Renewal Assessment Report

Bacillus thuringiensis subsp. *aizawai strain GC-91*

Volume 3 – B.7 Residues in or on treated products, food and feed

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B.7 Residues in or on treated products, food and feed

Bacillus thuringiensis subsp. *aizawai* GC-91 (in the following abbreviated as Bta GC-91) was included in Annex I of Council Directive 91/414/EEC in 2008. Subsequently Regulation (EC) No 1107/2009 repealed and replaced Directive 91/414/EEC and the active substance Bta GC-91 was deemed to be approved under that Regulation and included in the Annex to Regulation (EC) No 540/2011. EFSA delivered its conclusions on *Bacillus thuringiensis ssp. aizawai* (strains ABTS-1857, GC-91) on the 19 December 2012 (published January 2013). Based on this new information available, no need to change the conditions of approval of Bta GC-91 was identified. The Commission filed on 13 December 2013 an updated review report for Bta GC-91 to the Standing Committee on the Food Chain and Animal Health for examination.

The approval of Bta GC-91 under the Regulation (EC) No 1107/2009 expires 30 April 2019. In accordance with the same Regulation the original notifier Mitsui AgriScience International SA/NV has filed to the Commission an application for the renewal of the approval of the active substance Bta GC-91 on 30 April 2016.

Bta GC-91 is a transconjugant strain originating from a Bta and a Bt subsp. kurstaki strain. Bta in general occurs ubiquitous in soils on plants as well as in infested insects. Bta acts highly specific against insect species of the order Lepidoptera and is not expected to have any harmful effects on beneficials and other non-target species of other insect orders. The insecticidal activity of Bta is mainly attributed to spore bound insecticidal pro-toxins (Cry toxins) which are ingested by the target pests and activated under alkaline conditions in the midgut of the larvae. The first assessment of the strain proved that it does not have any harmful effects on human or animal health or on groundwater or any unacceptable influence on the environment. The overall conclusion from EFSA (2013) confirms that no critical areas of concern are identified within the framework of the use which was supported.
As the manufacturing process of Bta GC-91 has not been changed since original approval, all data submitted for the original approval of the strain are considered fully applicable for the current evaluation.

Besides new information, the submitted dossier includes all data, which have been presented in the DAR (Jan 2008) and DAR addendum (Nov 2012). This information is marked grey with a clear indication where the information is originating from.

Information from DAR and DAR addendum (May 2007, February 2013)

B. thuringiensis is a common, naturally occurring bacterium that is frequently isolated from soil, and from leaves, where it is regarded as a common part of the leaf microflora (i.e. phyllospheric).

The strain GC-91 is a product of a crossing or conjugation between two parental strains HD-191-A2 (flagella serotype *kurstaki)* and HD-135-S4 (flagella serotype *aizawai)*, which are derived from wild type strains and differ in their flagella serotype as well as in their δ-endotoxin genes. This is a natural process which allows one bacterium to donate genetic material to another. The resulting strain is called a transconjugant and has an optimal combination of the most active crystal proteins.

New information 2016

A literature search covering the last 10 years was performed (Cornelese, 2016, KMA 6/01) using the keywords *Bacillus thuringiensis* subsp. *kurstaki* OR Btk AND aizawai AND residue AND (consumer OR food OR feed OR risk OR bacillomycin OR cereulide OR crystal protein OR cry toxin OR cytotoxin OR enterotoxin OR entomocin OR fengycin OR cytolytic protein OR iturin, OR GC91 OR GC 91 OR GC-91 OR Turex OR Agree. Both active substance and registered products were included in the search.

An separate additional search was done for metabolites. The search terms with regard to metabolites/toxins for *Bacillus thuringiensis* spp. used were selected from the EFSA conclusion of *Bacillus thuringiensis* subsp. *aizawai* GC-91 (EFSA Journal 2013;11(1):3063) and a recent EFSA supporting publication on Literature search and

data collection on Risk Assessment for human health for microorganisms used as plant protection products (Evelyn Hackl et al. (2015), EFSA supporting publication 2015:EN-801. 173 pp).

The relevant literature found in the search is evaluated in the chapters below.

No specific MRL was fixed for the active substance under Reg. (EC) No 396/2005, according to Art. 18(1)(b) of that Regulation. Up till now *Bacillus thuringiensis* subsp. *aizawai* strain GC-91 is not included in Annex IV due to delay at EFSA. Moreover, the default MRL of 0.01 mg/kg is not applicable because agencies are not used to follow enforcement or maintenance procedures for micro-organisms.

Cited references

Report KMA 6/01 –Cornelese A. (2016). Literature review on *Bacillus thuringiensis* subsp. *aizawai* strain GC-91 and metabolites: Residues in or on treated products, food and feed
Unpublished report,
Rep. No.: 2281385-MA-06-01

Report KMA 6/02 –Cornelese A. (2016). Literature review on *Bacillus thuringiensis* subsp. *aizawai* strain GC-91 and metabolites: Residues in or on treated products, food and feed
Unpublished report,
Rep. No.: 2281385-MA-06-02

B.7.1 Persistence and likelihood of multiplication in or on crops, feedingstuffs or foodstuffs

Information from DAR and DAR addendum

Estimation of persistence/competitiveness of the micro-organism and relevant secondary metabolites (especially toxins) in or on the crop can be carried out as follows.

The strain GC-91 is a product of a crossing or conjugation between two parental strains HD-191-A2 (flagella serotype *kurstaki)* and HD-135-S4 (flagella serotype *aizawai)*, which are derived from wild type strains and differ in their flagella serotype as well as in their δ-endotoxin genes..

If one considers that the study results on Btk strains can be used also for the evaluation of the residual behaviour of the Bta strain GC-91, because the physiological, biochemical and biological properties of this *B. thuringiensis* subspecies are fully comparable to the ones of the commercially used strains of Btk, including the Btk strain HD-1, the most common commercial Btk strain, used as a standard in several toxicology studies;

If one considers that the spore counts reverted to the background levels after 28 days on soybean (Ignoffo et al., 1974), and after 14 days on cabbage (Pedersen et al., 1995), that Hostetter et al. (1975) determined the persistence of Bt spores on Juniperus virginiana following spraying of DiPel (containing *B. thuringiensis* ssp. *kurstaki* strain HD-1) and also found a half-life of less than 1 day, that the δ-endotoxins are rapidly degradable and endospores are rapidly inactivated when exposed to UV radiation (Pusztai et al., 1991);

Considering that, additionally, the sunlight-exposed leaves, since solar radiation is the key factor in reducing the persistence of populations and the activity of Bt preparations, modulated by rainfall and other environmental factors on the leaf surface (Pinnock et al., 1974);

If one considers that the populations following spraying were 8.9 x 10^6 CFU/g leaf. and viable spore numbers decreased rapidly, with remaining ratios of 19.8% after 1 day, 8.2% after 3 days, 1.6% after 7 days, 0.6% after 14 days, and 0.2% after 28 days, and that Griego & Spence (1978) measured the viability of *B. thuringiensis* ssp. *kurstaki* strain HD-1 after exposure to sunlight, and determined the half-life of spores on membrane filters was 10 minutes: It is possible to hypothesize that the half life of Bt spores on foliage does not exceed 1 to 3 days.

Multiplication of *B. thuringiensis* ssp. *aizawai* does not seem to play a role in natural environment. As seen from the data on persistence presented above, no multiplication occurs on the leaves due to sensitivity to solar radiation, foliage exudates and microbial competition. Spore germination was observed in sterile but not in natural soils.

Vegetative cells disappeared rapidly within 1-2 days after inoculation, but cells were able to form spores (Akiba, 1986). Germination of spores occurs only if the conditions are appropriate, that is only after ingestion by insects (Pedersen et al., 1995), or earthworms, or in the rhizosphere of several, but not all, plants (Hendriksen & Hansen, 2002). No proliferation of Bt occurred in forest soils after aerial spraying (Smith & Barry, 1998).

On the surface of the grape berries, which is a phyllospheric habitat, like on other phyllospheres, there can be interaction between the resident microflora (Bae et al., 2004). The persistence on the foliage also decreases due to the lack of resistibility to the foliage exudates and a high sensitivity to solar radiation and dehydration associated with the plasmids which are responsible for endotoxin expression (Benoit et al., 1990).

B. thuringiensis is a common bacterium that is frequently isolated from soil, but also from leaves, where it is regarded as a common part of the leaf microflora (Smith & Couche, 1991; Damgaard et al., 1997).

Indigenous Bt strains were found at amounts of 40 spores/g on soybean leaves (Ignoffo et al., 1974), at amounts between 3 CFU/cm^2 and 100 CFU/cm^2 on different deciduous trees (Smith & Couche, 1991), at amounts of 8 x 10^2 CFU/g on Juniperus virginiana (Hostetter et al., 1975), and at amounts of 3.9 x 10^2 CFU/g on cabbage leaves (Pedersen et al., 1995).

Bae et al. (2004) examined the occurrence and the significance of *Bacillus thuringiensis* on wine grapes. The insecticides gave total viable counts of 2.7 x 10^{10} and 1.9 x 10^{10} CFU/g, respectively. *B. thuringiensis* were applied four times during cultivation. *B. thuringiensis* was consistently isolated from all grape varieties in all vineyards throughout the period of grape cultivation. Its population varied between 10^2 – 10^6 CFU/g, with higher populations being found in the early stages of cultivation, when the berries were smaller and not bunched and, presumably, when the spray had greater access to individual berries.

There was no obvious influence of grape variety on the association of *B. thuringiensis*. At the time of harvest, B. thuringiensis populations on grapes varied between 10^2 and 10^4 CFU/g, depending on vineyard and corresponding to the natural occurrence of *B. thuringiensis* on leaves of different crops and plants (Ignoffo et al., 1974; Hostetter et al., 1975; Pedersen et al. 1995; Hendriksen & Hansen, 2002).

After flowering the Bt population is stabilizing at these concentrations during cultivation, independent from the timing of application. Even right after the pre-harvest application no increase of the B. thuringiensis population could be detected (Bae et al., 2004).

New information 2016

Information on persistence in the environment, also on vegetation, is provided in B.8.

In the Scientific Opinion of the EFSA BIOHAZ panel[1] information from literature on the fate of *B. thuringiensis* on plants after application as a MPCA has been published. This document includes information from studies that were considered during the original approval of Bta GC-91 as indicated above. Furthermore, the opinion contains more recent information on residues after application. Madsen et al. (2011, study in Danish) were able to detect between 100 and 1000 spores/g leaf 60 days after application, on white cabbage. In fields sprayed with a product containing *B. thuringiensis* serotype kurstaki, 2×10^4 spores/g were found in broccoli 1 week after the spraying and 8×10^3 and 2×10^3 spores/g in celery 1 and 2 months after the spraying, respectively. In an experiment where *B. thuringiensis* spores were spread on curly kale in a field 4 times, a decrease to detection level within 30 days after the first application was found, whereas this level was not reached for the last application in 120 days (Hendriksen, 2011 taken by EFSA panel from OECD ENV/JM/MONO(2011)42 published presentations).

Note RMS:

As *B. thuringiensis* and its secondary metabolites are not persistent in soil water and air, and spores are not persistent on crop, half-life less than 1 day, multiplication on crops is not expected. It is generally agreed that persistence of Bt populations on plant surfaces is low. Factors restricting field persistence are UV-mediated degradation of spores, rain fall and plant growth (dilution effects), lack of nutrients and low humidity. Natural levels of Bt on plant surfaces range between 3 and nearly 1000 CFU/g or cm^2 (Smith & Couche, 1991; Ignoffo et al., 1974; Hostetter et al., 1975) (see also B.2.8).

B.7.2 Because the microorganism is not infective or pathogenic and secondary metabolites are not expected on the crops no consumer risk is expected and no PHI is necessary. Further Information required - Exposure to consumers

The growth of endospores is initially dependent on the germination of the spore, followed by divisions of the

[1] EFSA SCIENTIFIC OPINION on Risks for public health related to the presence of *Bacillus cereus* and other *Bacillus* spp. including *Bacillus thuringiensis* in foodstuffs EFSA Panel on Biological Hazards (BIOHAZ), EFSA Journal 2016;14(7):4524

vegetative cell. On leaves, *B. thuringiensis* occurs mainly as spores, the concentration of nutrients of the leaf surface is insufficient to mediate growth of *B. thuringiensis*. As described in volume B.2.5 MA it is generally agreed that *B. thuringiensis* and its secondary metabolites are not persistent in soil water and air. The mobility of B. thuringiensis and the spores can be considered limited. Factors restricting field persistence are UV-mediated degradation of spores, rain fall and plant growth (dilution effects), lack of nutrients and low humidity.

Information from DAR and DAR addendum (May 2007, February 2013)

The currently available knowledge on the mammalian safety of *Bacillus thuringiensis* has been summarised (McClintock et al. 1995, Siegel 2001, Glare & O'Callaghan, 2000), demonstrating also the similarity of different *Bacillus thuringiensis* subspecies with regard to their lack of infectivity, toxicity and pathogenicity to mammals including humans. No toxicity and pathogenicity was observed in an acute toxicity study in rats. Bta strain GC-91 induced no signs of toxicity at a dose of 1.1 x 10^{10} CFU per kg b.w. administered to male and female rats, respectively (refer to Doc IIM, Section 3, Point IIM 5.3.2). In an acute oral toxicity study in rats, dosed up to 24 g Thuricide (2 x10^{12} spores *Bacillus thuringiensis kurstaki*) per kg b.w. (Fisher & Rosner -1959), no fatalities and no outward symptoms of toxicity occurred during observation for one week following dosing and gross and histological examinations revealed no abnormalities.

In a short term toxicity test in sheep, *Bacillus thuringiensis* (Bt) was administered in the diet for 5 months to six sheep (24-34 kg at the beginning of the study) at a dose of approximately 10^{12} spores per day (corresponding to ca. 3-4 x 10^{10} spores/kg b.w./day). The study gave no indication that *Bacillus thuringiensis* is a pathogen in sheep following oral ingestion of large daily doses for five months (Hadley et al., 1987).

In the medical records no adverse health effects attributable to *Bacillus thuringiensis* exposure were found in physical examinations in workers exposed to *Bacillus thuringiensis* during production, filling and packaging. No adverse health effects were noted upon occupational exposure in a production plant of *Bacillus thuringiensis*.

In a further study greenhouse workers were occupationally exposed to *Bacillus thuringiensis*. individual exposure estimated by using personal monitoring equipment was 3 x 10^5 CFU per person per working day (ranging from 0 to 3 x 10^6 CFU/d per person (referred to as *Bacillus cereus*-like CFU). This corresponded to aerial concentrations of 0 to 167 CFU/L. *Bacillus thuringiensis* (range 10^2 – 10^3 CFU/g) was detected in the faeces of 8 out of 20 workers, with approximately 10% of the CFU originating from spores. Furthermore, no health complaints were noted that could be causally related to exposure to *Bacillus thuringiensis*.

An AFLP-based phylogenetic comparison of B. thuringiensis, B. cereus and *B. anthracis*, prepared by Jackson et al. (2005), shows that the genetic distance between the toxigenic strains of Bt, Bc and Ba and the strain GC-91 is very wide and the cluster of GC-91 is far away from the hazardous and toxigenic strains. The quality controls, made regularly during the fermentation process, also show no production of toxic metabolites. In addition, no clear evidence of food poisoning has ever been reported during the use of the Bt products. General studies on humans and animals have shown that *B. thuringiensis* and the agricultural use of *B. thuringiensis* products have an excellent safety record. In the medical literature there is no case report associating commercially used *B. thuringiensis* directly with food poisoning (Siegel, 2001).

It is possible to say that on the whole, the use of *Bacillus thuringiensis* ssp. *aizawai* strain GC-91 is expected to be not hazardous to mammals, is not pathogenic and does not produce any known mammalian toxins.

B.7.2.1 Non-viable residues

Information from DAR and DAR addendum (May 2007, February 2013) Non-viable residues do not pose a risk to humans or the environment. Crystal proteins, the other major component in commercial Bt preparations apart from spores, are not toxic to mammals as indicated in different publications. In addition, crystal proteins are very unstable when exposed to light. The half-life of insecticidal activity of Bt preparations on cotton leaves as measured in bioassays using Trichoplusia ni or Heliothis virescens larvae was 34 to 47 hours following application (Beegle et al., 1981). If we consider sunlight is the decisive element in reduction of crystal protein activity there appear to be unlikely risk related to non-viable residues in southern European countries when adopting 14 days of Pre Harvest Interval (PHI).

New information 2016

The literature search covering the last 10 years (Cornelese, 2016) included a search for residues of known metabolites. No hits on information on metabolites were found in the search.

B.7.2.2 Viable residues

Information from DAR and DAR addendum (May 2007, February 2013)

The active components of commercial B. thuringiensis preparations, spores and crystal proteins, are not toxic to humans, plants, and most animals except target species belonging to the order Lepidoptera. The amount of spores used in acute oral toxicity tests (4 x 10^{10} CFU/kg b.w.) that did not show any toxic or pathogenic effect would correspond to about 10.8 kg of treated food commodities after harvest per kg b.w. according to the calculation of the pre-harvest application. Considering that both the components, spores and crystal proteins, are rapidly degraded in sunlight, and that the levels of strains introduced by applications decrease rapidly to background levels of indigenous Bacillus thuringiensis, one can assume residues of B. thuringiensis ssp. aizawai, following application of Agree 50 WP (according to the GAP and adopting a PHI of 14 days), are not expected to be of concern for human consumption.

New information 2016

The EFSA BIOHAZ panel on Risks for public health published a Scientific Opinion related to the presence of *Bacillus cereus* and other *Bacillus* spp. including *Bacillus thuringiensis* in foodstuffs[2]. Due to lack of available data, it was not possible to conduct a quantitative evaluation of the risk to public health arising from the presence of *B. thuringiensis* in food. Therefore, a qualitative evaluation has been done, considering relevant scientific literature and information from the background documents provided by the European Commission describing the specific cases in the alleged food poisoning due to *B. thuringiensis*. An evaluation of the occurrence and levels of *B. thuringiensis* in foods has been carried out, through an extensive literature review.

The BIOHAZ panel performed an extensive literature search in order to obtain information on the presence and levels of *B. thuringiensis* in food. Information on the presence and levels of *B. thuringiensis* in food extracted from the papers included in this search is difficult to summarise because very heterogeneous types of food (raw and cooked) have been analysed and in most of the cases details on measurements are missing. Additionally, the methodologies and techniques used to determine the presence and levels of *B. thuringiensis* in food samples are very diverse and in general, none of the analytical methodologies available and used in the selected research studies can be classified as 100% reliable. The levels of *B. thuringiensis* reported in food are very variable, in most cases below 10^3 CFU/g. *Bacillus thuringiensis* strains isolated from foods can in some cases be related to the use of biopesticides containing *B. thuringiensis*, but in most cases this possible relation has not been investigated.

Frederiksen et al. (2006), also cited in the opinion, investigated the occurrence of *Bacillus cereus* like strains on fresh fruits and vegetables. 128 isolated strains were characterized, of these 50 were classified as *B. thuringiensis* on the basis of cry genes. RAPD analysis and plasmid DNA profiling revealed that 23 strains were indistinguishable from the active organisms in commercially used products. It has to be underlined that these strains were indistinguishable using the available methods, which are not sufficiently reliable to unequivocally identify a strain. Moreover, this approach is heavily biased as the commercial strains are used as reference. Most probably, they are indistinguishable from commercial as well as various other Bc and Bt strains not considered. 14 isolates were indistinguishable from the *B. thuringiensis* subsp. *kurstaki* strain HD1and 9 isolates indistinguishable from the *B. thuringiensis* subsp. *aizawai* amongst which strain GC-91. The highest level measured was 10^4 CFU/g in cucumber and tomato. In the samples, also non-commercialised Bt strains were detected as well as other *B. cereus* like organisms.

Hendriksen et al. (2006) investigated the appearance of residues of *Bacillus thuringiensis subsp. kurstaki* HD1 on retail cabbage after the use as biopesticide. Extracted kale samples were grown on petri dishes. In total, 134 *B. cereus*-like colonies were isolated. The isolates were examined by phase-contrast microscopy for their ability to produce parasporal inclusion bodies (crystals) in the sporangium. Random amplification of polymorphic DNA (RAPD) method was used, and the authors report that common RAPD pattern was found for *Bt kurstaki* HD1 and *Bt aizawai* strains HD131, HD137, HD11, HD112 and HD283, and that this pattern was not found in 22 used *B. cereus* strains. These data were not shown however, and it is not described in Materials and Methods section if the RAPD was repeated. RAPD is a method that gives problems in experiment reproducibility and many scientific journals do not accept experiments based on this method. This is because RAPD primers are very short, which can result in alterations in their annealing behaviour to the template DNA and the resulting band profiles as a result of small deviations in experimental conditions. RAPD is especially difficult to apply to bacteria from *B. cereus* group due to extremely high similarity in their chromosomal DNA among them. It is possible that 72 isolates found on cabbage and broccoli shared the HD1 pattern (these data are also not shown in

[2] EFSA SCIENTIFIC OPINION on Risks for public health related to the presence of *Bacillus cereus* and other *Bacillus* spp. including *Bacillus thuringiensis* in foodstuffs EFSA Panel on Biological Hazards (BIOHAZ), EFSA Journal 2016;14(7):4524

the article), however most likely most of the *B. thuringiensis* strains found in the field as well as in the natural conditions would share this pattern. The authors reported that the maximum quantity of the Bt detected was 10^2 CFU/g cabbage. The half-life of Bt on cabbage was estimated at 16 h (Pedersen et al., 1995). Thus, even assuming that all the Bt present in the cabbage leaves originated from the biopesticide product used on the cabbage farm, the densities detected present no risk to public health.

Stephan et al. (2014) measured residues of Bt on tomato fruit after 5 times application of the commercial product XenTari. (*Bacillus thuringiensis* subspec. *aizawai*). Whole plants were treated in a greenhouse experiment with two types of tomatoes at a mean single application rate of 2.87×10^4 CFU/g fresh weight tomato (minimum 2.1×10^4 cfu/g fresh weight, maximum 4.7×10^4 cfu/g fresh weight). Fruits were sampled at different time steps just before and after the last application and after 1, 2, 3 and 7 days after the last application. For enumerating the Bt residues tomatoes were washed in sterile solution and a sample of the wash solution was plated on TSA and incubated for 20 h. at 25°C. The number of CFU per gram fresh weight was calculated. From each sample location five tomatoes were analysed. The number of CFU of each tomato was enumerated in triplicate.

Results of the greenhouse experiment show that a mean concentration of 63.9 CFU/g fresh weight in untreated control, with a maximal concentration of 8.7×10^2 CFU/g fresh weight, was observed throughout the entire experiment. When XenTari® was applied five-times at weekly intervals, the mean concentration of colony forming units on tomato fruits ranged between 4.9×10^4 and 8.5×10^4 CFU/g fresh weight. The concentration of Bt spores decreased during 7 days after the last application to between 46% and 77% of the initial spore concentration immediately after the last spray. Comparable results were achieved at an experiment carried out at the commercial farm. In the untreated control a maximum concentration of 1.9×10^2 CFU/g fresh weight was achieved, whereas a single application of XenTari® on the whole plant resulted in a concentration of 2.1×10^4 CFU/g fresh weight. Within one week the concentration declined to 1.3×10^4 CFU/g fresh weight.

Zhou et al. (2008) investigated treated food for the occurrence of Bt like strains and a total of 19 *Bacillus thuringiensis*-like strains were isolated. Pre-treated samples of different food items were plated and incubated. Bacterial colonies were subcultured and those isolates producing a parasporal body observed und light microscope were preliminary identified as *Bacillus thuringiensis* and further characterized by a serotyping test, SDS-PAGE, random amplified polymorphic DNA, and enterotoxin gene PCR analysis. As mentioned above RAPD is not a reliable method for bacteria identification. Serotyping is still the most widely accepted subspecific classification method for Bt, however it is now known that strains within the same Bt serovar often do not share biochemical, genetic and toxicological attributes. SDS-PAGE analysis can provide the idea of proteins contained in the spores/crystals, but the pattern will to high extent depend on the sample preparation (for example pH so important for solubilisation of Bt crystals, and only soluble proteins are visible on SDS-PAGE gel). By no means can SDS-PAGE protein pattern serve for Bt strains identification or even grouping. As for the enterotoxin genes PCR method, it is now known that most of the *B. cereus* group strains have enterotoxin genes present on their genome and some are even expressed. In the study, a total of 19 *B. thuringiensis* strains were isolated from food and green-tea beverages. The authors reported that by using the described methods five strains isolated, two from pasteurized full fat milk and three from green-tea beverages, were indistinguishable from commercialized B. *thuringiensis* subsp. *kurstaki* isolated from biopesticides. It has to be emphasized that by using these methods most of the Bt strains are indistinguishable from commercial Bt strains, due to a high similarity among *B. cereus* group strains and even higher between different Btk strains. However, only very low levels were detected with a maximum of 3.6 CFU/mL. A summary table with 'background levels' in various commodities is provided below.

Bt as a species occurs naturally in a range of environmental compartments such as soils, plant surfaces and infected insects.

A literature review aiming to define background levels of Bt in the environment was done within the frame of the preparation of the EFSA Scientific opinion on the Risk for public health related to *B. cereus* and other *Bacillus* spp. including *B. thuringiensis* in food stuff published in 2016[3].

Confirming information already presented during fist evaluation of the strain, it was concluded that members of the *B. cereus* group occur ubiquitous in the environment in soil, plants, sediments, water, invertebrates and mammals. In soils, 0 - 50% of the *B. cereus* group isolates affiliate with Bt reaching levels of up to 5×10^5 CFU/g soil. On plants, the populations vary between 0 and 6×10^4 CFU/g with a mean density of 100 CFU/g in areas not previously treated with Bt. A summary of recorded background levels can be found in **Table B.7.2.2-1.**

Table B.7.2.2-1 Natural background levels of Bt in different environmental compartments

[3] EFSA SCIENTIFIC OPINION on Risks for public health related to the presence of *Bacillus cereus* and other *Bacillus* spp. including *Bacillus thuringiensis* in foodstuffs EFSA Panel on Biological Hazards (BIOHAZ), EFSA Journal 2016;14(7):4524

Environmental compartment*	**Density of *B. cereus* group members including Bt**	**Reference**
Soil		
Cultivated soils UK	2×10^4 CFU/g	Collier et al. (2005, cited in EFSA Scientific Opinion)
Cultivated soils Denmark	2×10^5 CFU/g	Hendriksen et al. (2006, cited in EFSA Scientific Opinion)
Cultivated soils Denmark to be checked	5×10^5 CFU/g	Raynond et al. (2010, cited in EFSA Scientific Opinion)
Agricultural soils France	4.43 - 5.23 log CFU/g	Brillard et al. (2015, cited in EFSA Scientific Opinion)
Danish soils	4×10^4 - 2×10^5 CFU/g, mean 105 CFU/g	Hendriksen et al. (2011, cited in EFSA Scientific Opinion)
Rice field	4.23 - 6.52 10^5 CFU/g	Chatterjee et al. (2007, cited in EFSA Scientific Opinion
Water		
Rainwater	1.16 - 2.5 log CFU/L	Brillard et al. (2015, cited in EFSA Scientific Opinion)
Groundwater	0.17 - 0.96 log CFU/L	
Plants/crops		
Broad-leaf dock	2×10^4 CFU/g	Collier et al. (2005, cited in EFSA Scientific Opinion)
Curly kale	Max. 6×10^4 CFU/g, mean 3×10^2 CFU/g	Hendriksen et al. (2011)**
Cauliflower leaves	80 – 1700 CFU/cm^2 leaf	Damgaard et al. (1994, cited in EFSA Scientific Opinion)
Rice	Max. 23 CFU/g	Ankolekar et al. (2009, cited in EFSA Scientific Opinion)
Maize and bean leaves	0.46 - 1.5 spores/cm^2	Jara et al. (2006, cited in EFSA Scientific Opinion)
Rice	2 - 11.2 CFU/g	Kim et al. (2014, cited in EFSA Scientific Opinion)
Food		
Vegetables and fruits	10 – 11000 CFU/g	Frederiksen et al. (2006, cited in EFSA Scientific Opinion)
Ready to eat food (48,901 samples)	0 - 10^4 CFU/g, usually below 10^3 CFU/g CFU/g	Rosenquist et al. (2005, cited in EFSA Scientific Opinion)
Spices, paprika, allspice, peppercorns, and mixed spices	3 to 240 MPN/g	Hariram and Labbé (2015, cited in EFSA Scientific Opinion)

EFSA SCIENTIFIC OPINION on Risks for public health related to the presence of *Bacillus cereus* and other *Bacillus* spp. including *Bacillus thuringiensis* in foodstuffs EFSA Panel on Biological Hazards (BIOHAZ), EFSA Journal 2016;14(7):4524

*References were also provided for diary products but were not included here, as this is not of relevance for use of the strain for pest control in agriculture

**Presentation of data shown at Second Biopesticides Steering Group Seminar on the Fate in the Environment of Microbial Control Agents and Their Effects on Non-target Organisms, summarized in Series of Pesticides No. 64. At page 125 of the cited paper for the presentation: Fate of microbials in the environment, A structural model for explanation of the fate By Niels Bohse Hendriksen.

An overview of available information on persistence of Bt strains in the environment, also on crop, is provided in Volume 3 B.8.

Taking into account the available information, the EFSA BIOHAZ panel on Risks for public health concluded, that most cases of food-borne outbreaks caused by the *B. cereus* group have been associated with bacterial concentrations above 10^5 CFU/g foodstuff. However, in some cases both emetic and diarrhoeal illness have been reported, involving concentrations between 10^3 and 10^5 CFU/g of *B. cereus*. Following these considerations, the Panel concluded that, taking the enterotoxigenic potential into account as well as that *B. thuringiensis* cannot be distinguished from *B. cereus* at the chromosomal level, the levels of *B. cereus* that can be considered as a risk for consumers are also likely to be valid for *B. thuringiensis*. There is, however, no evidence that *B. thuringiensis* has the genetic determinants for the emetic toxin cereulide. With reference to B. 2.8, the authors of the Opinion[2] came to the conclusion that neither the emetic toxin of *B. cereus* nor the highly cytotoxic form of CytK, namely CytK2, are produced by Bt. All other enterotoxins could be potentially produced by members of this species. Other virulence factors such as sphingomyelase or Haemolysin II have so far not been detected in Bt.

However, whether the conclusion of the Panel is justified is questionable as it mainly refers to shortcomings of clinical diagnostic methods not able to distinguish between *B. cereus* group members leading to uncertainties which role Bt actually played in the recorded outbrakes. It does not take into account that:

- insecticidal Bt strains differ significantly from pathogenic *B. cereus* with regard to their physiological properties (less stress resistant spores, lower germination and growth rates, less well growing at high temperature and under microaerobic conditions), ecology and environmental behaviour (highly adapted to their insect hosts) and toxigenic properties (lower potential for surface attachment and less aggressive against human cell lines, production of lower amounts of enterotoxins in the lab)
- despite a certain toxigenic potential, indicated by the presence of enterotoxin genes in their genome, there is no hint that commercial Bt strains, including strain Bta GC-91, will fulfil all prerequisites required to exhibit pathogenicity in humans. This includes persistence on treated crops and harvested good until the time of consumption, the ability to survive the gastric passage, to germinate and grow in the human intestine, to attach and invade epithelium cells and to produce the respective enterotoxins, at relevant levels, under these conditions. For more details, please refer to B.2.8.
- pathogenicity is strongly strain specific and only if there is evidence that strains are sufficiently similar with regard to properties of potential relevance for human health, a read across in the pathogenicity assessment is eventually possible (SANCO/10754/2005 rev.5, 2005).
- It has to be emphasized that analytical methods used in literature references were not specific enough to distinguish strains of Bt from commercial Bt strains due to a high similarity among *B. cereus* group strains and even higher similarity between different Btk strains. Currently strain specific methods are available that should be used instead to determine residue levels in food.

From the information provided above and the step approach described in B.2.8 it is clear, that a commercial Bt strain, such as Bta GC-91 can hardly be compared to a pathogenic *B. cereus* strain. Therefore, any prediction of a safety level for commercial Bt strains based on information of pathogenic *B. cereus* isolates is not reasonable.

Taken together all information about Bta CG-91, the risk for consumers due to use of the strain for pest control in agricultural settings appears to be acceptable.

Note RMS: The QPS list (see Scientific Opinion 2017*) considered the notification of Bta CG-91 as not appropriate for QPS until the respective dossier (including the literature review) is received. However, the arguments above which are based on the updated dossier will exclude the risk for consumers of the strain for pest control in agricultural settings.

* Scientific Opinion on the update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA. Scientific Opinion, EFSA Journal 2017; 15(3); 4664.

Cited references

Report KMA 6.2.2/01 – Frederiksen, K., Rosenquist, H., Jørgensen, K., Wilcks, A., 2006. Occurrence of natural *Bacillus thuringiensis* contaminants and residues of *Bacillus thuringiensis*-based insecticides on fresh fruits and vegetables.

Published report,

Applied and Environmental Microbiology, 72(5):435–3440

Abstract: A total of 128 Bacillus cereus-like strains isolated from fresh fruits and vegetables for sale in retail shops in Denmark were characterized. Of these strains, 39% (50/128) were classified as *Bacillus thuringiensis* on the basis of their content of cry genes determined by PCR or crystal proteins visualized by microscopy. Random amplified polymorphic DNA analysis and plasmid profiling indicated that 23 of the 50 B. thuringiensis strains were of the same subtype as *B. thuringiensis* strains used as commercial bioinsecticides. Fourteen isolates were indistinguishable from *B. thuringiensis* subsp. *kurstaki* HD1 present in the products Dipel, Biobit, and Foray, and nine isolates grouped with *B. thuringiensis* subsp. *aizawai* present in Turex. The commercial strains were primarily isolated from samples of tomatoes, cucumbers, and peppers. A multiplex PCR method was developed to simultaneously detect all three genes in the enterotoxin hemolysin BL (HBL) and the nonhemolytic enterotoxin (NHE), respectively. This revealed that the frequency of these enterotoxin genes was higher among the strains indistinguishable from the commercial strains than among the other *B. thuringiensis* and *B. cereus*-like strains isolated from fruits and vegetables. The same was seen for a third enterotoxin, CytK. In conclusion, the present study strongly indicates that residues of *B. thuringiensis*-based insecticides can be found on fresh fruits and vegetables and that these are potentially enterotoxigenic.

Report KMA 6.2.2/02 – Hendriksen, N.B., Hansen, B.M., 2006. Detection of *Bacillus thuringiensis kurstaki* HD1 on cabbage for human consumption.

Published report,

FEMS Microbiol Lett., 257(1):106–111

Abstract: The objectives of the study were to develop a specific procedure for quantification and identification of *Bacillus thuringiensis kurstaki* HD1, which is used as a biopesticide, and to quantify its presence in different kinds of cabbage for human consumption. We found that *B. thuringiensis kurstaki* HD1 can be distinguished from other *B. thuringiensis* strains by its unique random amplification of polymophic DNA-PCR pattern with the OPA9 primer and the presence of the flagellin genes, as detected by the primers FLAB1 and FLAB2. We detected from one to 100 *Bacillus cereus*-like bacteria in 10 batches of five different cabbage products for consumption. As many as 73 out of 134 isolates (53.7%) were identical with *B. thuringiensis kurstaki* HD1. The results show that *B. thuringiensis kurstaki* HD1 from biopesticides can be found in vegetables for human consumption. The authors conclude that it is unlikely that *B. thuringiensis kurstaki* HD1 occurring on cabbage products, at the densities found in their study, is of any concern in relation to public health.

Report KMA 6.2.2/03 – Stephan, S., Scholz-Döbelin, H., Reintges, T., Pelz, J., Jehle, J.A. Keßler, J., 2014 Investigations on residues of XenTari® (*Bacillus thuringiensis* subspec. *aizawai*) on greenhouse tomatoes.

Published report,

Journal für Kulturpflanzen 66 (9): 312–318

XenTari® (*Bacillus thuringiensis* (*B.t.*) subspecies *aizawai*)is an important biological plant protection agent for the control of Noctuidae larva on tomato fruits in greenhouses and belongs to the group of presumptive *Bacillus cereus* species. In general, food control agencies do not routinely differentiate between *B.t.* and *B. cereus* and a threshold of 10^5 colony forming units (cfu)/g fresh weight is applied
for presumptive *B. cereus* in official food control. As no data exists on the expected residues of *B.t.* spores after application, residual experiments were conducted on tomatoes in greenhouses. In the greenhouse experiment, five applications of XenTari® were applied at weekly intervals. The concentration of *B.t.* spores on the tomato fruits ranged in all experiments between 4.9×10^4 and 8.5×10^4 cfu/g fresh weight. For single application of *B.t.*, a maximum spore concentration of 4.7×10^4 cfu/g fresh weight was measured. None of the experiments reached the threshold for *B. cereus* of 1×10^5 cfu/g, although treatments were applied in a very narrow window. The findings were confirmed by additional laboratory experiments and by experiments conducted on a commercial tomato farm. To prove the degradation of *B.t.* spores under protected greenhouse conditions over time, a series of samples was taken after the last application over one week. Over all, the experiments demonstrated that the con-

centration of *B.t.* spores was reduced within one week to between 46% and 77% of the initial spore concentration. Therefore, in comparison to open field condition the degradation of *B.t.* spores under greenhouse condition was limited. When only the upper parts of the tomato plant were treated with XenTari® a distinct reduction of *B.t.* spores of up to 90% of *B.t.* spores with a concentration of 1.85×10^3 cfu/g fresh weight on the marketable tomatoes was achieved.

Report KMA 6.2.2/04 – Zhou, G., Yan, J., Dasheng, Z., Zhou, X., Yuan, Z., 2008. The residual occurrences of *Bacillus thuringiensis* biopesticides in food and beverages.

Published report,

International Journal of Food Microbiology, 127(1–2):68–72

Abstract: In 2006, 54 pasteurized full fat milk samples, 40 ice-cream samples, and two green-tea beverage samples were analyzed and a total of *19 Bacillus thuringiensis*-like strains were isolated, nine from seven pasteurized milks, one from an ice-cream with peach pulp and juice, and nine from two green-tea beverages. These strains were classified as *B. thuringiensis*, contained the cry1A gene and produced crystal inclusions during sporulation. All strains were characterized by a serotyping test, SDS-PAGE, random amplified polymorphic DNA, and enterotoxic gene PCR analysis. Most isolates produced bipyramidal crystals and belonged to serotypes H3a3b, H5a5b, or H7. Furthermore, two strains from pasteurized full fat milks and three strains from green-tea beverages were indistinguishable from the *B. thuringiensis* subsp. kurstaki strains isolated from commercial biopesticides (Kaiyan®, Qiangdi®, Lvpuan® and Sutai®), suggesting the residual occurrences of *B. thuringiensis* from biopesticides in food and beverages.

B.7.3 Summary and evaluation of residue behaviour

Information from DAR and DAR addendum (May 2007, February 2013) The strain GC-91 is a product of a crossing or conjugation between two parental strains HD-191-A2 (flagella serotype *kurstaki*) and HD-135-S4 (flagella serotype *aizawai*), which are derived from wild type strains and differ in their flagella serotype as well as in their δ-endotoxin genes..

The theoretical maximum population of *B. thuringiensis* ssp. *aizawai* strain GC-91 after application of Agree 50 WP is a cumulative population of 1.9×10^{14} CFU/ha, when assuming no decrease in the population between applications. Applying an interception factor of 0.85 (FOCUS, 2000) for fully developed grapes, that we can consider worst case, consequently, 1.6×10^{14} CFU are applied on grapevine plants per ha. The average yield for grape is assumed to be 11 t/ha (for Germany) and 5.5 t/ha (for Italy): this results in a final content of 1.48 x 107 CFU/g grape in the most favourable case or a double amount in the worst case.

If one considers that the persistence of Bt spores, following spraying of DiPel (containing *B. thuringiensis* ssp. *kurstaki* strain HD-1), is less than 1 day and that the δ-endotoxins are rapidly degradable, and endospores are rapidly inactivated when exposed to UV radiation, modulated by rainfall and other environmental factors on the leaf surface, it is possible to hypothesize that the half life of Bt spores on foliage is not exceeding 1 to 3 days.

There was no obvious influence of grape variety on the association of *B. thuringiensis*. At the time of harvest, *B. thuringiensis* populations on grapes varied between 10^2 and 10^4 CFU/g, depending on vineyard and corresponding to the natural occurrence of *B. thuringiensis* on the leaves of different crops and plants. After flowering the Bt population is stabilizing at these concentrations during cultivation, independent from the timing of application. Even right after the pre-harvest application, no increase of the *B. thuringiensis* population could be detected (Bae et al., 2004).

In comprehensive reviews, the existing knowledge on the mammalian safety of *Bacillus thuringiensis* has been summarised, demonstrating also the similarity of different *Bacillus thuringiensis* subspecies with regard to their lack of infectivity, toxicity and pathogenicity to mammals including humans.

In the medical records no adverse health effects attributable to *Bacillus thuringiensis* exposure were found in physical examinations in workers exposed to *Bacillus thuringiensis* during production, filling and packaging. No adverse health effects were noted upon occupational exposure in a production plant of *Bacillus thuringiensis*.

According to Rosenquist et al. (2005), a population of up to 10^3 CFU/g *B. cereus*-like cells is “satisfactory” for ready-to-eat food: a pre harvest interval of 14 days can be considered a good safety period to theoretically reduce

the population of *B. thuringiensis* ssp. *aizawai* applied at the security level of 10^3 CFU. Further information with regard to residues of *B. cereus* like cells on food is provided in this dossier for re-registration of bel *Bacillus thuringiensis* ssp. *Aizawai* below.

Levels of strains introduced by applications decrease rapidly to background levels of indigenous Bacillus thuringiensis. Therefore, any residues of *B. thuringiensis* ssp. *aizawai*, following application of Agree 50 WP according to GAP, are not expected to be of concern for human consumption.

New information 2016

Residues of *B. thuringiensis* subsp. *aizawaii* on crop may be expected after spray application. Initial decay on leaves occurs rapidly with some tailing thereafter. The growth of endospores is dependent on the germination of the spore, followed by divisions of the vegetative cell. On leaves, *B. thuringiensis* occurs mainly as spores, the concentration of nutrients of the leaf surface is insufficient to mediate growth of *B. thuringiensis*.

A number of studies monitored the occurrence of Btk on food. The cited publications report findings on fresh food of strains of Bt that are used commercially. These results have to be considered with care. In all studies the methods of identification are molecular methods that are not suitable to unequivocally distinguish closely related strains within the group of *Bacillus* spp. Moreover, in all studies, the strains from commercially known products were used as reference and therefore biased results to a large extend. The EFSA BIOHAZ panel indicates that most cases of food-borne outbreaks caused by the *B. cereus* group have been associated with concentrations above 10^5 CFU/g and that the levels of *B. cereus* that can be considered as a risk for consumers might be also valid for *B. thuringiensis*. However, this approach is not justified as pathogenic *B. cereus* strains differ significantly from commercial Bt strains in the physiological requirements, environmental behaviour and their toxigenic potential. Based on available information it can be concluded that the risk for consumers due to possible exposure of Bta CG-91 is acceptable.

B.7.4 References relied on

Literature search
A literature search with regard to human toxicology was performed

This report summarizes the search and selection process of open peer-reviewed literature for *Bacillus thuringiensis* subsp. *aizawai* strain GC-91 and metabolites.

The search strategy was based on a multi-concept approach. For details regarding the search strategy and the results obtained, please refer to Point 4 and Point 5 of this Literature Review Report.

The selection process resulted in the classification of the available reports in the four categories recommended by the EFSA (2011)[4] guideline:

1) Studies that are relevant to the data requirement and that provide data for establishing or refining risk assessment parameters. These studies should be considered for reliability.
2) Studies that are relevant to the data requirement, but in the opinion of the applicant provide only supplementary information that does not alter existing risk assessment parameters.
3) Studies for which relevance cannot be clearly determined.
4) Studies of no relevance

The relevance criteria applied are reported in Point 3.2 of this Literature Review Report.
The reliability assessment for relevant studies was done according to the recommendations of the EFSA (2011)[1].

The overall results are shown below on **Table 2-1**.

Table 2-1 Results of the study selection process for Section 6

Data requirement capture in the search:	**n**
Total number of summary records retrieved after all searches of peer-reviewed literature	40
Number of summary records excluded from the search after rapid assessment for relevance	34
Total number of full-text documents assessed in detail	6
Number of studies excluded from further consideration after detailed assessment of relevance	1
Number of studies not excluded for relevance after detailed assessment	5

[4] Guidance of EFSA: Submission of scientific peer-reviewed open literature for the approval of pesticide active substances under Regulation (EC) No 1107/2009. EFSA Journal 2011;9(2):2092

3 Protocol: objective of the review and criteria used

3.1 Objective

The review was made in order to identify scientific peer-reviewed open literature on the active substance *Bacillus thuringiensis* subsp. *aizawai* strain GC-91 and its metabolites which may affect the assessment on human health, animal health and/or the environment, with the special consideration of residues in or on treated products.

3.2 Criteria for relevance and reliability

The criteria for relevance and reliability used are summarised below in **Table 3.2-1.** Only studies that were considered relevant were assessed for reliability.

Table 3.2-1 Criteria for relevance and reliability used in the review

Relevance criteria
• Property investigated was relevant for data requirements of Regulation (EC) No 1107/2009 • Subject relevant for residues of *Bacillus thuringiensis* subsp. *aizawai* strain GC-91 analysed on products, food and feed • Subject relevant for residues of *Bacillus thuringiensis* subsp. *aizawai* strain GC-91 occurrence on plants • Test species/system relevant to the residues on food and feed • Application on crops and consumer risk • Relevant crop / trial location
Reliability criteria
Minimum information reported e.g.: • Test item or related compound • Test species relevant • Clear and comprehensive description of material and methods, incl. duration, replicates, test conditions; appropriate statistical methods used • Conclusions robust and clear • Definition of endpoints • Presentation of result • Guideline compliance

4 Search methods and results

The literature research was conducted on the DIMDI database provided by the German Institute of Medical Documentation and comprised searches in MEDLINE; BIOSIS, CAB and SCISEARCH databases. Search strategy aimed to find all recent (from 2006 – 2016) references that are relevant for residues.

Keywords used:

Active substance *Bacillus thuringiensis* OR Btk AND *kurstaki*# AND *aizawai*# AND (residue?) AND (consumer OR food OR feed OR risk OR bacillomycin OR cereulide OR crystal protein# OR cry? toxin# OR cytotoxin OR

enterotoxin OR entomocin OR fengycin OR cytolytic protein# OR iturin, OR GC91 OR CG 91 OR CG-91 OR Turex OR Agree.

An additional search was performed for all known metabolites from micro organisms used for plant protection searching for leucocidin, mycosubtilin nhe, surfactin, thuringiensin, vegetative insecticida, cry?,zwittermycin, bacillomycin, cereulide, chi?, crystal protein#, cytotoxin, enterotoxin, entomocin, fengycin, glucanase, hbl, hemoly?, cytolytic protein#, or iturin

Obtained references were first subjected to a *rapid assessment* based on title and the abstract. Summary records that appeared to be relevant passed to a second step in which a detailed assessment of full text documents was conducted.

Details on the search strategies are presented in **Table 4-1**. Results are listed in **Table 4-2**.

Table 4-1 Search process for peer-reviewed open literature in bibliographic databases

Database	BA00	CV72	SCISEARCH	MEDLINE
Justification for choosing the source	BIOSIS Previews covers worldwide literature in the field of biology (zoology, botany, microbiology), human and veterinary medicine, biochemistry, pharmacology, toxicology, and environmental sciences, especially from Northern America and Europe. It corresponds to the printed Biological Abstracts and Biological Abstracts/RRM (Reports, Reviews, Meetings). BA00: 2000 to date	CAB Abstracts covers worldwide literature of agriculture and related sciences including biotechnology, veterinary medicine, nutrition, medicine and forestry sciences. Sources include approx. 9000 international journals, books, conference proceedings, and patents. CV72: 1972 to present	SciSearch covers worldwide literature in the fields of science, technology, and medicine. The database contains all citations published in "Science Citation Index Expanded". Sources include approx. 6650 international journals of 150 disciplines including Clinical Medicine and Life Sciences. IS00: 2000 to date	MEDLINE (Medical Literature Analysis and Retrieval System Online) covers worldwide literature on every area of medicine, including dental medicine, veterinary medicine, psychology, and public health. The database corresponds to the printed "Index Medicus" and to some other printed material. Sources include approx. 4800 international journals. ME00: 2000 to date
Date of the search	01.07.2016			
Date span of the search	2006 - 2016			
Language limit	English – Spanish – French – German - Italian			
Other limits set	Available abstracts			
Search type	Advanced Search			
Search strategy	Search term 1:	(((FT=Bacillus thuringiensis OR FT=Bt) AND FT=kurstaki# AND (residue?) AND (consumer OR food OR feed OR risk)))		
	Search term 2:	(((FT=Bacillus thuringiensis OR FT=Bt) AND (FT=kurstaki# OR aizawai #) AND (residue?) AND (consumer OR food OR feed OR risk)))		
	Search term 3:	(((FT=bacillomycin OR FT=cereulide OR FT=crystal protein# OR cry? toxin# OR FT=cytotoxin OR FT=enterotoxin OR FT=entomocin OR FT=fengycin OR FT=cytolytic protein# OR FT=iturin) AND (residue?) AND (consumer OR food OR feed OR risk)))		
	Search term 4	Search term 2 AND search term 3		
	Search term 5:	(((FT=Bacillus thuringiensis OR FT=Bt) AND FT=aizawai# AND (residue?) AND (consumer OR food OR feed OR risk)))		
	Search term 6	(((((((FT=GC-91 OR FT=GC 91) OR FT=GC91) OR FT=Turex) OR FT=Agree) AND (FT=Bacillus thuringiensis)) AND (consumer OR food OR feed OR risk)))		

Table 4-2 Search results

Search strategy	Total number of records	Total number after removal of duplicates
Search term 1	40	31
Search term 2	45	36
Search term 3	1311	Not assessed
Search term 4	10	6
Search term 5	13	9
Search term 6	12	6
Total number of hits:		**88*** **40****

= maximum truncation; ? = variable truncation
* Duplicate references identified by the title in several databases were deleted from the list.
** Duplicate references identified by the title in several databases and through several searches were deleted from the list.

Table 4-3 Search process for peer-reviewed open literature in bibliographic databases for metabolites as from the EFSA conclusion of *Bacillus thuringiensis* subsp. *aizawai* GC-91 and EFSA supporting publication on Literature search and data collection on Risk Assessment for human health for microorganisms used as plant protection products.

Search strategy	Search term 1:	(((((((FT=leucocidin OR FT=mycosubtilin) OR FT=nhe) OR FT=surfactin) OR FT=thuringiensin) OR FT=vegetative insecticida) OR FT=cry?) OR FT=zwittermycin)
	Search term 2:	(((((((((((((FT=bacillomycin OR FT=cereulide) OR FT=chi?) OR FT=crystal protein#) OR FT=cytotoxin) OR FT=enterotoxin) OR FT=entomocin) OR FT=fengycin) OR FT=glucanase) OR FT=hbl) OR FT=hemoly?) OR FT=cytolytic protein#) OR FT=iturin))
	Search term 3:	1 OR 2
	Search term 4	(((FT=Bacillus thuringiensis OR FT=Bt) AND (FT=aizawai# OR FT=kurstaki#)
	Search term 5:	((FT=residue?) AND (FT=consumer OR FT=food OR FT=feed OR FT=risk))
	Search term 6	4 AND 5 AND 3

Table 4-4 Search results for metabolites

Search strategy	Total number of records	Total number after removal of duplicates
Search term 1	1503675	
Search term 2	4914206	
Search term 3	6293439	
Search term 4	2331	
Search term 5	151363	
Search term 6	21	16
Total number of hits:		**16*** **0****

* Duplicate references identified by the title in several databases were deleted from the list.
** Duplicate references identified by the title in several databases and through several searches were deleted from the list

5 Results of the study selection process

The relevance criteria were applied in the order presented in **Table 3.2-1** to sort out references that could be relevant (*rapid assessment* based on titles and abstracts).

Results were selected by evaluating and sorting all entries.

After the *rapid assessment*, 5 references were identified as being potentially relevant to subjected to a *detailed assessment* of the full-text documents.

The overall results of the literature research are presented in **Table 5-1.**

Table 5-1 Results of the study selection process

Data requirement capture in the search:	**n**
Total number of summary records retrieved after all searches of peer-reviewed literature	40 (88)
Number of summary records excluded from the search after rapid assessment for relevance	34 (82)
Total number of full-text documents assessed in detail	6
Number of studies excluded from further consideration after detailed assessment of relevance	1
Number of studies not excluded for relevance after detailed assessment	5

Table 5-2 and Table 5-3 below present all relevant studies subjected to a detailed assessment of full-text documents (n = 4) by data requirement and by author, respectively.

Table 5-4 presents all the studies excluded from the risk assessment after detailed assessment of full-text documents.

Table 5-2 **List of bibliographical references identified as potentially relevant and studies of unclear relevance included in the dossier after detailed assessment of full-text documents for relevance: ordered by data requirements**

EU point	Author	Year	Title	Source	Relevant to the data requirement? Yes/No	Does the new information alter the risk assessments or List of Endpoints? Yes/No	Reliability Yes/No
MA 6	EFSA peer review	2006	Conclusion on the peer review of the pesticide risk assessment of the active substance Bacillus thuringiensis subsp. aizawai (strains ABTS 1857, GC-91).	JOURNAL OF PESTICIDE REFORM/ SUMMER 2006 • VOL. 26, NO.2	Yes	No	Yes
MA 6.2.1/6.2.2	Frederiksen, K., Rosenquist, H., Jørgensen, K., Wilcks, A.	2006	Occurrence of natural *Bacillus thuringiensis* contaminants and residues of *Bacillus thuringiensis*-based insecticides on fresh fruits and vegetables	Applied and Environmental Microbiology, 72(5):3435–3440	Yes	No	Yes
MA 6.2.2	Zhou, G., Yan, J., Dasheng, Z., Zhou, X., Yuan, Z.	2008	The residual occurrences of *Bacillus thuringiensis* biopesticides in food and beverages	International Journal of Food Microbiology, 127(1–2):68–72	Yes	No	Yes
MA 6.2.2	Hendriksen, N.B., Hansen, B.M.	2006	Detection of *Bacillus thuringiensis kurstaki* HD1 on cabbage for human consumption	FEMS Microbiol Lett., 257(1):106–111	Yes	No	Yes
MA 6.4.2	Stephan, D., Scholz-Döbelin, H., Reintges, T., Pelz, J., Jehle, J.A., Keßler, J.	2014	Investigations on residues of XenTari® (*Bacillus thuringiensis* subspec. *aizawai*) on greenhouse tomatoes	Journal für Kulturpflanzen, 66(9):312-318	Yes	No	Yes

Table 5-3 List of bibliographical references identified as potentially relevant and studies of unclear relevance included in the dossier after detailed assessment of full-text documents for relevance: ordered by authors

Author	EU point	Year	Title	Source	Relevant to the data requirement? Yes/No	Does the new information alter the risk assessments or List of Endpoints? Yes/No	Reliability Yes/No
EFSA peer review	MA 6	2006	Conclusion on the peer review of the pesticide risk assessment of the active substance Bacillus thuringiensis subsp. aizawai (strains ABTS 1857, GC-91).	JOURNAL OF PESTICIDE REFORM/ SUMMER 2006 • VOL. 26, NO.2	Yes	No	Yes
Frederiksen, K., Rosenquist, H., Jørgensen, K., Wilcks, A.	MA 6.2.1/6.2.2	2006	Occurrence of natural *Bacillus thuringiensis* contaminants and residues of *Bacillus thuringiensis*-based insecticides on fresh fruits and vegetables	Applied and Environmental Microbiology, 72(5):435–3440	Yes	No	Yes
Hendriksen, N.B., Hansen, B.M.	MA 6.2.2	2006	Detection of *Bacillus thuringiensis kurstaki* HD1 on cabbage for human consumption	FEMS Microbiol Lett., 257(1):106–111	Yes	No	Yes
Stephan, D., Scholz-Döbelin, H., Reintges, T., Pelz, J., Jehle, J.A., Keßler, J.	MA 6.4.2	2014	Investigations on residues of XenTari® (*Bacillus thuringiensis* subspec. *aizawai*) on greenhouse tomatoes	Journal für Kulturpflanzen, 66(9):312-318			
Zhou, G., Yan, J., Dasheng, Z., Zhou, X., Yuan, Z.	MA 6.2.2	2008	The residual occurrences of *Bacillus thuringiensis* biopesticides in food and beverages	International Journal of Food Microbiology, 127(1–2):68–72	Yes	No	Yes

Table 5-4 List of bibliographical references excluded from the dossier after detailed assessment of full-text documents for relevance: ordered by authors

Author	Year	Title	Source	Relevant to the data requirements? Y/N/unclear	Does the new information alter the risk assessments or List of Endpoints? Y/N/unclear	Reliability Y/N/unclear	Justification
Ginsburg, C.	2006	Aerial spraying of *Bacillus thuringiensis kurstaki* (Btk)	Journal of Pesticide Reform, 26(2):13-16	N	N	N	The article is about the potential and theoretical risk to people with large scale applications of Btk in aerial spray programme. It does not provide specific information on exposure, it does not report any measurements on residues.

Data point	**Author(s)**	**Year**	**Title** **Owner, Report No.** **Source (where different from owner)** **GLP or GEP status** **Published or not**	**Vertebrate study Y/N**	**Data protection claimed Y/N**	**Justification if data protection is claimed**	**Owner**	**Previously submitted Y/N*** **If Y => old data point**
KMA 6/01	Cornelese A.	2016	LITERATURE REVIEW ON BACILLUS THURINGIENSIS SUBSP. AIZAWAI STRAIN CG-91 AND METABOLITES: RESIDUES IN OR ON TREATED PRODUCTS, FOOD AND FEED Certis USA LLC, 2281385-MA-06-01 GAB Consulting GmbH, Heidelberg, Germany GLP/GEP: no Published: no	no	yes	protected	CEU	N
KMA 6/02 1. additional submission	A.Cornelese	2016	LITERATURE REVIEW ON BACILLUS THURINGIENSIS SUBSP. AIZAWAI STRAIN GC-91 AND METABOLITES: RESIDUES IN OR ON TREATED PRODUCTS, FOOD AND FEED Certis USA LLC, 2281385-MA-06-02 GAB Consulting GmbH, Heidelberg, Germany GLP/GEP: no Published: no	no	yes	protected	CEU	N
KMA 6.1/01	Hadley, W.M., Burchiel, S.W., McDowell, T.D., Thilsted, J.P., Hibbs, C.M., Whorton, J.A., Day, P.W., Friedman, M.B., Stoll, R.E.	1987	FIVE-MONTH ORAL (DIET) TOXICITY/INFECTIVITY STUDY OF BACILLUS THURINGIENSIS INSECTICIDES IN SHEEP not available, not applicable Fundam Appl Toxicol, 8, 236-242 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 6.1

Data point	Author(s)	Year	Title Owner, Report No. Source (where different from owner) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously submitted Y/N* If Y => old data point
KMA 6.1/05	Siegel, J.P.	2001	THE MAMMALIAN SAFETY OF BACILLUS THURINGIENSIS-BASED INSECTICIDES not available, not applicable Journal of invertebrate Pathology, 77, 13-21 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 6.1
KMA 6.1/12	Pusztai, M., Fast, P., Gringorten, L., Kaplan, H., Lessard, T., Carey, P.R.	1991	THE MECHANISM OF SUNLIGHT-MEDIATED INACTIVATION OF BACILLUS THURINGIENSIS CRYSTALS not available, not applicable Biochemical Journal, 273, 43-47 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 6.3
KMA 6.1/13	Pinnock, D.E., Brand, R.J., Jackson, K.L., Milstead, J.E.	1974	THE FIELD PERSISTENCE OF BACILLUS THURINGIENSIS SPORES ON CERCIS OCCIDENTALIS LEAVES not available, not applicable Journal of invertebrate Pathology, 23, 341-346 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 6.3
KMA 6.1/14	Ignoffo, C.M., Hostetter, D.L., Pinnell, R.E.	1974	STABILITY OF BACILLUS THURINGIENSIS AND BACULOVIRUS HELIOTHIS ON SOYBEAN FOLIAGE not available, not applicable Environ Entomol, 3, 117-119 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 6.3
KMA 6.1/15	Pedersen, J.C., Damgaard, P.H., Eilenberg, J., Hansen, B.M.	1995	DISPERSAL OF BACILLUS THURINGIENSIS VAR. KURSTAKI IN AN EXPERIMENTAL CABBAGE FIELD not available, not applicable Canadian Journal of Microbiology, 41, 118-125 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 6.3

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KMA 6.1/16	Hostetter, D.L., Ignoffo, C.M., Kearby, W.H.	1975	PERSISTENCE OF FORMULATIONS OF BACILLUS THURINGIENSIS SPORES AND CRYSTALS ON EASTERN RED CEDAR FOLIAGE IN MISSOURI not available, not applicable Journal of the Kansas Entomological Society, 48, 189-193 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 6.3
KMA 6.1/18	Smith, R.A., Barry, J.W.	1998	ENVIRONMENTAL PERSISTENCE OF BACILLUS THURINGIENSIS SPORES FOLLOWING AERIAL APPLICATION not available, not applicable Journal of invertebrate Pathology, 71, 263-267 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 6.3
KMA 6.1/19	Akiba, Y.	1986	MICROBIAL ECOLOGY OF BACILLUS THURINGIENSIS VI. GERMINATION OF BACILLUS THURINGIENSIS SPORES IN THE SOIL not available, not applicable Japanese Journal of Applied Entomology and Zoology, 21, 76-80 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 6.3
KMA 6.1/20	Hendriksen, N.B., Hansen, B.M.	2002	LONG-TERM SURVIVAL AND GERMINATION OF BACILLUS THURINGIENSIS VAR. KURSTAKI IN A FIELD TRIAL not available, not applicable Canadian Journal of Microbiology, 48, 256-261 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 6.3

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KMA 6.1/21	Bae, S., Fleet, G.H., Heard, G.M.	2004	OCCURRENCE AND SIGNIFICANCE OF BACILLUS THURINGIENSIS ON WINE GRAPES not available, not applicable Int J Food Microbiology, 94, 301-312 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 6.3
KMA 6.1/23	Benoit, T.G., Wilson, G.R., Bull, D.L., Aronson, A.I.	1990	PLASMID-ASSOCIATED SENSITIVITY OF BACILLUS THURINGIENSIS TO UV LIGHT not available, not applicable Applied and Environmental Microbiology, 56, 2282-2286 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 6.3
KMA 6.1/24	Smith, R.A., Couche, G.A.	1991	THE PHYLLOPLANE AS A SOURCE OF BACILLUS THURINGIENSIS VARIANTS not available, not applicable Applied and Environmental Microbiology, 57, 311-315 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 6.3
KMA 6.1/25	Damgaard, P.H., Hansen, B.M., Pedersen, J.C., Eilenberg, J.	1997	NATURAL OCCURRENCE OF BACILLUS THURINGIENSIS ON CABBAGE FOLIAGE AND IN INSECTS ASSOCIATED WITH CABBAGE CROPS not available, not applicable Journal of Applied Microbiology, 82, 253-258 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 6.3
KMA 6.1/26	Rosenquist, H., Smidt, L., Andersen, S.R., Jensen, G.B., Wilcks, A.	2005	OCCURRENCE AND SIGNIFICANCE OF BACILLUS CEREUS AND BACILLUS THURINGIENSIS IN READY-TO-EAT FOOD not available, not applicable FEMS Microbiology Letters, 250, 129-136 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 6.3

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KMA 6.2.1/01	Beegle, C.C., Dulmage, H.T., Wolfenbarger, D.A., Martinez, E.	1981	PERSISTENCE OF BACILLUS THURINGIENSIS BERLINER INSECTICIDAL ACTIVITY ON COTTON FOLIAGE not available, not applicable Environ Entomol, 10, 400-401 GLP/GEP: no Published: yes	no	no	not protected	-	Y KIIM 6.4.1
KMA 6.2.2/01	Frederiksen, K., Rosenquist, H., Jorgensen, K., Wilcks, A.	2006	OCCURRENCE OF NATURAL BACILLUS THURINGIENSIS CONTAMINANTS AND RESIDUES OF BACILLUS THURINGIENIS-BASED INSECTICIDES ON FRESH FRUITS AND VEGETABLES not available, not applicable Applied and Environmental Microbiology, 72, 3435-3440 GLP/GEP: no Published: yes	no	no	not protected	-	N
KMA 6.2.2/02	Hendriksen, N. B., Hansen, B. M.	2006	DETECTION OF BACILLUS THURINGIENSIS KURSTAKI HD1 ON CABBAGE FOR HUMAN CONSUMPTION not available, not applicable FEMS Microbiology Letters, 257, pp. 106-111 GLP/GEP: no Published: yes	no	no	not protected	-	N
KMA 6.2.2/03	Stephan, S., Scholz-Döbelin, H., Reintges, T., Pelz, J., Jehle, J.A. Keßler, J.	2014	INVESTIGATIONS ON RESIDUES OF XENTARI® BACILLUS THURINGIENSIS SUBSPEC. AIZAWAI) ON GREENHOUSE TOMATOES not available, ISSN 1867-0911 Journal für Kulturpflanzen, 66, 312 - 318 GLP/GEP: no Published: yes	no	no	not protected	-	N

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KMA 6.2.2/04	Zhou, G., Yan, J., Dasheng, Z., Zhou, X., Yuan, Z.	2008	THE RESIDUAL OCCURRENCES OF BACILLUS THURINGIENSIS BIOPESTICIDES IN FOOD AND BEVERAGES not available, not applicable International Journal of Food Microbiology, 127, 68-72 GLP/GEP: no Published: yes	no	no	not protected	-	N