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Toxicity Assessment and Clearance of Brazilian Microbial Pest Control Agents in Mice

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Abstract The environmental toxicology of chemical pesticides have increased interest in the development and use of microbial pest control agents. In the present study four new Brazilian strains of *Bacillus* and one fungus were tested to evaluate the acute oral toxicity and clearance of these microbials in C57BL6 mice. No mortality was observed after exposure for any of the microorganisms tested. Clearance was significant after 30 days but for one strain of *B. thuringiensis* and one of *B. sphaericus* this time was not enough to completely eliminate the spores.

Keywords *Bacillus thuringiensis* · *Bacillus sphaericus* · Mammalian toxicity · Biopesticides

Microbial pest control agents (MPCAs) have been considered an interesting ecological alternative to chemical pesticides. Because of their host specificity and nature, MPCAs are generally regarded as potentially less harmful and thus are seen as attractive alternatives to some chemical applications within an integrated control program. Although several species of microorganisms have been registered for use in many countries, new strains with new toxins need to be evaluated to guarantee their safety to the environment and human health. There are controversial works on this subject. While McClintock et al. (1995) and Siegel (2001) present reviews about *Bacillus thuringiensis* showing that laboratory animals and human epidemiology studies present no evidence of adverse effects, authors such as Green et al. (1990) and Bernstein et al. (1999) have reported adverse effects in exposed workers. Studies evaluating adverse effects of the bacteria *B. sphaericus* are scarce and, with some fungi, are inexistent. As well as chemical pesticides, biopesticides safety is a major factor in gaining public acceptance and use, and this is of course very important when it comes to registration. In Brazil, the Normative Instruction n° 03/06 was recently published on the registration of microbial pesticides (Brazil 2006). This Brazilian Directive requires several toxicity assays to be performed before registration can take place. One of these studies is the acute oral toxicity and pathogenicity evaluation, standardized by the United States Environmental Protection Agency (USEPA 1996) and widely performed all around the world. In Brazil, the Brazilian Agricultural Research Corporation (Embrapa) has developed and discovered several new strains of MPCAs to control insects in agriculture and public health. The purpose of the present study was to evaluate the acute oral toxicity and pathogenicity, besides the clearance rate, of five Brazilian entomopathogens to C57BL/6 mice, generating initial data on potential health effects and guiding the choice for a future biopesticide.

Materials and Methods

Five Brazilian entomopathogenic microbials were tested in this study. Two *Bacillus thuringiensis* strains of different

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serotype: *B. thuringiensis* serotype *kurstaki*, encoding Cry1Aa, Cry1Ab, Cry1Ac and Cry1B proteins, toxic to lepidopteran larvae (Monnerat et al. 2007), *B. thuringiensis* serotype *israelensis,* encoding Cry4A, Cry4B, Cry11 and cyt1 proteins, toxic to dipteran larvae (Monnerat et al. 2005), two strains of *B. sphaericus* serotype H5 encoding 51 and 42 kDa proteins, toxic to dipteran larvae (Monnerat et al. 2004), and the fungus *Sporothrix insectorum* (Hoog & Evans), toxic to the rubber tree lacebug, *Leptopharsa heveae* (Alves et al. 2003). These strains were isolated from Brazilian soils and are stored at a Collection of Entomopathogenic *Bacillus* spp. at Embrapa Genetic Resources and Biotechnology, and in a Collection of Entomopathogenic Fungi at Embrapa Cerrados, Brazil.

Male and female C57BL/6 mice from the breeding stock of Brasilia University Center (Labocien-UniCEUB) were used. This strain of mouse, also called C57 black 6 or black 6 is the most widely used of all inbred strains and is an animal model that is very susceptible to several bacterial diseases (Cohen et al. 1995; Grandgirard et al. 2007). Young males and females (12 weeks old) were used, with weight variation not exceeding 20% of the mean of each sex. Mice were maintained individually in plastic cages, on a 12-h light/dark cycle and environmental temperature of 22 ± 3°C. The evaluation of acute oral toxicity and pathogenicity were performed based on the United States Environmental Protection Agency guideline (USEPA 1996). Mice were divided into ten groups. Five were negative control groups with three males and three females in each one, which received 100 µL of dechlorinated and filtered tapwater and were maintained near the tested groups; and five were tested groups that received a single dose (100 µL) of a cell suspension containing 10^8–10^9 spores per millilitre (CFU/mL) of *Bacillus* strains tested, growth in NYSM medium (Yousten 1984) in shaker at 200 rpm, 30°C for 72 h, and of a concentration containing 10^9 spores per millilitre (CFU/mL), in dechlorinated water, of lyophilized *S. insectorum* spores. Oral administration was performed by gavage for all animals.

The observation period lasted 30 days, with careful external examination and recording of any symptoms of adverse effects in skin, eyes, respiration and behaviour, including observation of tremors, convulsions, diarrhoea, lethargy, salivation, sleep, coma and differences in weight registered before administration and after 30 days. To estimate the clearance of MPCAs, feces of mice were collected weekly. In order to determine the presence and the amount of bacteria a pool of 1 g of feces for each animal was collected, diluted in 1 mL of water, submitted to a heat shock (80° for 12 min), diluted in serial decimal dilutions and plated in NYSM agar containing 100 mg/L of penicillin (*B. thuringiensis*) or streptomycin (*B. sphaericus*), in petri dishes and incubated at 30°C for 24 h (Silva et al. 2002; WHO 1985). Then the colonies were counted and analyzed by morphology through a phase contrast microscope to confirm the presence of the respective strain. Potato Dextrose Agar medium (PDA) was used to determine the growth of *S. insectorum*. After 30 days animals were killed by CO_2 asphyxiation and a necropsy was performed on each animal to evaluate infectivity and persistence of microbial strain in selected organs. For each tested group the presence of MPCAs in lungs, small intestine, cecum and large intestine were quantified, after dilution in saline/peptone and growth in the respective culture media. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Brasilia (UnB).

Differences in the body weight gain, as well as proportions of clearance between groups were evaluated by one-way ANOVA followed by Dunnett's multiple comparison test.

Results and Discussion

For 30 days after initial administration of MPCAs no mortality nor any sign or symptom of disease was registered. None of the animals exposed to the negative control (water) or to the MPCAs showed behavioral or clinical abnormalities. There were no statistical differences in body weight gain between controls and tested groups; these data are presented in Table 1.

Clearance of the microorganisms quantified in feces is presented in Fig. 1. These observations reveal a low recuperation of spores in feces, showing the theoretical fast elimination or degradation of most inoculated spores. The presence of spores in 1–3 animals of the control groups should be noted, and this was considered as results from keeping the animals in the same environment, probably involving aerial transportation of the spores in dust, because the individual plastic cages were maintained side by side.

Table 1 Body weight gain (Δg) of mice in controls and treated by the oral route with MPCAs after 30 days

MPCAs	Body weight gain (day 30–day 1, Δg)	
	Controls (mean ± SD)	Tested (mean ± SD)
B. thuringiensis kurstaki	5.61 ± 1.14	5.40 ± 1.19
B. thuringiensis israelensis	5.24 ± 0.78	5.85 ± 1.81
B. sphaericus strain 1	5.50 ± 1.18	5.69 ± 1.12
B. sphaericus strain 2	5.14 ± 0.80	5.59 ± 1.21
S. insectorum	5.64 ± 0.75	5.14 ± 0.91

Values are expressed as mean ± standard deviation. Data were evaluated by ANOVA followed by Dunnett's multiple comparison test ($p < 0.05$)

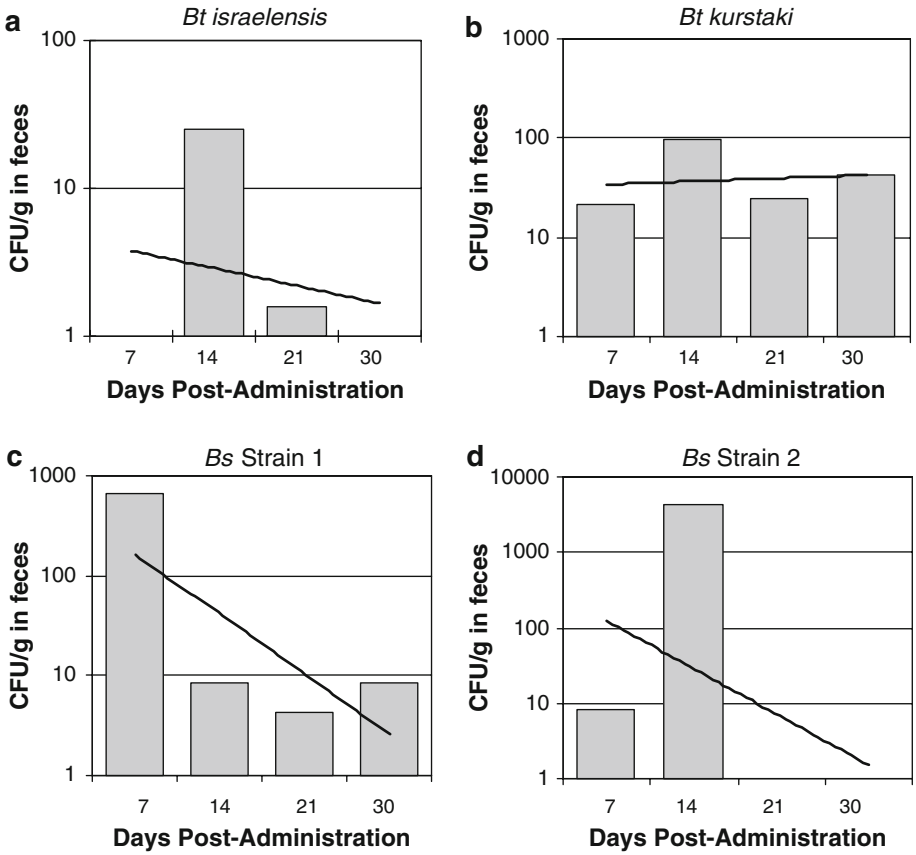

Fig. 1 Four-week quantification of spores from tested microorganisms (CFU/g) in feces of inoculated animals until 30 days. Values are expressed as a mean of CFU/g of feces of the six individuals in each group. The trends for the initial inoculums of each strain were **a** *Bacillus thuringiensis kurstaki* (8.7×10^8 spores per animal); **b** *Bacillus thuringiensis israelensis* (5.9×10^8 spores per animal); **c** *Bacillus sphaericus* strain 1 (8.9×10^9 spores per animal); **d** *Bacillus sphaericus* strain 2 (1.8×10^9 spores per animal)

The clearance of *Sporothrix insectorum* could not be detected because there is no selective medium for isolating the spores of this species. In addition, the technique used for bacterium strains was impossible because of the great variety of microorganisms and fungi types in feces of mice.

The decrease of spores in feces of the animals for all *Bacillus* tested was very significant at 30 days after inoculation (Fig. 1); both the contaminated controls and the inoculated animals were almost totally clear at this point. In 7 days the recovery of spores in inoculated animals was from seven to eightfold less (10^9–10^2, 10^8–10^0). A variation in these levels was observed in the following weeks, but the reduction was evident from seven to ninefold less (10^8–10^1, 10^9–10^0), depending of the initial inoculum.

The gross necropsy showed no visible damage to organs, but spores of *Bt kurstaki* and *Bs* strain 1 were isolated in selected organs, with slightly more recorded in the large intestine (Table 2). This may explain the presence of spores of these strains in feces until the thirtieth day after administration (Table 1).

Several studies have showed the presence of *B. thuringiensis* in food, beverages and environments (Hongyu et al. 2000; Zhou et al. 2008a, b). Besides, the improvement of biological control methods has raised more concern about biosafety and the use of these products. The present study has shown that the spores from the potential microbial pesticides, including two strains of *B. thuringiensis*, administered orally, did not cause any adverse effect in

Table 2 Quantification of the tested microorganisms (CFU/organ) isolated from selected organs at the end of the 30th day after administration

MPCAs	Initial inoculum p/animal	Organs			
		Lungs	Small intestine	Cecum	Large intestine
Bt kurstaki	8.7×10^8	ND	ND	ND	1.2 ± 0.8
Bt israelensis	5.9×10^8	ND	ND	ND	ND
Bs strain 1	8.9×10^9	<1	<1	<1	1.2 ± 1.2
Bs strain 2	1.8×10^9	<1	ND	ND	<1

Values are expressed as mean ± standard error by organ of the six animals tested

ND not detected

mice, after 30 days of inoculation. This absence of adverse effects has been observed by several authors. Innes and Bendell (1989) evaluated, at 90 days, the effects of a commercial formulation of *B. thuringiensis kurstaki* on populations of wild mammals. These authors observed that the ingestion of contaminated insects did not generate any damage in these populations.

In a significant review of *Bacillus thuringiensis* safety, McClintock et al. (1995) present a retrospective of the evaluations performed by USEPA until the year 1989. In this work all evaluations with *B. thuringiensis israelensis* and *B. thuringiensis kurstaki*, show clearly that tested microbials did not demonstrate infective or pathogenic effects in rodents. Lethal effects were observed only at higher doses, up to 2.0×10^9 CFU per animal in rabbits and 2.3×10^{10} to higher than 4.7×10^{11} spores per kilogram in rats. For *Bt israelensis* (Fig. 1b) and *Bs* strain 2 (Fig. 1d), 30 days seemed to be enough for total clearance of the microorganism from mice. This can also be observed in Table 2, where spores of these strains were scarcely detected in the organs of animals at this time. On the other hand, *Bt kurstaki* (Fig. 1a) and *Bs* strain 1 (Fig. 1c) were still present in feces of mice after 30 days, showing that this time period was not enough for the total clearance of these strains, although for *Bs* strain 1 (Fig. 1c) the trend showed a clear reduction in spore number. This persistence has been observed in several studies. Siegel and Shadduck (1990) affirm that the simple introduction of entomopathogens into mammals leads to disturbances in the normal flora and the recovery of some portion of the inoculums from the host may occur for a variable length of time. Working with CD-1 mice, these authors recovered *B. thuringiensis israelensis* for a period of 80 days and *B. sphaericus* for 67 days, after administration by intraperitoneal injection. As regards *Bacillus sphaericus*, Saik et al. (1990) present a review indicating that several strains of this bacterium were inoculated by different routes in many mammalian species, with few effects observed, even after administration of activated and inactivated strains. The authors reported that the observed effects were caused by physical damage. In a recent report Wilcks et al. (2006) have detected spores of *B. thuringiensis kurstaki* in Sprague–Dawley rats, at 10^3–10^4 CFU/g of fecal and intestinal samples, at 2 weeks after the last dosage. In this study the animals received 10^8 spores on each of the four initial days. In comparison, the present study has detected that the amount of spores in feces was around 10^2 CFU/g at 2 weeks, after a single dose. As observed in our data, the presence of 10 CFU of any of the tested strains in an animal seems to be normal and it should not be considered an important health concern. The detection of spores in animals from the control group was similar to the levels observed in tested ones, and this result may demonstrate the high vulnerability of aerial contamination with the dust generated by mammalian breeding. To show this possibility in humans, Jensen et al. (2002) worked with 20 agricultural workers who had to spray *B. thuringiensis*-based products. The authors showed that 40% of the workers presented spores in feces, at 10^2–10^3 CFU/g, without evidence of any ill effects on the workers' health. As proposed by Siegel and Shadduck (1990) and reinforced by Siegel (1997, 2001) the differences between persistence and infection should be observed. For these authors persistence is normal because the clearance process is slow and needs to be evaluated case by case, but infection is defined as disruption to the host caused by multiplication of the inoculums in tissue, toxin production, or both, and this was not observed in the present investigation. It should be noted, however, that new strains of potential MPCAs have been found regularly all around the world and their toxic and pathogenic properties need to be evaluated to guarantee the safety of human and environmental health.

The results obtained by the present study indicate an absence of toxicity and pathogenicity of all bacterial strains tested to mice, and this information gained by following international rules is one of the first steps in supporting the registration of these entomopathogens to be used in the environment. It should be pointed out that although it was not possible to detect *S. insectorum* in feces and to establish its persistence, the data at 30 days post-dosage indicate absence of oral acute toxicity, and this is a first step towards the safe use of this fungus species.

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