pest of soybean. From the wireworm *Agriotes lineatus*, a serious agricultural pest of various vegetables and fruits, *Bacillus* sp. next to other microorganisms were isolated ([redacted] et al., (2012). *B. subtilis* has also been reported to be prevalent in the gut of the cola nut weevil, [redacted] in Nigeria ([redacted] & [redacted], 2010).

In conclusion, *Bacillus* sp. are ubiquitous, worldwide distributed bacteria. The use of *B. amyloliquefaciens* and *B. subtilis* as active substances for biological control of pests and diseases is not new. Thus, several publications report the examination of *Bacillus* sp. strains for the control of pests and diseases, where alternatives to chemical products are still not registered. The identification of numerous publications dealing with the isolation and identification of *B. subtilis* is therefore not unusual. Several publications (not all stated, please refer to the literature review report, submitted under Point IIM 8), deal with the insecticidal activity against mosquitos to identify sustainable and effective methods for the control of mosquito-transferrable diseases, as Malaria or Dengue fever. These studies identified *B. subtilis* species with mosquitocidal activity. Nevertheless, it has to be considered, that toxicity on arthropods is in general strain specific. Moreover, phylogeny from *B. amyloliquefaciens* QST 713, clearly differ from *B. subtilis* (please refer to Annex II, Doc IIM, Point IIM 1.3.1).

Nevertheless, some articles studied the use of *B. amyloliquefaciens* QST 713 products against other diseases under consideration of their toxic potential on non-target arthropods. Compendiously, the impact on beneficial organisms as predatory mites, or coleopteran species is very low.

<u>Cited references (abstracts):</u>

Report: IIM 8.8/06 – [redacted], L.P., [redacted], H.M. (2011), Alternative Organic Fungicides for Apple Scab Management and Their Non-target Effects

Published report
HortScience, 46, 1254-1259
M-518628-01-1

Abstract: A major challenge in organic apple production in humid production regions is the available fungicide options for apple scab [*Venturia inaequalis* (Cooke) Wint.] management. The standard sulfur/lime sulfur fungicide program can be injurious to the applicator, the apple ecosystem, and the apple tree itself. The objectives of this study were to compare the efficacy of three potential alternative fungicides [potassium bicarbonate (PB), neem oil (NO), and *Bacillus subtilis* (Bs)] with a standard organic sulfur/lime sulfur (SLS) fungicide program and a non-treated control (NTC) for management of apple scab and to evaluate potential non-target impacts on pest and beneficial arthropod populations. The five treatments were applied to 'Empire' trees arranged in a completely randomized design with five single-tree replications at the University of Vermont Horticultural Research Center in South Burlington, VT. Fungicides were applied with a handgun to drip using maximum label rates. Applications began on 26 Apr. 2007 and 23 Apr. 2008 and continued on approximately a weekly schedule through the end of June and then every 2 weeks through 23 July 2007 and 17 July 2008, respectively. The standard SLS treatment resulted in the best scab control in both years. The NO treatment reduced foliar and fruit scab compared with the NTC and the other alternatives at the end of the 2008 growing season and had insecticidal activity. However, both the SLS and NO treatments had disadvantages, including phytotoxic burning on the fruit and/or significantly more russeting on the fruit at harvest. In each year of the study, one or more of the alternative treatments, particularly Bs, resulted in higher insect damage than the non-fungicide-treated control. This research showed that PB, Bs, and NO do not offer advantages over the standard SLS fungicide program in organic apple production and in some cases offer distinct disadvantages in terms of non-target impacts. Chemical names used: potassium bicarbonate (Armicarb "O"), *Bacillus subtilis* (Serenade MAX), neem oil (Trilogy), sulfur (Microthiol Sulfur)/lime sulfur (Miller Lime Sulfur)

Report: IIM 8.8/07 – [redacted], [redacted], [redacted], [redacted], [redacted], [redacted], Y (2008), Integrating biocontrol agents in strawberry powdery mildew control strategies in high tunnel growing systems.
Published report
Crop Protection, 27, 622–631
M-520060-01-1

Abstract: Strawberry powdery mildew is a serious disease and its control is based on chemical fungicides. The efficacy of alternatives to chemical fungicides was evaluated and their integration into strategies against strawberry powdery mildew to reduce pesticide residues on fruits was also tested. Bicarbonates and mineral oils were poorly effective. Biocontrol agents (BCAs), like *Ampelomyces quisqualis*, *Bacillus subtilis* and *Trichoderma harzianum* T39, controlled the disease, but to a lesser extent than chemical fungicides. When BCAs were alternated with chemicals, a significant reduction of chemical fungicide residues was achieved while maintaining good powdery mildew control. None of the tested strategies increased populations of the pest *Tetranychus urticae*, nor had side effects on the beneficial predatory mite *Amblyseius andersoni* populations and, consequently, they can be regarded as safe for the ecological balance of arthropods in strawberry greenhouses.

Report: IIM 8.8/08 – [redacted], [redacted], [redacted], [redacted] (2004), Evaluation of the effects of biocontrol agents (BCA) on the beneficial *Amblyseius andersoni* and the parasite *Tetranychus urticae* mites.
IOBC/wprs Bulletin, 27, 193-196
M-530440-01-1

Abstract: The effect of commercial and experimental biocontrol preparations (AQ 10, Serenade, TRICHODEX and *Beauveria bassiana* strains B1 and B3) and of some fungicides (Ortiva, Tiovit jet) were tested in laboratory trials on the predatory mite *Amblyseius andersoni* Chant and on the spider mite *Tetranichus urticae* Koch. Direct and residual toxicity tests with females of the phytoseid showed that all the tested BCA based preparations and Ortiva are harmless (<30% of

toxicity). Tiovit jet (31.61%) was slightly harmful. In contrast, the reference Pyrethrum showed a high toxicity level (100% mortality). Moreover, the tested BCAs did not interfer with the *T. urticae* spider mite populations, neither in term of adult survival nor in term of demographic parameters.

Report: IIM 8.8/09 – [redacted]., [redacted], W.D., [redacted], M.P. (2010), Effects of fungicides on a mycophagous coccinellid may represent integration failure in disease management
Published report
Biological Control, 54, 292-299
M-518665-01-1

Abstract: The adults and larvae of halyziine coccinellids (Coleoptera: Coccinellidae: Halyzini) are obligate mycophages on hyphae and conidia of powdery mildew (PM) (Erysiphales) fungi, that are plant pathogens warranting chemical control in many managed systems. These insects have been observed to reduce PM severity through consumption. Fungicide applications, however, may interfere with this ecological service. Five commercial fungicides were topically applied to the mycophagous coccinellid, *Psyllobora vigintimaculata*, in the laboratory to gauge contact toxicity. In order to detect interference in the field, population density of naturally occurring *P. vigintimaculata* was assessed weekly in a northern California vineyard (*Vitis vinifera*, cultivar "Chardonnay") over 3 years in relation to PM (*Erysiphe necator*) severity and in the presence of various fungicides. Wettable sulfur was toxic to adults in the laboratory, resulting in complete cohort mortality 24 h after application. Topical applications of a strobilurin fungicide (trifloxystrobin) and a demethylation inhibitor fungicide (myclobutanil) also resulted in significant adult mortality. Rapid and complete larval mortality was observed in the laboratory after contact with wettable sulfur and myclobutanil. There was no effect on survival after contact with the PM-antagonistic bacterium, *Bacillus subtilis*. Vineyard density of *P. vigintimaculata* was reduced in vines receiving applications of sulfur and myclobutanil, even when considering the covariate PM severity. The microbial antagonist, *Streptomyces lydicus*, did not significantly affect insect density. Our study questions the integration of chemical disease management with naturally occurring mycophagous agents in some agricultural systems.

Report: IIM 8.8/10 – [redacted], [redacted], [redacted], [redacted], [redacted], [redacted], [redacted] (2012), Isolation of entomopathogenic *Bacillus* from a biodynamic olive farm and their pathogenicity to lepidopteran and coleopteran insect pests
Published report
Crop Protection, 31, 72-77.
M-520066-01-1

Abstract: The occurrence of *Bacillus* entomopathogenic bacteria on a Tunisian biodynamic farm was determined by examining 75 samples from olive tree (*Olea europaea* L.) habitats. A total of 40 *Bacillus* isolates were characterized according to their phenotypic, physiological and biochemical parameters. Isolates of the species *Bacillus subtilis*, *Bacillus mycoides*, *Brevibacillus brevis*, *Paenibacillus polymyxa*, *Bacillus licheniformis*, *Bacillus* sp. (1), *Bacillus* sp. (2) and a standard strain Btk HD-1 were used separately in feeding bioassays on fresh artificial diet against larvae of lepidopterans *Prays oleae* ([redacted]) and *Palpita unionalis* (Hübner) and coleopterans *Hylesinus oleiperda* (F.) and *Phloeotribus scarabaeoides* (Bernard), which are olive tree pests. Larvae were successfully reared on an artificial diet with 25 g powdered olive tree leaves. Compared to the control data, only Btk and the isolates of *B. licheniformis*, *P. polymyxa* and *B. brevis* were entomopathogenic. Larval mortality assessed 7 days post-treatment showed high mortality rates with Btk to lepidopteran larvae (86.6% for *P. oleae* and 80.9% for *P. unionalis*) and low mortality against coleopteran pests. *B. brevis* isolates showed high mortality rates against *P. oleae* (up to 67.9%). *B. licheniformis* isolates caused up to 59.2% larval mortality for *P. oleae* and 43.6% for *P. unionalis*. Highest coleopteran mortality was achieved by *P. polymyxa* isolates (up to 55%). According to the 16S rDNA results, isolates of each of the three entomopathogenic strains were similar. Proteins in the strain supernatants were toxic to *P. oleae* larvae with LC50 values of 10.0 (*B. brevis*), 12.5 (*B. licheniformis*) and 37.6 µg/ml (*P. polymyxa*). Also, *P. polymyxa* showed an LC50 of 12.4 mg/l against *P. scarabaeoides*. Our results suggest that entomopathogenic *Bacillus* present locally in the biodynamic farm could be used in biological control programmes of olive tree pests.

Report: IIM 8.8/11 – [redacted]., [redacted], [redacted], [redacted] (2008), Isolation and identification of bacteria from *Thaumetopoea pityocampa* Den. and Schiff. (Lep., *Thaumetopoeidae*) and determination of their biocontrol potential
Published report
World Journal of Microbiology and Biotechnology , 24, 3005-3015
M-520064-01-1

Abstract: The pine processionary moth *Thaumetopoea pityocampa* (Den. and Schiff.) is one of the most harmful insect pest for pine species in Mediterranean countries including Turkey. The objective of the present study is to find a more effective and safe biological control agent against *T. pityocampa*. Thus, we investigated the bacterial flora of the pest insect, collected from the Middle Black Sea Region of Turkey from 2003 to 2004. Based on morphological, physiological, biochemical and molecular methods, 14 different bacterial isolates were determined. The identified bacterial flora of *T. pityocampa* consisted of bacteria belonging to the Enterobacteriaceae (Tp1), *Arthrobacter* sp. (Tp2), *Staphylococcus* spp. (Tp3 and 10), *Bacillus subtilis* (Tp4), *Serratia liquefaciens* (Tp5), *Bacillus thuringiensis* subsp. *morrisoni* (Tp6 and 14), an acrystalliferous form *Bacillus thuringiensis* (Tp7), *Staphylococcus cohnii* (Tp8), *Bacillus licheniformis* (Tp9), *Bacillus pumilus* (Tp11), *Brevibacterium* sp. (Tp12) and *Bacillus simplex* (Tp13). After analysing the conclusions of conventional and molecular tests Tp1 (Enterobacteriaceae), Tp2 (*Arthrobacter* sp.) and Tp12 (*Brevibacterium* sp.) were assigned as novel bacterial species. Isolate Tp5 had a promising insecticidal effect on third instar larvae of *T. pityocampa* (up to 70% mortality within 10 days).

Report: IIM 8.8/12 – [redacted], [redacted], [redacted], [redacted], K.F., [redacted], [redacted], A.S., [redacted], S.K. (2012), Biocontrol potential of a *Bacillus subtilis* strain against *Bactrocera oleae*
Published report
Annals of Microbiology, 62, 211-216
M-530336-01-1

Abstract: Within the Mediterranean basin, pest infestation of the olive tree especially by *Bactrocera oleae* is a serious economic problem. In this study, we have isolated 115 bacterial strains from various ecological niches, and tested their ability to protect the olive fruits against *Bactrocera oleae*. Among these strains, culture supernatant (CS) of one bacterial strain displayed the highest rate of larval mortality, and was identified as *Bacillus subtilis* by 16S rRNA molecular analysis. Further characterization of the CS of the *Bacillus* sp. strain showed that the highest insecticidal activity against third instar larvae occurs at pH 7. Our results indicate that this bacteria strain may be a prospective alternative in pest control programs

Report: IIM 8.8/13 – [redacted], [redacted], [redacted], T (2014), Combination of endophytic *Bacillus* and *Beauveria* for the management of *Fusarium* wilt and fruit borer in tomato
Published report
Pest Manag Sci, 70, 1742-1750
M-530346-01-1

Abstract:
Background: Most of the approaches for biocontrol of pests and diseases have used a single biocontrol agent as antagonist to a single pest or pathogen. This accounts for the inconsistency in the performance of biocontrol agents. The development of a bioformulation possessing a mixture of bioagents could be a viable option for the management of major pests and diseases in crop plants.
Results: A bioformulation containing a mixture of *Beauveria bassiana* (B2) and *Bacillus subtilis* (EPC8) was tested against Fusarium wilt and fruit borer in tomato under glasshouse and field conditions. The bioformulation with B2 and EPC8 isolates effectively reduced the incidence of Fusarium wilt (*Fusarium oxysporum* f. sp. *lycopersici*) and fruit borer (*Helicoverpa armigera*) under glasshouse and field conditions compared with the individual application of B2 and EPC8

isolates and control treatments. In vitro studies showed a higher larval mortality of *H. armigera* when fed with B2 + EPC8-treated leaves. Further, plants treated with the B2 + EPC8 combination showed a greater accumulation of defence enzymes such as lipoxygenase, peroxidase and polyphenol oxidase against wilt pathogen and fruit borer pest than the other treatments. Moreover, a significant increase in growth parameters and yield was observed in tomato plants treated with B2 + EPC8 compared with the individual bioformulations and untreated control.

Conclusion: The combined application of *Beauveria* and *Bacillus* isolates B2 and EPC8 effectively reduced wilt disease and fruit borer attack in tomato plants. Results show the possibility of synchronous management of tomato fruit borer pest and wilt disease in a sustainable manner.

Report: IIM 8.8/14 – [redacted]., [redacted], A.M., [redacted] (2011), Potency of *Bacillus thuringiensis* and *Bacillus subtilis* against the cotton leafworm, *Spodoptera littoralis* (Bosid.) larvae.

Published report

Archives of Phytopathology and Plant Protection, 44, 204-215

M-529977-01-1

Abstract: The biological activities of two species of bacteria isolated from soil of cotton fields identified as *Bacillus subtilis* strain NRC313 (*BS* NRC313) and *Bacillus thuringiensis* strain NRC335 (*BT* NRC335) were evaluated against the third larval instar of the cotton leafworm, *Spodoptera littoralis* (Boisd.). The different entomopathogenic bacteria of *BS* NRC313and *BT* NRC335 contained 10 × 10^8 cell/ml, and caused mortality of 100 and 97.3% for the above mentioned strains, respectively. Concentrations of 2.5 × 10^8 to 10 × 10^8 cell/ml of strains *BS* NRC313 and *BT* NRC335 were applied to the larvae: LC_{50} were 3.3 × 10^8 and 3.9 × 10^8 cell/ml respectively. The influence of exposure to toxin concentrations manifested in terms of decreasing the adult emergence and prolongation of the generation period. The percentage of larvae that survived and succeeded to pupate increased by decreasing the concentration. The longevity of adult emergence that resulted from larvae treated with *Bacillus subtilis* were 6.0 ± 0.51 and 9.0 ± 0.63 days at 5 × 10^8 and 2.5 × 10^8 cell/ml, respectively compared with 9.8 ± 0.47 in control. The results indicated that *Bacillus subtilis* was more potent than *Bacillus thuringiensis*. Field applications of *B. thuringiensis*, *B. subtilis* and Reldan achieved 55.6, 67.4 and 89.4% reduction of the cotton leafworm larvae *Spodoptera littoralis* in clover plants under field conditions.

Report: IIM 8.8/15 – [redacted], [redacted], [redacted], [redacted], [redacted], S., [redacted] (2012a), The impact of the *Bacillus subtilis* SPB1 biosurfactant on the midgut histology of *Spodoptera littoralis* (*Lepidoptera*: Noctuidae) and determination of its putative receptor.

Published report

J Invertebr Pathol, 109, 183-186

M-530107-01-1

Abstract: SPB1 is a Bacillus subtilis strain producing a lipopeptide biosurfactant. The insecticidal activity of this biosurfactant was evaluated against the Egyptian cotton leaf worm (*Spodoptera littoralis*). It displayed toxicity with an LC(50) of 251 ng/cm(2). The histopathological changes occurred in the larval midgut of *S. littoralis* treated with B. subtilis SPB1 biosurfactant were vesicle formation in the apical region, cellular vacuolization and destruction of epithelial cells and their boundaries. Ligand-blotting experiments with *S. littoralis* brush border membrane vesicles showed binding of SPB1 biosurfactant to a protein of 45 kDa corresponding to its putative receptor. The latter differs in molecular size from those recognized by Bacillus thuringiensis Vip3A and Cry1C toxins, commonly known by their activity against *S. littoralis*. This result wires the application of B. subtilis biosurfactant for effective control of *S. littoralis* larvae, particularly in the cases where *S. littoralis* will develop resistance against B. thuringiensis toxins.

Report: IIM 8.8/16 – [redacted], [redacted], [redacted], [redacted], [redacted] (2012b), Histopathological effects of *Bacillus subtilis* SPB1 biosurfactant in the midgut *of Ephestia kuehniella* (*Lepidoptera*: *Pyralidae*) and improvement of its insecticidal

efficiency.
Published report
Journal of Plant Diseases and Protection, 119, 24-29
M-530121-01-1

Abstract: The present investigation aimed to throw light on the effect of *Bacillus subtilis* SPB1 biosurfactant on the third larvalinstars of the Mediterranean flour moth, *Ephestia kuehniella* , under laboratory conditions. The toxicity of this compound was investigated with emphasis on histopathological effects in the midgut of larvae. The tested dose levels showed stronghistopathological disturbances in the midgut of this pest. The most frequently observed effects were cell vacuolisation, microvilli damage and epithelium cell contents passing into the midgut lumen. The present study was also extended to improve the insecticidal activity of the SPB1 biosurfactant against *E. kuehniella* larvae through optimisation of the pH of the biosurfactant solution and the incubation temperature using Central Composite Design. The experimental results were fitted to a second-order polynomial model that yielded a determination coefficient of $R^2 = 0.998$. The optimal insecticidal activity conditions were found to be a temperature of 33.20°C and pH of 6.66. The predicted andobserved responses were 200.4 ng mg^{-1} and 182.57 ng mg^{-1}, respectively. In comparison to the original LC_{50} level, a 30% decrease was obtained.

Report: IIM 8.8/17 – [redacted], [redacted], A.M. (2008), Mosquito pupicidal toxin production by *Bacillus subtilis* subsp. *subtilis*
Published report
Biological Control, 44, 242-247
M-530103-01-1

Abstract: A strain of *Bacillus subtilis* exhibiting mosquito larvicidal and pupicidal activity was identified as *B. subtilis* subsp. *subtilis* by partial *gyrA* sequence. A study of the association of growth and sporulation in the production of the mosquitocidal toxin and the susceptibility status of different mosquito species is presented here. The pupal stages of *Anopheles stephensi, Culex quinquefasciatus* and *Aedes aegypti* [LC_{50} (µg/ml) 2, 7.3 and 11.8, respectively] were found to be more susceptible to the Crude Mosquitocidal Toxin (CMT) than larval stages [LC_{50} (µg/ml) 19, 23 and 34, respectively] with *An. stephensi* being the most susceptible species. The LT_{50} of freshly molted pupae of *An. stephensi*, when exposed to an LC_{90} (6.82 µg/ml) dosage of CMT, was found to be 1.17 h. Maximum biomass production was achieved at 48 h (15.46 g/l) and maximum production of the CMT was observed at 24 h (1.12 g/l) of growth. Mosquitocidal toxin production was found to be associated with vegetative growth of the organism rather than with sporulation since the mosquitocidal activity was initiated after the lag phase and the maximum mosquitocidal activity was obtained at 12 h, much earlier than the initiation of sporulation. The mosquitocidal toxins of *B. subtilis* may be a prospective alternative in mosquito control programs involving bacterial biopesticides.

Report: IIM 8.8/18 – [redacted] (2013), Evaluation of the potency of some entomopathogenic bacteria isolated from insect cadavers on *Anopheles arabiensis* Giles (Order: *Diptera*; Family: *Culicidae*) mosquito larvae in Nigeria.
Published report
African journal of microbiology research, 7, 4877-4881
M-520067-01-1

Abstract: The laboratory evaluation of larvicidal activities of some bacteria namely; *Pseudomonas aeruginosa, Bacillus polymyxa* and and *Bacillus subtilis* was assessed against the second and fourth instar of *Anopheles arabiensis* collected from South West Nigeria. Concentrations ranging from 1.3 x 10^7cfu/mL to 6.5 x 10^7 cfu/mL were tested on the larvae for a period of 48 h. The disparity in the activities was monitored with *B. subtilis* displaying the highest activity in both the second and fourth instar with LC_{50} of 0.865 and 2.361 mg/mL respectively. *P. aeruginosa* showed LC_{50} of 1.931 and 4.205 mg/mL while the least activity was recorded in *B. polymyxa* with LC_{50} of 5.776 and 7.403 mg/mL. There were significant differences in the LC_{50} value of the bacteria on the tested instars. Values obtained from the fourth instar group were

significantly different from those obtained from the second instar group in all the treatments. Finding from this study show that *B. subtilis* may be a potential biocontrol agent of *A. arabiensis,* the main malaria vector in Nigeria.

Report: IIM 8.8/19 – [redacted], [redacted], K.P., [redacted]. (2012), Mosquito adulticidal activity of a biosurfactant produced by *Bacillus subtilis* subsp. *subtilis*.
Published report
Pest Manag Sci, 68, 1447-1450
M-530108-01-1

Abstract:

Background: A biosurfactant, surfactin, produced by a strain of Bacillus subtilis subsp. subtilis (VCRC B471), was effective in killing mosquito larval and pupal stages. As it was lethal to the non-feeding pupal stage, it was presumed that it could kill the adult mosquitoes also. In this study, the adulticidal effect of the biosurfactant was assessed in the laboratory against a malaria vector, Anopheles stephensi.

Results: The biosurfactant surfactin, separated from the culture supernatant of the production strain, showed mosquito adulticidal activity when tested as ultralow-volume (ULV) spray in a Peet-Grady chamber. Knockdown activity and mortality were found to increase with increasing surfactin dosage. Knockdown dosage (KD) and lethal dosage (LD) were calculated by statistical analysis. The KD(50) and KD(90) dosages were 10.73 and 26.39 mg m(-3) respectively. The LD(50) and LD(90) dosages were 16.13 and 39.21 mg m(-3) . The average droplet size of B. subtilis surfactin was 17.5 ± 1.07 µm.

Conclusion: The present study indicates that the biosurfactant surfactin, produced by B. subtilis subsp. subtilis (VCRC B471), is a potential bioadulticide for ULV spray against malaria-transmitting Anopheles stephensi mosquitoes. This is the first report of a mosquito adulticide from a microbial source.

Report: IIM 8.8/20 – [redacted], A.V., [redacted], D.C., [redacted], [redacted], A.M., [redacted], [redacted], A.M., Alves [redacted], F.J. (2007), First isolation of microorganisms from the gut diverticulum of *Aedes aegypti* (*Diptera* : *Culicidae*): new perspectives for an insect-bacteria association
Published report
Mem Inst Oswaldo Cruz, 102, 919-924
M-520063-01-1

Abstract: We show for the first time that the ventral diverticulum of the mosquito gut (impermeable sugar storage organ) harbors microorganisms. The gut diverticulum from newly emerged and non-fed *Aedes aegypti* was dissected under aseptic conditions, homogenized and plated on BHI medium. Microbial isolates were identified by sequencing of 16S rDNA for bacteria and 28S rDNA for yeast. A direct DNA extraction from *Ae. aegypti* gut diverticulum was also performed. The bacterial isolates were: *Bacillus sp., Bacillus subtilis* and *Serratia sp*. The latter was the predominant bacteria found in our isolations. The yeast species identified was *Pichia caribbica*.

Report: IIM 8.8/21 – [redacted]. (2006), *Bacillus* species in the intestine of termites and other soil invertebrates,
Published report
Journal of applied Microbiology, 101, 620-627
M-518910-01-1

Abstract: Soil invertebrates harbour a complex microbial community in their intestinal system. The total number of microbes in the hindgut of soil invertebrates can reach a titre of 10(11) ml(-1). The gut microbes play an indispensable role in the digestion of food and are of ecological importance in the global carbon cycle. The gut microbiota can include a variety of micro-organisms from the three domains Bacteria, Archaea and Eucarya. The bacterial groups from the intestinal systems are mainly affiliated to the proteobacteria, the gram-positive groups Firmicutes and Actinobacteria, the Bacteroides/Flavobacterium branch and the spirochetes. The Archaea are represented by methanogens. The eukaryotic groups consist of protozoa, yeasts and fungi. Intestinal

bacteria are involved in the degradation of cellulose, hemicellulose and aromatic compounds as well as nitrogen fixation. They also contribute to the redox status of the gut. Bacilli form a significant portion of the intestinal microbial community of soil invertebrates, especially among cellulose degraders. The diversity and function of bacilli in soil invertebrates will be discussed in this paper.

Report: IIM 8.8/22 – [redacted], [redacted], [redacted], [redacted], [redacted], [redacted], [redacted], [redacted], [redacted], M.R. (2012), Diversity of the Bacterial and Fungal Microflora from the Midgut and Cuticle of Phlebotomine Sand Flies Collected in North-Western Iran

Published report

PLoS ONE, 7, 1-10

M-518925-01-1

Abstract:

Background: Phlebotomine sand flies are the vectors of the leishmaniases, parasitic diseases caused by *Leishmania* spp. Little is known about the prevalence and diversity of sand fly microflora colonizing the midgut or the cuticle. Particularly, there is little information on the fungal diversity. This information is important for development of vector control strategies.

Methodology/Prinicpal Findings: FIVE SAND FLY SPECIES: *Phlebotomus papatasi*, *P. sergenti*, *P. kandelakii*, *P. perfiliewi* and *P. halepensis* were caught in [redacted] and [redacted] in North-Western Iran that are located in endemic foci of visceral leishmaniasis. A total of 35 specimens were processed. Bacterial and fungal strains were identified by routine microbiological methods. We characterized 39 fungal isolates from the cuticle and/or the midgut. They belong to six different genera including *Penicillium* (17 isolates), *Aspergillus* (14), *Acremonium* (5), *Fusarium* (1), *Geotrichum* (1) and *Candida* (1). We identified 33 Gram-negative bacteria: *Serratia marcescens* (9 isolates), *Enterobacter cloacae* (6), *Pseudomonas fluorescens* (6), *Klebsiella ozaenae* (4), *Acinetobacter* sp. (3), *Escherichia* coli (3), *Asaia* sp. (1) and *Pantoea* sp. (1) as well as Gram-positive bacteria *Bacillus subtilis* (5) and *Micrococcus luteus* (5) in 10 isolates.

Conclusion/Significance: Our study provides new data on the microbiotic diversity of field-collected sand flies and for the first time, evidence of the presence of *Asaia* sp. in sand flies. We have also found a link between physiological stages (unfed, fresh fed, semi gravid and gravid) of sand flies and number of bacteria that they carry. Interestingly *Pantoea* sp. and *Klebsiella ozaenae* have been isolated in Old World sand fly species. The presence of latter species on sand fly cuticle and in the female midgut suggests a role for this arthropod in dissemination of these pathogenic bacteria in endemic areas. Further experiments are required to clearly delineate the vectorial role (passive or active) of sand flies.

Report: IIM 8.8/23 – [redacted]., [redacted], M.G., [redacted], A.O., [redacted], T.R., [redacted], R.N. (2009), Characterization and Identification of Proteolytic Bacteria From the Gut of the Velvetbean Caterpillar (*Lepidoptera: Noctuidae*)

Published report

Environ Entomol, 38, 1078-1085

M-520008-01-1

Abstract: The characterization and identification of proteolytic bacteria from the gut of the velvetbean caterpillar (*Anticarsia gemmatalis*) were the objectives of this study. Twelve aerobic and anaerobic isolates of proteolytic bacteria were obtained from the caterpillar gut in calcium caseinate agar. The number of colony forming units (CFUs) of proteolytic bacteria was higher when the bacteria were extracted from caterpillars reared on artificial diet rather than on soybean leaves (1.73 +/- 0.35 x 10(3) and 0.55 +/- 0.22 x 10(3) CFU/mg gut, respectively). The isolated bacteria were divided into five distinct groups, according to their polymerase chain reaction-restriction fragment-length polymorphism profiles. After molecular analysis, biochemical tests and fatty acid profile determination, the bacteria were identified as *Bacillus subtilis*, *Bacillus cereus*, *Enterococcus gallinarum*, *Enterococcus mundtii*, and *Staphylococcus xylosus*. Bacterial proteolytic activity was assessed through in vitro colorimetric assays for (general) proteases, serine proteases, and cysteine proteases. The isolated bacteria were able of hydrolyzing all tested substrates, except

Staphylococcus xylosus, which did not exhibit serine protease activity. This study provides support for the hypothesis that gut proteases from velvetbean caterpillar are not exclusively secreted by the insect cells but also by their symbiotic gut bacteria. The proteolytic activity from gut symbionts of the velvetbean caterpillar is suggestive of their potential role minimizing the potentially harmful consequences of protease inhibitors from some of this insect host plants, such as soybean, with implications for the management of this insect pest species.

Report: IIM 8.8/24 – [redacted], [redacted], [redacted], [redacted] (2012), An investigation on the bacterial flora of *Agriotes lineatus* (*Coleoptera*: *Elateridae*) and pathogenicity of the flora members
Published report
Crop Protection, 40, 1-7
M-518924-01-1

Abstract: The wireworm *Agriotes lineatus* (L.) (Coleoptera: Elateridae) is a serious agricultural pest of various vegetables and fruits throughout the world. To find an effective and safe biological control agent against this pest, we investigated the bacterial flora of *A. lineatus*. Nineteen different bacterial strains were isolated and identified as *Paenibacillus* sp. (Ag1), *Cellulomonas* sp. (Ag2), *Bacillus subtilis* (Ag3), *Staphylococcus* sp. (Ag4), *Enterococcus mundtii* (Ag5), *Staphylococcus* sp. (Ag6), *Sphingobacterium* sp. (Ag7), *Staphylococcus pasteuri* (Ag8), *Arthrobacter gandensis* (Ag9), *Bacillus* sp. (Ag10), *Chryseobacterium* sp. (Ag11), *Streptomyces* sp. (Ag12), *Oerskovia turbata* (Ag13), *Bacillus thuringiensis* (Ag14), *Pseudomonas fluorescens* (Ag15), *Oerskovia jenensis* (Ag16), *Arthrobacter gandavensis* (Ag17), *B. thuringiensis* (Ag18), and *Pseudomonas plecoglossicida* (Ag19) based on conventional and molecular tests. *A. gandavensis* and *P. plecoglossicida* were isolated for the first time from any insect. The insecticidal effects of these 19 bacterial isolates and the additional 11 isolates belonging to *Bacillus* genus isolated from different hosts were tested on third instar larvae of *A. lineatus*. Ag17 (*A. gandavensis*), Ag18 (*B. thuringiensis*), and Ag19 (*P. plecoglossicida*) from the bacterial flora of *A. lineatus*, and two *Bacillus* isolates (*Bacillus circulans* Ar1 from *Anoplus roboris* and *B. thuringiensis* subsp. *kurstaki* BnBt from *Balanicus nucum*) showed 100% mortality 10 days after treatment. Our results indicate that the bacterial isolates tested in this study may be considered as a possible microbial control agent against *A. lineatus*.

Report: IIM 8.8/25 – [redacted], [redacted], A.G. (2010), Microbiology of the gut of the kola nut weevil, *Balanogastris kolae*
Published report
J Insect Sci. 12, 1-6
M-520054-01-1

Abstract: Reports have shown that many insects have microbes in their gut system. Gut microbes are very important for insect vitality and much of their nutrition is derived from products of microbial metabolism. The habitat of *Balanogastris kolae* (Desbrocher des Loges) (*Coleoptera*: *Curculionidae*) suggests that they possess the ability to digest varieties of sugars particularly starch and protein materials present in the kola nut, *Cola nitida* Schott & Endlicher (*Malvales*: *Malvaceae*). The aim of this study was to characterize the gut bacterial communities of the kola weevil, *B. kolae*. To ascertain this, the gut bacterial community of a kola nut-feeding weevil, *B. kolae* was characterized using culture-dependent methods. The bacterial counts in the foregut, midgut and hindgut were 7.14 ± 0.11 × 10(6)cfu ml(-1), 2.68 ± 0.13 × 10(7) cfu ml(-1) and 1.43 ± 0.20 × 10(6) cfu ml(-1) respectively. There were no significant differences in the total bacterial count of the foregut, midgut and hindgut. The bacterial species were identified to be *Fusobacterium nucleatum*, *Staphylococcus aureus*, *Bacillus subtilis*, *Corynebacterium fascians*, *Arthrobacter globiformis*, *Serratia marcescens*, *Bacillus brevis*, *Vibrio haemolyticus* and *Flavobacterium breve*. The majority of these isolates were demonstrated to have both proteolytic and amylolytic activities.

IIM 8.9 Effects on other terrestrial invertebrates

IIM 8.9.1 Effects on earthworms

Since *B. amyloliquefaciens* is ubiquitous in soil ecosystems, intake of bacteria during ingestion of soil by earthworms is a natural process. Moreover, earthworms are able to utilize soil microbes at their foods or even proliferate selective in the gut. Therefore, side effects are negligible. For background information, please refer to the baseline dossier.

For confirmation, a literature search was conducted on the DIMDI database provided by the German Institute of Medical Documentation and comprised of searches in MEDLINE, BIOSIS, CAB and SCISEARCH databases. The search terms "earthworm?" and "*Bacillus amyloliquefaciens*" or "*Bacillus subtilis*" revealed in only few relevant hits. Please refer to literature research report submitted in Point IIM 8.

Since *B. subtilis* are naturally present in soil, earthworms ingest *Bacilli* and *Paenibacilli*. Moreover, gut-associated microorganisms are responsible for the capacity of earthworms to emit nitrous oxide (N_2O) ([redacted], 2005).

[redacted] et al. (2007) studied the microbe-earthworm relationship of the four earthworm species *Eudrilus eugeniae*, *Lampito mauratii*, *Eisenia fetida* and *Perionyx excavates*. Thus, earthworms were maintained in in three different substrates: clay loam soil, cowdung and cane sugar mill waste. Of each substrate, fungal, bacterial, actinomycete and yeast population was determined. *B. subtilis* was one of the dominant bacteria in the provided feed with 5×10^6 CFU/g in clay loam soil, 1.6×10^7 CFU/g in cow dung and 1.8×10^7 CFU/g in press mud. Although it was shown that bacterial populations increased through earthworm passage particularly in the cast, *B. subtilis* was neither detected in the gut nor in the vermicompost of any of the investigated earthworm species suggesting that the ingested populations were completely digested. The same applied for most gram positive bacteria including *B. cereus*, while some gram negatives e.g. *Klebsiella pneumoniae* and *Enterobacter chloacae* readily proliferated in the earthworms. These findings indicate that *B. subtilis* and probably all bacilli do not grow inside the earthworm gut but are readily digested.

However, biocontrol bacterial *B. subtilis* and *B. amyloliquefaciens* showed low mortality on *Eisenia foetida* earthworms when applied at 10^8 CFU/mL ([redacted] et al., 2014). In this study, several microbial biocontrol strains (fungi and bacteria) were tested for their potential in pest and disease management and their environmental safety on *Daphnia magna* and *E. foetida*. Therefore, 10^8 CFU/mL of each tested bacterial strain (*B. subtilis* B49b, 98a and Us.a2, *B. amyloliquefaciens* OS17) was applied (375 mL) to an artificial soil in which *E. foetida* were placed 300-600 mg b.w.). After 7 days, no mortality was registered, whereas low mortality (7.5-17.5%) was shown 14 days of incubation, in comparison to the untreated control (5% mortality).

<u>Cited references (abstracts):</u>

Report: IIM 8.9.1/07 – [redacted]. (2005), *Bacillus* species in the intestine of termites and other soil invertebrates.
Published report
Journal of applied Microbiology, 101, 620-627
M-518910-01-1

Abstract: Soil invertebrates harbour a complex microbial community in their intestinal system. The total number of microbes in the hindgut of soil invertebrates can reach a titre of 10(11) ml(-1). The gut microbes play an indispensable role in the digestion of food and are of ecological importance in the global carbon cycle. The gut microbiota can include a variety of micro-organisms from the three domains8.9. Bacteria, Archaea and Eucarya. The bacterial groups from the intestinal systems are mainly affiliated to the proteobacteria, the gram-positive groups Firmicutes and Actinobacteria, the Bacteroides/Flavobacterium branch and the spirochetes. The Archaea are represented by methanogens. The eukaryotic groups consist of protozoa, yeasts and fungi. Intestinal bacteria are involved in the degradation of cellulose, hemicellulose and aromatic compounds as well as nitrogen fixation. They also contribute to the redox status of the gut. Bacilli form a significant portion of the intestinal microbial community of soil invertebrates, especially among cellulose degraders. The diversity and function of bacilli in soil invertebrates will be discussed in

this paper.

Report: IIM 8.9.1/08 – [redacted], [redacted] LS, [redacted] V, [redacted] J. (2007) Diversity of microflora in the gut and casts of tropical composting earthworms reared on different substrates, published report.
J Environ Biol 28, 87-97
M-518911-01-1

Abstract: The diversity of fungi, bacteria, yeast, actinomycetes and protozoa were analysed in the gut and casts of *Eudrilus eugeniae*, *Lampito mauritii, Eisenia fetida* and *Perionyx excavatus*, both qualitatively and quantitatively as influenced by different feed substrates like clay loam soil, cowdung and pressmud. While actinomycetes (*Streptomyces albus, S. somaliensis, Nocardia asteroides, N. caviae* and *Saccharomonospora*) were not digested by any of these species of worms, protozoa (*Amoeba proteus, A. terricola, Paramecium trichium, Euglena viridis, E. orientalis, Vorticella picta* and *Trichomonas hominis*) and yeast (*Candida tropicalis, C. krusei C. albicans* and *Cryptococcus neoformans*) were totally digested. Certain species of fungi (*Saksenae vasiformis, Mucor plumbeus, Cladosporium carrionii, C. herbacium, Alternaria sp., Cunninghamella echinulata, Mycetia sterila, Syncephalostrum racemosum, Curvalaria lunata, C. geniculata* and *Geotrichum candidum*) and bacteria (*Pseudomonas aeruginosa, Bacterium antitratum, Mima polymorpha, Enterobacter derogenes, E. cloacae, Proteus vulgaris, P. mirabilis, P. rettgeri, Escherichia coli, Staphylococus citreus, Bacillus subtilis, B. cereus, Enterococci* and *Micrococci*) were completely digested. Certain other species were not digested fungi like *Aspergillus fumigatus*, *A. flavus, A. ochraceous, Trichoderma koningii* (except by *E. eugeniae*), *Fusarium moniliforme* (except by *E. eugeniae*) and *Rhizopus* sp., and bacteria like *Klebsiella pneumoniae* and *Morganella morganii*) and these were multiplied during the transit of the organic residues through the gut of worms. The microbial proliferation was more in the casts, due to the environment prevailing--rich in nutrient supply and large surface area available for growth and reproduction of the microbes that lead to enhanced microbial activity and humic acid contents in the casts.

Report: IIM 8.9.1/09 – [redacted]., [redacted], [redacted], [redacted], [redacted], [redacted], [redacted] (2014) Pests and diseases management using compatible bacteria and entomopathogenic fungal strains, published report
Scientific Bulletin Series F, Biotechnologies, 18, 66-72.
M-535539-01-1

Abstract: Pest and disease management using biocontrol microbial strains is a request of the organic agriculture or a phytosanitary alternative that can decrease chemical inputs in the integrated agricultural systems. Biocontrol bacteria of *Bacillus* spp. proved to suppress soil-borne phytopathogenic fungi. RDIPP selected strains of *Bacillus amyloliquefaciens; B. licheniformis* and *B. subtilis* provided to be useful in plant protection and formulated them as bioproducts for seed and soil treatments. For pest biological control, entomopathogenic fungi such as *Beauveria bassiana, B. brongniartii, Isaria farinosa, Metarhizium anisopliae and Verticillium lecanii* are known as efficient. For this reason, the aim of our work was to select compatible microbial strains of biocontrol bacteria and entomopathogenic fungi that could be applied together, as simultaneously treatments, for suppressing diseases and pests attack. Results revealed in vitro compatibility of *Bacillus licheniformis* 77.1s biocontrol strain with *Beauveria spp.* entomopathogenic fungi. These biological control microorganisms could be used in combination to prevent in the same time pests and diseases.

As a requirement for environmental safety, the selected microbial strains were ecotoxicologicaly tested according to the GLP principles (Good Laboratory Practices) and OECD guidelines. Results proved that the selected strains were nontoxic for non-target species of the aquatic and soil macrofauna: *Daphnia magna* (crustacean) and *Eisenia foetida* (earth worm) respectively.

IIM 8.9.2 Effects on other terrestrial invertebrates

No EC data requirement.

IIM 8.10 Effects on soil micro-organisms

B. *amyloliquefaciens* QST 713 may be washed to soil ecosystems (please refer to Annex III, Section 5). Therefore, *B. amyloliquefaciens* QST 713 may affect soil miroorganisms. Nevertheless, *B. amyloliquefaciens* are ubiquitous in soil ecosystems.

Please refer to the baseline dossier for the background information.

According to the Working Document to the Environmental Safety Evaluation of Microbial Biocontrol Agents (SANCO/12117/2012-rev. 0, September 2012)[1]. tests assessing possible effects of microbial pesticides on soil micro-organisms are not stringently significant for the following reasons:

- Microorganisms may be affected by almost everything that is added to the soil. Interpretation of test results is therefore often ambiguous.
- Risk caused by introduction of microorganisms to the soil microbial community is minimal, because soil microflora naturally fluctuates in time and space. The natural populations are well adapted to their habitat and exhibit many defence mechanisms in order to assure their survival.
- Soil microbial communities show good resilience, and populations are able to recover even upon extreme decimation e.g. by methyl bromide.

A literature search has been performed in the DIMDI database provided by the German Institute of Medical Documentation and comprised of searches in MEDLINE, BIOSIS, CAB and SCISEARCH databases, using search terms “soil microorg?” and “*Bacillus amyloliquefaciens*” or “*Bacillus subtilis*”. Very few articles were identified. For details on the literature search, please refer to the Literature Review Report submitted in Point IIM 8.

[redacted] et al. (2012) studied the influence of three different sustainable disease management practices on soil microbial communities. Therefore, impact of compost amendment, crop rotation and application of biocontrol organisms (*B. subtilis* formulation Kodiak®, *Trichoderma virens* formulation SoilGuard® and *Rhizoctonia solani* hypovirulent strain Rhs1A1) were tested over three years experimental period on two testing locations. *B. subtilis* formulation was applied at 2 g/L at a rate of 300 mL/row. It was shown that compost amendment and rapeseed rotation had a much higher impact on soil microbial community characteristics, in comparison to application of microbial biocontrol agents. Moreover, application of *B. subtilis* formulation exerted relatively minor effects on both testing sites. In contrast, consistent overall effects were observed by application of the fungal microorganisms *T. virens* and *R. solani*.

Nevertheless, *B. amyloliquefaciens* is reported to produce metabolites that may affect other microorganisms (please refer to Annex II, Doc IIM, Point 2.7.2). Thus, produced substances are widely tested against microbial plant pathogens (e.g. *Fusarium* sp. *Phytium ultimum*, *Alternaria* sp.) and fungicidal potential was demonstrated. Thus, non-target fungi may be affected. However, effect is assumed to be much lower than by other chemical fungicides, since *B. amyloliquefaciens* naturally occur in soils. This was demonstrated by [redacted] et al. (2009) when studying the impact of *B. amyloliquefaciens* BNM122 application on rhizosphere and soil microbial communities. Thereby, 20 soybean seeds were inoculated with 20 mL of bacterial suspension containing 3×10^8 CFU/mL. Seeds were incubated in the suspension for 2 h and sown in soil. Alternatively, seeds were treated with the fungicids thiram (35%) and carbendazim (15%) at doses recommended by the manufacturer. To assess the impact on soil communities, cell counts on TSA media were obtained. Moreover, soybean root nodulation by Bradyrhizobium japonicum and mycorrhization by arbuscular mycorrhizal fungi were determined to evaluate the effect on rhizosphere. It was shown, that neither genetic structure of rhizosphere bacterial community was affected by *B. amyloliquefaciens* BNM122, nor nodulation of soybean. However, soybean mycorrhization significantly decreased. Nevertheless, much higher negative effect was observed when plants were treated with thiram and carbendazim.

[1] Working Document to the Environmental Safety Evaluation of Microbial Biocontrol Agents, SANCO/12117/2012-rev.0, September 2012, EUROPEAN COMMISSION HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL Directorate E – Safety of the food chain Unit E.3 – Chemicals, contaminants, pesticides.

Cited references (abstracts):

Report: IIM 8.10/02 – [redacted], [redacted], R.P., [redacted], [redacted], M.S., [redacted], [redacted], [redacted], [redacted], S.D. (2012), Compost, rapeseed rotation, and biocontrol agents significantly impact soil microbial communities in organic and conventional potato production systems
Published report
Applied Soil Ecology, 52, 29-41
M-518913-01-1

Abstract: Cultural practices such as organic amendments, rotations, and use of biological control organisms are regularly investigated for their effects on controlling plant diseases but their effects on soil microbial populations are often unexplored. In this study, three different sustainable disease management practices, use of compost amendment, biocontrol organisms, and a potentially disease-suppressive rotation, were established in potato field trials at two sites under different management regimes and histories, and evaluated over three potato cropping seasons for their effects on soil microbial communities. Specific management factors assessed included the presence or absence of a conifer-based compost amendment, addition of one of three different biocontrol organisms (*Trichoderma virens*, *Bacillus subtilis*, and *Rhizoctonia solani* isolate *Rhs1A1*), and a *Brassica napus* (rapeseed) green manure rotation crop preceding potato, and treatments were assessed in all factorial combinations. The two farm sites represented organic and conventional potato production practices in [redacted] County, Maine. Compost amendment and rapeseed rotation had the greatest impacts on soil microbial communities, with both treatments increasing total populations of culturable bacteria at both sites over the course of the study, as well as causing detectable shifts in soil microbial community characteristics as determined by sole carbon-source substrate utilization and fatty acid methyl ester (FAME) profiles. Compost amendment generally led to increased utilization of complex substrates and increased levels of Gram-positive bacteria and fungi, and compost effects were more pronounced at the conventional site. Rapeseed rotation often resulted in somewhat different effects at the two different sites. Consistent overall effects were observed with the biocontrol amendments *Rhs1A1* and *T. virens*, including increased microbial activity and bacterial populations. Combined effects of multiple treatments were greater than those of individual treatments and were generally additive. These results indicate that each treatment factor had significant and specific effects on soil microbial communities, and that combined effects tended to be complementary, suggesting the potential of combining multiple compatible management practices and their associated changes in soil microbial communities.

Report: IIM 8.10/03 [redacted], M.S., [redacted], M.F., [redacted]., [redacted], N.L., [redacted], N.L., [redacted], A.F. (2009), *Bacillus amyloliquefaciens* BNM122, a potential microbial biocontrol agent applied on soybean seeds, causes a minor impact on rhizosphere and soil microbial communities
Published report
Applied Soil Ecology, 41, 185-194
M-530023-01-1

Abstract: The increase in soybean productivity has contributed to a greater use of agrochemicals, which cause major problems, such as soil and water pollution and reduction of biodiversity, and have a negative impact on non-target species. The development of microbial biocontrol agents for soybean diseases can help to reduce pesticide abuse. *Bacillus amyloliquefaciens* BNM122 is a potential microbial biocontrol agent able to control the damping-off caused by *Rhizoctonia solani* when inoculated in soybean seeds, both in a plant growth chamber and in a greenhouse. In this study, we report the effect of soybean seed treatments with strain BNM122 or with two fungicides (thiram and carbendazim) on the structure and function of the bacterial community that colonizes the soybean rhizosphere. Also, soybean root nodulation by *Bradyrhizobium japonicum*, mycorrhization by arbuscular mycorrhizal fungi and plant growth were evaluated. We used the *r*- and *K*-strategist concept to evaluate the ecophysiological structure of the culturable bacterial community, community-level physiological profiles (CLPP) in Biolog™ EcoPlates to study bacterial functionality, and the patterns of 16S RNA genes amplified by PCR and separated by

denaturing gradient gel electrophoresis (PCR–DGGE) to assess the genetic structure of the bacterial community. Neither the ecophysiological structure nor the physiological profiles of the soybean rhizosphere bacterial community showed important changes after seed inoculation with strain BNM122. On the contrary, seed treatment with fungicides increased the proportions of *r*-strategists and altered the metabolic profiles of the rhizosphere culturable bacterial community. The genetic structure of the rhizosphere bacterial community did not show perceptible changes between treated and non-treated seeds. Regarding the bacterial and fungal symbioses, seed treatments did not affect soybean nodulation, whereas soybean mycorrhization significantly decreased ($P < 0.05$) in plants obtained from seeds treated with strain BNM122 or with the fungicides. However, a higher negative effect was observed in plants which seeds were treated with the fungicides. Plant growth was not affected by seed treatments.

It can be concluded that soybean seed treatment with *B. amyloliquefaciens* BNM122 had a lesser effect on soil microbial community than that with the fungicides, and that these differences may be attributed to the less environmental persistence and toxic effects of the strain, which deserve further studies in order to develop commercial formulations.

IIM 8.11 Other/special studies

The information presented in Points IIM 8.1 to 8.10 is considered sufficient to evaluate the impact of the *B. amyloliquefaciens* QST 713 on non-target species. Therefore, no other studies are required.

B. amyloliquefaciens QST 713 mode of action includes the production of antimicrobial compounds. Nevertheless, it is not expected that these compounds will have any adverse effects on the environment or on non-target organisms for the following reasons: they do not occur independently from the microorganism in the environment and are produced only by vegetative cells, often in the moment and area of contact between bacteria and fungi. Moreover, compared to background levels of antimicrobial substances in soils that can even provide a kind of natural control of bacterial and fungal pathogens e.g. in suppressive soils, the effects of a short-term increase in the levels of antimicrobial substances due to the application of *B. amyloliquefaciens* QST 713 in the environment appears to be negligible.