

Hematotoxicity of *Bacillus thuringiensis* as Spore-crystal Strains Cry1Aa, Cry1Ab, Cry1Ac or Cry2Aa in Swiss Albino Mice

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Abstract

Formulated and sporulated cultures of *Bacillus thuringiensis* (Bt) have been widely used against insect pests, but after the advent of genetically modified plants expressing δ -endotoxins, the bioavailability of Cry proteins has been increased. For biosafety reasons their adverse effects should be studied, mainly for non-target organisms. Thus, we evaluated, in Swiss albino mice, the hematotoxicity and genotoxicity of four Bt spore-crystals genetically modified to express individually Cry1Aa, Cry1Ab, Cry1Ac or Cry2Aa, administered alone by gavage with a single dose of 27 mg/Kg, 136 mg/Kg or 270 mg/Kg, 24 h, 72 h or 7 days before euthanasia. Binary combinations of these four spore-crystal proteins were also assayed at 270 mg/Kg with a single administration 24 h before euthanasia. Control mice received filtered water or cyclophosphamide at 27 mg/kg. For hematotoxicity evaluations, blood samples were drawn by cardiac puncture and processed in a multiple automated hematology analyzer; for genotoxicity analyses, micronucleus test was carried out in mice bone marrow cells. Spore-crystal administrations provoked selective hematotoxicity for the 3 exposure times, particularly for erythroid lineage. A significant reduction in bone marrow cell proliferation demonstrated cytotoxic but not genotoxic effects. These effects persisted for all exposure times, becoming more evident at 7 days. Similar results were observed for binary combinations at 24 h, suggesting that further studies are required to clarify the mechanism involved in the hematotoxicity found in mice, and to establish the toxicological risks to non-target organisms, especially mammals, before concluding that these microbiological control agents are safe for mammals.

Keywords: *Bacillus thuringiensis*, δ -endotoxins, Cry, Biosafety, Non-target organisms, Bioinsecticide

Introduction

Agricultural production has been boosted by continued and indiscriminate applications of pesticides, mainly from the 1940s to the 1960s [1]. Unfortunately, this pest control management resulted in harmful outcomes such as the selection of resistant populations of insect pests, compromising the efficiency of control and forcing farmers to use increasingly high doses of pesticides [2-4]. Moreover, these chemical insecticides have harmful effects on human health and wildlife, leaving residues in food and the environment [2,3].

Among the viable alternatives for the replacement of these synthetic pesticides, entomopathogenic biological agents show potential for use in biological control programs and integrated production, because they leave few human side effects and have low impact on natural enemies and the environment [3,5,6]. In this context, *Bacillus thuringiensis* (Bt), a gram-positive, rod-shaped, spore-forming bacterium, is the most important biopesticide sold worldwide [7,8], having been used for over 40 years by organic farmers who spray it as a foliar insecticide [7,9].

Bt is a microbial control agent (MCA) that produces a range of entomopathogenic toxins [10,11]. The most prominent feature of Bt is that during sporulation it synthesizes δ -endotoxins or insecticidal crystal proteins (ICPs), which are parasporal crystalline protein inclusions containing crystal proteins (Cry proteins or Cry toxins) as their major constituent [12-15]. These are toxic to larvae of susceptible insects and small invertebrates [1,16], and their use in combating predators from the Hymenoptera, Homoptera, Orthoptera, Coleoptera, Diptera and Lepidoptera Orders, the main cause of damage to agriculture, has been effective [7,17].

Apart from the wide use of formulated and sporulated cultures of Bt as foliar sprays, forming part of integrated pest management strategies against insect pests of agricultural crops [11,18], advances in biotechnology have allowed the development of many genetically modified plants expressing Bt δ -endotoxins [8,19,20]. Consequently, this gene has been widely cloned in different crops and then large

amounts of such toxins are released into the environment. However, its adverse effects on non-target organisms are poorly understood [7,9,20].

The primary threat to the effectiveness of long-term use of Bt toxins is the evolution of resistance by pests [21], and one of the strategies to delay the emergence of resistant pests is the combined use of Cry toxins that are effective for the same target species. The simultaneous expression of binary combinations of Cry toxins minimizes the chance of insect resistance to Bt-plants [22]. In addition to the binary combinations, advances in genetic engineering promise the expression of multiple Cry toxins in Bt-plants, known as gene pyramiding [23]. Therefore, studies on non-target species are requirements of international protocols to verify the adverse effects of these toxins, ensuring human and environmental biosafety [8].

Due to its growing use in agricultural activities, Bt presence has already been detected in different environmental compartments such as soil and water [8]. Consequently, the bioavailability of Cry proteins has increased, and for biosafety reasons their adverse effects might be studied, mainly for non-target organisms. Studies are therefore needed to evaluate (i) Bt toxicity to non-target organisms [7,9]; (ii) the

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persistence of *Bt* toxin and its stability in aquatic environments [24]; and (iii) the risks to humans and animals exposed to potentially toxic levels of *Bt* through their diet [25].

Thus, we aimed to evaluate, in Swiss albino mice, the hematotoxicity and genotoxicity of four *Bt* spore-crystals genetically modified to express individually Cry1Aa, Cry1Ab, Cry1Ac or Cry2A, administered alone by gavage with a single dose of 27 mg/Kg, 136 mg/Kg or 270 mg/Kg, 24 h, 72 h or 7 days before euthanasia, as well as their Cry binary combinations at 270 mg/Kg with a single administration 24 h before euthanasia.

Materials and Methods

Bt spore-crystal toxins

The spore-crystals Cry1Aa, Cry1Ab, Cry1Ac and Cry2Aa from *B. thuringiensis* var. *kurstaki* were obtained in lyophilized form from the Germplasm Bank of the Brazilian Agricultural Research Corporation (Embrapa) through its National Genetic Resource and Biotechnology Research Center (Cenargen), Brasília/DF, Brazil. These strains were genetically modified to express individually Cry1Aa, Cry1Ab, Cry1Ac or Cry2A, and bioassays using the purified proteins from these genetically modified (GM) *Bt* spore-crystals have been published [26].

Animals and experimental design

Swiss albino mice of both genders obtained from the animal facilities of the Faculty of the University of São Paulo (Ribeirão Preto/SP, Brazil) were kept in the animal facility of the Laboratory of Genetics of the University of Brasília (Brasília/Brazil), housed in plastic cages at room temperature ($22^{\circ}\text{C} \pm 2^{\circ}\text{C}$) in a 12 h light/dark cycle with lights on at 6 a.m., and with free access to food and water. The period of acclimatization of the animals was at least seven days. Because quantitative differences in micronucleus induction have been identified between the sexes, but no qualitative differences have been described [27], and hematology reference values for mice in the veterinary therapeutic guidelines do not differentiate sexes [28], a sample size (N) of 6 mice aged approximately three months, 50% male and 50% female, was used.

Four strains of lyophilized *Bt* spore-crystals, Cry1Aa, Cry1Ab, Cry1Ac and Cry2Aa, were resuspended in distilled water at 37°C , agitated for 10 minutes and administered orally by gavage, with a single dose of 27 mg/Kg, 136 mg/Kg or 270 mg/Kg, 24 h, 72 h or 7 days before euthanasia. Cry binary combinations (Cry1Aa+1Ab, Cry1Aa+1Ac, Cry1Aa+2Aa, Cry1Ab+1Ac, Cry1Ab+2Aa, Cry1Ac+2Aa) were also assayed at 270 mg/Kg with a single administration 24 h before euthanasia. Control mice received filtered water (negative control) or cyclophosphamide (CP, positive control) at 27 mg/kg. The minimum dose of *Bt* spore-crystal toxins was of 27 mg/Kg; the maximum dose (270 mg/Kg) was 10 times greater than the minimum dose, while the intermediate dose (136 mg/Kg) was equivalent to about half the maximum dose. In our previous experiments, exposures greater than 270 mg/Kg had caused signs of toxicity and death, so this concentration was considered the maximum tolerated.

The animals were anesthetized by an intraperitoneal administration of ketamine (80 mg/kg) plus xylazine (10 mg/kg). Blood samples collected by cardiac puncture (400 μL), using an insulin syringe containing EDTA as anticoagulant, were used to carry out hemogram in a multiple automated hematology analyzer for veterinary use, Sysmex pocH-100iV Diff (Curitiba/Paraná, Brazil) calibrated for mice. Blood smear slides were also prepared and stained with Giemsa for visual assessments of anisocytosis (variation in size), poikilocytosis (change in

shape of red blood cells - RBC), polychromasia (variation in erythrocyte coloration related to the maturation of RBC), hemagglutination and erythrocyte rouleaux. After euthanasia by cervical dislocation, bone marrow cells were surgically removed and the slides for the micronucleus (MN) test were prepared according to a standard method [29]. The genotoxic potential of spore-crystal toxins was assessed by quantification of MN in polychromatic erythrocytes (PCE) and normochromatic erythrocytes (NCE), and their possible cytotoxicity was evaluated by calculating the percentage of PCE. A total of 4000 cells was analyzed in light microscopy (1000X magnification), using a Zeiss Axioskop 2 microscope. For the 24-hour exposure, circulating blood and bone marrow of the mice were collected; for the 72-hour and 7-day exposure, only circulating blood was collected.

All procedures were reviewed and approved by the institutional Ethics Committee for Animal Research (Institute of Biological Science, University of Brasília), number 32942/2009.

Colony Forming Units (CFU)

In order to quantify the number of viable *Bt* spore-crystals, the colony forming units test (CFU) was performed according to Alves and Moraes (1998) [30]. For this, 0.1 g of each lyophilized spore-crystal was diluted and homogenized with a vortex in 10 mL of sterile distilled water. Thenceforward, five successive dilutions were made, using in each one 0.1 mL of previous dilution to 9.9 mL of sterile distilled water. Then, 0.1 mL of each one of the last three dilutions was plated in three replicates on a conventional culture medium (NYSM) and placed in an incubator at 30°C for 14 hours. The colonies of each replicate were counted and the average was calculated, with the result given in cells/mL (Table 5).

Statistical analysis

Statistical analysis was carried out using SPSS (Statistical Package for the Social Sciences) version 15.0. Data were expressed as mean \pm SEM (standard error of mean) and values of $p < 0.05$ were considered statistically significant. The continuous variables were tested for normal distribution with Shapiro-Wilk. Possible differences among the groups analyzed were investigated through Kruskal-Wallis test, since the data were not normally distributed. For significant Kruskal-Wallis results, Mann-Whitney U test was performed to verify differences between the treatments (2-to-2 comparisons). P-values with statistical significance ($p < 0.05$) were only considered when they also presented biological significance, according to the following criteria: (1) group C (negative control) compared to all groups; (2) group CP (positive control) compared to the treatments with the same exposure time or between CP of different exposure times; (3) the same toxins compared in the same exposure time but in different doses (dose-effect) or at 270 mg/Kg in different exposure times (24 h, 72 h, 7 days); (4) different toxins compared to each other at 270 mg/Kg in the 24 h, 72 h and 7-day exposure; (5) binary combinations compared to single doses of those Cry present in the combinations at 270 mg/Kg, at 24 h of exposure; (6) binary combinations compared to each other.

Results

Erythrogram (Table 1)

24 hours of exposure: Oral administrations of single doses of Cry1Ab 136 mg/Kg ($p=0.006$), Cry1Ac 270 mg/Kg ($p=0.011$) and Cry2Aa 27 mg/Kg ($p=0.006$) significantly reduced MCH values, while Cry1Aa 27 mg/Kg ($p=0.034$) increased MCHC values. All *Bt* spore-crystals promoted significant reductions in MCV values ($p=0.004$ for Cry1Aa 27 mg/Kg, $p=0.003$ for Cry1Ac 27 mg/Kg, and $p=0.000$ for the

G	Treatment	RBC (x 10 ⁶ /μL)	HGB (g/dL)	HCT (%)	MCH (pg)	MCHC (g/dL)	MCV (fL)	RDW (%)
1	Filtered water,	7.53 ± 0.28	11.90 ± 0.44	30.38 ± 1.02	15.83 ± 0.09	39.12 ± 0.41	40.40 ± 0.40	17.83 ± 0.41
2	CP 27 mg/Kg, 24 h	7.60 ± 0.12	12.05 ± 0.26	30.57 ± 0.66	15.85 ± 0.16	39.45 ± 0.75	40.22 ± 0.46	16.80 ± 0.60
3	Cry1Aa 27 mg/Kg, 24 h	8.25 ± 0.13	12.78 ± 0.21	30.90 ± 0.36	15.50 ± 0.25	41.36 ± 0.38*	37.50 ± 0.43**	14.94 ± 0.54**
4	Cry1Aa 136 mg/Kg, 24 h	7.92 ± 0.14	12.19 ± 0.31	29.84 ± 0.68	15.37 ± 0.19	40.81 ± 0.32	37.64 ± 0.28**	14.16 ± 0.38**
5	Cry1Aa 270 mg/Kg, 24 h	8.35 ± 0.20	12.40 ± 0.29	31.08 ± 0.71	14.85 ± 0.16	39.88 ± 0.34	37.22 ± 0.12**	15.38 ± 0.21**
6	Cry1Ab 27 mg/Kg, 24 h	8.17 ± 0.22	12.55 ± 0.42	31.13 ± 0.89	15.35 ± 0.22	40.30 ± 0.38	38.12 ± 0.21**	15.35 ± 0.31*
7	Cry1Ab 136 mg/Kg, 24 h	7.69 ± 0.09	11.23 ± 0.25	28.55 ± 0.62	14.62 ± 0.21**	39.35 ± 0.26	37.12 ± 0.42**	16.65 ± 0.56**
8	Cry1Ab 270 mg/Kg, 24 h	8.19 ± 0.16	12.63 ± 0.20	30.77 ± 0.53	15.45 ± 0.18	41.07 ± 0.33	37.60 ± 0.17**	15.70 ± 0.36**
9	Cry1Ac 27 mg/Kg, 24 h	7.98 ± 0.12	12.25 ± 0.24	30.42 ± 0.56	15.33 ± 0.20	40.28 ± 0.56	38.08 ± 0.22**	16.93 ± 0.36
10	Cry1Ac 136 mg/Kg, 24 h	8.11 ± 0.32	12.12 ± 0.40	29.92 ± 1.02	14.98 ± 0.22	40.53 ± 0.47	36.93 ± 0.23**	16.18 ± 0.67*
11	Cry1Ac 270 mg/Kg, 24 h	8.17 ± 0.35	11.98 ± 0.39	29.68 ± 1.07	14.67 ± 0.21*	40.42 ± 0.32	36.37 ± 0.35**	16.17 ± 0.67
12	Cry2Aa 27 mg/Kg, 24 h	8.24 ± 0.18	12.02 ± 0.15	29.95 ± 0.53	14.62 ± 0.22**	40.15 ± 0.35	36.40 ± 0.36**	15.02 ± 0.52**
13	Cry2Aa 136 mg/Kg, 24 h	7.86 ± 0.23	11.68 ± 0.33	29.05 ± 0.77	14.87 ± 0.10	40.22 ± 0.18	36.97 ± 0.31**	15.47 ± 0.30**
14	Cry2Aa 270 mg/Kg, 24 h	7.75 ± 0.27	11.82 ± 0.37	28.97 ± 0.89	15.27 ± 0.17	40.82 ± 0.42	37.42 ± 0.29**	15.62 ± 0.34**
15	Cry1Aa+1Ab 270 mg/Kg, 24 h	8.30 ± 0.18	12.45 ± 0.30	31.15 ± 0.78	15.00 ± 0.10*	40.00 ± 0.23	37.53 ± 0.28**	14.87 ± 0.12**
16	Cry1Aa+1Ac 270 mg/Kg, 24 h	8.47 ± 0.42	12.93 ± 0.63	31.78 ± 1.25	15.30 ± 0.13	40.60 ± 0.48	37.63 ± 0.39**	14.85 ± 0.46**
17	Cry1Aa+2Aa 270 mg/Kg, 24 h	8.46 ± 0.14	12.70 ± 0.09	31.77 ± 0.24	15.03 ± 0.19	39.98 ± 0.27	37.57 ± 0.47**	14.95 ± 0.32**
18	Cry1Ab+1Ac 270 mg/Kg, 24 h	8.03 ± 0.15	12.17 ± 0.29	29.63 ± 0.78	15.15 ± 0.21	41.08 ± 0.28	36.88 ± 0.47**	15.48 ± 0.39**
19	Cry1Ab+2Aa 270 mg/Kg, 24 h	8.20 ± 0.16	11.95 ± 0.10	29.83 ± 0.35	14.58 ± 0.23**	40.07 ± 0.24	36.42 ± 0.48**	15.38 ± 0.43**
20	Cry1Ac+2Aa 270 mg/Kg, 24 h	8.33 ± 0.17	11.95 ± 0.21	30.50 ± 0.47	14.37 ± 0.33**	39.18 ± 0.48	36.70 ± 0.53**	15.82 ± 0.37**
P-values 24 h		0.075	0.072	0.179	0.000	0.002	0.000	0.000
21	CP 27 mg/Kg, 72 h	7.72 ± 0.28	11.65 ± 0.28	29.30 ± 0.76	15.13 ± 0.34	39.75 ± 0.27	38.03 ± 0.60*	15.02 ± 0.22**
22	Cry1Aa 270 mg/Kg, 72 h	6.86 ± 0.47 ^a	10.77 ± 0.65 ^a	26.73 ± 1.59 ^a	15.77 ± 0.29	40.28 ± 0.45	39.15 ± 0.54	16.57 ± 0.67
23	Cry1Ab 270 mg/Kg, 72 h	7.92 ± 0.29	11.87 ± 0.26 ^b	29.83 ± 0.81	15.02 ± 0.31	39.82 ± 0.44	37.75 ± 0.47**	15.40 ± 0.62**
24	Cry1Ac 270 mg/Kg, 72 h	8.17 ± 0.19	11.97 ± 0.44	30.28 ± 0.88	14.63 ± 0.27*	39.48 ± 0.46	37.07 ± 0.56**	16.40 ± 0.36 ^{-o}
25	Cry2Aa 270 mg/Kg, 72 h	8.30 ± 0.22	12.38 ± 0.31	30.67 ± 0.78	14.92 ± 0.16	40.38 ± 0.22	36.95 ± 0.52**	15.82 ± 0.10 ^{-o}
P-values 72 h		0.027	0.300	0.213	0.014	0.211	0.002	0.004
26	CP 27 mg/Kg, 7 days	8.10 ± 0.28	12.25 ± 0.34	30.82 ± 0.87	15.15 ± 0.13	39.75 ± 0.25	38.13 ± 0.41*	16.32 ± 0.59*
27	Cry1Aa 270 mg/Kg, 7 days	7.12 ± 0.30	10.77 ± 0.44 ^a	26.88 ± 0.99 ^a	15.13 ± 0.26	40.00 ± 0.25	37.80 ± 0.57**	16.38 ± 0.46 ^{-a}
28	Cry1Ab 270 mg/Kg, 7 days	6.53 ± 0.38 ^b	9.68 ± 0.58 ^{b,b'}	24.55 ± 1.36 ^{b,b'}	14.83 ± 0.15**	39.38 ± 0.40	37.63 ± 0.37**	17.02 ± 0.54
29	Cry1Ac 270 mg/Kg, 7 days	8.16 ± 0.10	12.17 ± 0.26	30.38 ± 0.48	14.92 ± 0.26*	40.03 ± 0.31	37.27 ± 0.47**	16.03 ± 0.33**
30	Cry2Aa 270 mg/Kg, 7 days	7.99 ± 0.20	11.90 ± 0.31	30.25 ± 0.77	14.88 ± 0.09*	39.33 ± 0.34	37.85 ± 0.28**	15.92 ± 0.41**
P-values 7 days		0.001	0.000	0.000	0.004	0.293	0.000	0.059
Total p-values		0.000	0.002	0.007	0.000	0.001	0.000	0.000

The data correspond to the means and to the standard error of mean (SEM). RBC=Red Blood Cells; HGB=Hemoglobin; HCT=Hematocrit; MCV=Mean Corpuscular volume; MCH=Mean Corpuscular hemoglobin; MCHC=Mean corpuscular hemoglobin concentration; RDW=Red cell distribution width (represents an indication of the amount of variation – anisocytosis – in cell size); g/dL=grams per deciliter; fL=femtoliters; pg=picograms. P-values were generated by the Kruskal-Wallis test. Asterisks indicate significant (p<0.05) and highly significant (p<0.01) differences detected by the Mann Whitney U test in the comparisons with the negative control. The following symbols represent significant differences in respect to: ●=CP 24 h; ○=CP 72 h; †= dose-effect for the dose of 27 mg/Kg; ‡=dose-effect for the dose of 136 mg/Kg; a= Cry1Aa 270 mg/Kg, 24 h; b=Cry1Ab 270 mg/Kg, 24 h; b'=Cry1Ab 270 mg/Kg, 72 h

Table 1: Results of erythrogram of Swiss albino mice treated with *Bt* spore-crystal Cry1Aa, Cry1Ab, Cry1Ac and Cry2Aa administered orally (*per os*, p.o.) 24 h, 72 h and 7 days before euthanasia. Control mice received filtered water (negative control) or cyclophosphamide (CP – positive control) at 27 mg/Kg.

others). For the visually assessed slides, microcytosis and hypochromia particularly increased for the treatments with Cry1Ab at 27 and 136 mg/Kg, with hypochromia being more severe for the dose of 136 mg/Kg; spherocytes were also very prevalent in these slides. Microcytosis with spherocyte presence was also observed in slides of Cry2Aa 270 mg/Kg. Significantly decreased RDW were also observed for most Cry treatments, except for Cry1Ac at 27 and 270 mg/Kg (p=0.041 for Cry1Ab and Cry1Ac at 136 mg/Kg, p=0.002 for the other treatments). In the dose-effect evaluations, values for RDW were significantly higher for Cry1Aa at 270 mg/Kg than at 136 mg/Kg (p=0.004). Binary combinations containing Cry1Ab+Cry2Aa (p=0.004) and Cry1Ac+Cry2Aa (p=0.000) caused significant reductions in MCH values, and all Cry combinations promoted significant reductions in VCM and RDW. For the visually assessed slides, a higher hypochromia to Cry1Ac+2Aa was observed. Among different binary combinations, Cry1Ac+Cry2Aa showed a higher RDW compared to Cry1Aa+Cry1Ab

and Cry1Aa+Cry1Ac (p=0.041), which also occurred with Cry1Aa in respect to Cry1Aa+Cry1Ab (p=0.041).

72 hours of exposure: Compared to negative control, Cry1Ac 270 mg/Kg continued to significantly reduce MHC values (p=0.040), while MCV and RDW were significantly reduced after treatment with CP, Cry1Ab, Cry1Ac and Cry2Aa. Among toxins, Cry1Aa showed a significant reduction in RBC (p=0.030), HGB (p=0.041) and HCT (p=0.015) values compared to Cry2Aa, and increased MCV compared to Cry2Aa (p=0.009) and Cry1Ac (p=0.026).

7 days of exposure: Cry1Ab 270 mg/Kg resulted in significantly reduced HGB (p=0.009) and HCT (p=0.002) values, while all Cry administrations significantly reduced MCH and MCV; the same occurred with MCV after CP treatment and with RDW after CP and all Cry administrations, except the Cry1Ab. For the visually assessed slides

a higher hypochromia was observed for Cry1Aa, Cry1Ac, and Cry2Aa, more pronounced in the two first treatments.

Comparing toxins, RBC, HGB and HCT were significantly lower for Cry1Ab than for Cry1Ac and Cry2Aa.

Leukogram (Table 2)

24 hours of exposure: In single doses only Cry1Ab 27 mg/Kg promoted a significant increase in lymphocyte frequency in comparison to the negative control ($p=0.041$). Cry1Ab significantly increased total leukocytes (white blood cells or WBC) and lymphocytes for the dose of 27 mg/Kg in respect to the doses of 136 mg/Kg ($p=0.015$ for WBC, $p=0.002$ for lymphocytes) and 270 mg/Kg ($p=0.026$ for total WBC, $p=0.015$ for lymphocytes), and increased neutrophils+monocytes for 136 mg/Kg in respect to 270 mg/Kg ($p=0.009$).

72 hours of exposure: Compared to negative control, only Cry1Aa

caused significantly increased neutrophils+monocytes ($p=0.003$). Cry1Aa also showed increased WBC compared to Cry1Ab ($p=0.015$) and Cry1Ac ($p=0.009$), as well as neutrophils+monocytes in respect to Cry1Ab ($p=0.016$), Cry1Ac ($p=0.004$) and Cry2Aa ($p=0.002$).

7 days of exposure: Compared to the negative control, CP ($p=0.026$), Cry1Ac and Cry2Aa ($p=0.041$ for both) promoted a significant increase in WBC, and this result was related to increased neutrophils+monocytes ($p=0.004$ for CP; $p=0.026$ for Cry1Ac and Cry2Aa). Cry1Ab ($p=0.002$) caused a significant reduction in lymphocytes, while Cry2Aa ($p=0.026$) resulted in a significant increase in their number. Also, Cry1Aa ($p=0.041$) and Cry1Ab ($p=0.026$) caused a significant increase in eosinophils. Cry1A ($p=0.009$) and Cry1Ab ($p=0.015$) also showed significantly increased eosinophils compared to Cry2Aa. WBC and lymphocytes were also significantly reduced for Cry1Ab in respect to Cry1Ac and Cry2Aa.

G	Treatment	WBC ($\times 10^3/\mu\text{L}$)	Lymphocytes ($\times 10^3/\mu\text{L}$)	Neutrophils + Monocytes ($\times 10^3/\mu\text{L}$)	Eosinophils ($\times 10^3/\mu\text{L}$)
1	Filtered water	4.63 \pm 0.74	3.35 \pm 0.55	1.22 \pm 0.20	0.07 \pm 0.05
2	CP 27 mg/Kg, 24 h	5.37 \pm 0.77	3.73 \pm 0.53	1.58 \pm 0.34	0.05 \pm 0.03
3	Cry1Aa 27 mg/Kg, 24 h	6.32 \pm 0.85	4.94 \pm 0.73	1.38 \pm 0.14	0.00 \pm 0.00
4	Cry1Aa 136 mg/Kg, 24 h	5.53 \pm 0.52	4.20 \pm 0.45	1.27 \pm 0.11	0.06 \pm 0.04
5	Cry1Aa 270 mg/Kg, 24 h	5.47 \pm 0.77	3.93 \pm 0.77	1.47 \pm 0.31	0.07 \pm 0.03
6	Cry1Ab 27 mg/Kg, 24 h	6.82 \pm 0.62	5.13 \pm 0.33 [*]	1.68 \pm 0.33	0.00 \pm 0.00
7	Cry1Ab 136 mg/Kg, 24 h	4.60 \pm 0.49 [†]	2.60 \pm 0.42 [†]	1.92 \pm 0.45	0.08 \pm 0.03
8	Cry1Ab 270 mg/Kg, 24 h	4.60 \pm 0.47 [†]	3.53 \pm 0.43 [†]	0.98 \pm 0.10 [‡]	0.08 \pm 0.04
9	Cry1Ac 27 mg/Kg, 24 h	6.90 \pm 0.89	4.25 \pm 0.45	2.52 \pm 0.66	0.13 \pm 0.10
10	Cry1Ac 136 mg/Kg, 24 h	6.78 \pm 1.66	4.00 \pm 0.97	2.48 \pm 0.56	0.30 \pm 0.24
11	Cry1Ac 270 mg/Kg, 24 h	5.17 \pm 0.66	3.55 \pm 0.66	1.60 \pm 0.37	0.02 \pm 0.02
12	Cry2Aa 27 mg/Kg, 24 h	4.38 \pm 0.63	2.43 \pm 0.36	1.90 \pm 0.53	0.05 \pm 0.02
13	Cry2Aa 136 mg/Kg, 24 h	4.68 \pm 1.06	3.05 \pm 0.91	1.50 \pm 0.32	0.13 \pm 0.06
14	Cry2Aa 270 mg/Kg, 24 h	5.03 \pm 0.78	2.98 \pm 0.28	2.02 \pm 0.73	0.03 \pm 0.02
15	Cry1Aa+1Ab 270 mg/Kg, 24 h	5.97 \pm 0.52	4.28 \pm 0.54	1.67 \pm 0.08 ^b	0.02 \pm 0.02
16	Cry1Aa+1Ac 270 mg/Kg, 24 h	4.10 \pm 0.69	2.85 \pm 0.58	1.20 \pm 0.28	0.05 \pm 0.02
17	Cry1Aa+2Aa 270 mg/Kg, 24 h	4.92 \pm 0.84	3.38 \pm 0.84	1.43 \pm 0.52	0.10 \pm 0.05
18	Cry1Ab+1Ac 270 mg/Kg, 24 h	4.72 \pm 0.59	3.18 \pm 0.51	1.42 \pm 0.13 ^b	0.12 \pm 0.08
19	Cry1Ab+2Aa 270 mg/Kg, 24 h	4.78 \pm 0.54	3.47 \pm 0.48	1.28 \pm 0.24	0.03 \pm 0.02
20	Cry1Ac+2Aa 270 mg/Kg, 24 h	4.63 \pm 0.70	3.13 \pm 0.51	1.48 \pm 0.40	0.02 \pm 0.02
	P-values 24 hours	0.454	0.095	0.758	0.486
21	CP 27 mg/Kg, 72 h	3.37 \pm 0.78	2.40 \pm 0.46	0.95 \pm 0.41	0.02 \pm 0.02
22	Cry1Aa 270 mg/Kg, 72 h	6.53 \pm 0.82 [°]	3.42 \pm 0.41	2.98 \pm 0.43 ^{°a}	0.13 \pm 0.08
23	Cry1Ab 270 mg/Kg, 72 h	4.27 \pm 0.36	2.72 \pm 0.16	1.48 \pm 0.29	0.07 \pm 0.03
24	Cry1Ac 270 mg/Kg, 72 h	3.72 \pm 0.35	2.37 \pm 0.35	1.25 \pm 0.13	0.10 \pm 0.06
25	Cry2Aa 270 mg/Kg, 72 h	4.80 \pm 0.57	3.58 \pm 0.46	1.18 \pm 0.15	0.03 \pm 0.02
	P-values 72 hours	0.059	0.331	0.000	0.557
26	CP 27 mg/Kg, 7 days	7.22 \pm 0.68 ^{°°}	4.33 \pm 0.40 [°]	2.82 \pm 0.35 ^{°°}	0.07 \pm 0.03
27	Cry1Aa 270 mg/Kg, 7 days	6.70 \pm 1.83	3.30 \pm 0.77	3.15 \pm 1.20	0.25 \pm 0.08 ^{°a}
28	Cry1Ab 270 mg/Kg, 7 days	3.90 \pm 0.58 [°]	1.85 \pm 0.10 ^{°°b.b'}	1.77 \pm 0.42	0.28 \pm 0.08 [°]
29	Cry1Ac 270 mg/Kg, 7 days	6.58 \pm 0.50 ^{°c'}	3.85 \pm 0.62	2.63 \pm 0.60 [°]	0.10 \pm 0.04
30	Cry2Aa 270 mg/Kg, 7 days	7.85 \pm 1.37 [°]	5.33 \pm 0.89 ^{°d}	2.48 \pm 0.55 ^{°d}	0.03 \pm 0.02
	P-values 7 days	0.034	0.006	0.092	0.015
	Total P-values	0.015	0.004	0.034	0.024

G= group. WBC= White Blood Cells. The data correspond to the means and to the standard error of mean (SEM). P-values were generated by the Kruskal-Wallis test. Asterisks indicate significant ($p<0.05$) and highly significant ($p<0.01$) differences detected by the Mann Whitney U test in the comparisons with the negative control. The following symbols represent significant differences in respect to: °=CP 24 h; °°=CP 72 h; °°°=CP 7 days; †=dose-effect for the dose of 27 mg/Kg; ‡=dose-effect for the dose of 136 mg/Kg; a=Cry1Aa 270 mg/Kg, 24 h; b=Cry1Ab 270 mg/Kg, 24 h; b'=Cry1Ab 270 mg/Kg, 72 h; c=Cry1Ac 270 mg/Kg, 24 h; c'=Cry1Ac 270 mg/Kg, 72h; d=Cry2Aa 270 mg/Kg, 24 h; d'=Cry2Aa 270 mg/Kg, 72 h.

Table 2: Results of leukogram of Swiss albino mice treated with *Bt* spore-crystal Cry1Aa, Cry1Ab, Cry1Ac and Cry2Aa administered orally (*per os*, p.o.) 24 h, 72 h, and 7 days before euthanasia. Control mice received filtered water (negative control) or cyclophosphamide (CP – positive control) at 27 mg/Kg.

Plateletgram (Table 3)

24 hours of exposure: In comparison with negative control, CP promoted a significant fall in platelet number (PLT, $p=0.026$), while a single dose of Cry1Aa 27 mg/Kg significantly increased PLT ($p=0.009$), decreased mean platelet volume (MPV, $p=0.015$) and platelet large cell ratio (P-LCR, $p=0.026$); the same decreased P-LCR occurring at 136 mg/Kg ($p=0.041$). Cry1Ac 136 mg/Kg significantly increased MPV ($p=0.019$) and P-LCR ($p=0.038$), while Cry2Aa at 270 mg/Kg significantly decreased platelet distribution width (PDW, $p=0.026$). Regarding dose-effect, Cry1Aa at 136 mg/Kg showed reduced PLT ($p=0.015$) compared to 27 mg/Kg and reduced PDW ($p=0.009$) compared to 270 mg/Kg. Significantly increased values of MPV, P-LCR and PDW were verified for Cry1Ac at 136 mg/Kg compared to 27 mg/Kg and 270 mg/Kg (at 27 mg/Kg, $p=0.010$; at 270 mg/Kg, $p=0.029$). Cry1Ac at 136 mg/Kg also showed a significantly increased PDW in

respect to the dose of 270 mg/Kg ($p=0.038$). Among toxins, at 270 mg/Kg, PLT was significantly higher after treatment with Cry1Aa compared to Cry2Aa ($p=0.041$), while PDW was significantly higher for Cry1Ab than for Cry1A ($p=0.041$).

As regards the negative control, binary combinations significantly reduced levels as follows: Cry1Aa+Cry2Aa for PLT ($p=0.002$), Cry1Ab+Cry2Aa for MPV ($p=0.024$), and Cry1Ab+Cry1Ac for PDW ($p=0.030$). Between combinations, Cry1Aa+Cry2Aa showed a lower PLT compared to Cry1Ab+Cry1Ac ($p=0.015$), and Cry1Aa+Cry2Aa presented higher MPV and RDW ($p=0.036$) in comparison with Cry1Ab+Cry2Aa.

Cry1Aa+Cry2Aa showed significantly reduced PLT ($p=0.009$) and increased PDW ($p=0.009$) compared to Cry1Aa, which also presented lower PDW compared to Cry1Aa+Cry1Ab and Cry1Aa+Cry1Ac ($p=0.015$ for both), and Cry1Ab showed significantly higher PDW

Group	Treatment	PLT ($\times 10^3/\mu\text{L}$)	MPV (fl)	P-LCR (%)	PDW (fl)
1	Filtered water	1219.00 \pm 56.64	6.93 \pm 0.10	10.88 \pm 0.81	6.95 \pm 0.11
2	CP 27 mg/Kg, 24 h	978.67 \pm 93.57*	7.10 \pm 0.30	11.23 \pm 2.33	7.25 \pm 0.24
3	Cry1Aa 27 mg/Kg, 24 h	1431.60 \pm 27.40**	6.38 \pm 0.15*	7.32 \pm 1.15*	6.74 \pm 0.05*
4	Cry1Aa 136 mg/Kg, 24 h	1243.57 \pm 113.56†	6.56 \pm 0.14	7.91 \pm 1.14*	6.77 \pm 0.07
5	Cry1Aa 270 mg/Kg, 24 h	1292.00 \pm 74.24*	6.75 \pm 0.15	11.15 \pm 1.68	6.53 \pm 0.07*+
6	Cry1Ab 27 mg/Kg, 24 h	1168.00 \pm 73.22	6.62 \pm 0.17	9.93 \pm 1.10	6.63 \pm 0.10*
7	Cry1Ab 136 mg/Kg, 24 h	1076.00 \pm 148.54	6.85 \pm 0.22	10.73 \pm 1.74	6.75 \pm 0.16
8	Cry1Ab 270 mg/Kg, 24 h	1020.50 \pm 113.38	7.05 \pm 0.16	12.33 \pm 1.35	6.87 \pm 0.11
9	Cry1Ac 27 mg/Kg, 24 h	1224.67 \pm 58.93*	6.90 \pm 0.09	9.63 \pm 0.88	7.03 \pm 0.10
10	Cry1Ac 136 mg/Kg, 24 h	955.83 \pm 183.68	7.48 \pm 0.10*†	15.35 \pm 0.80*†	7.13 \pm 0.09†
11	Cry1Ac 270 mg/Kg, 24 h	1213.50 \pm 133.20	6.83 \pm 0.16‡	10.78 \pm 0.78‡	6.63 \pm 0.08*‡
12	Cry2Aa 27 mg/Kg, 24 h	1282.33 \pm 110.10*	6.90 \pm 0.16	10.24 \pm 0.87	6.92 \pm 0.14
13	Cry2Aa 136 mg/Kg, 24 h	1146.83 \pm 62.99	6.97 \pm 0.09	11.13 \pm 0.94	6.9 \pm 0.07
14	Cry2Aa 270 mg/Kg, 24 h	1057.50 \pm 65.31	7.00 \pm 0.10	11.78 \pm 0.42	6.80 \pm 0.13*
15	Cry1Aa+1Ab 270 mg/Kg, 24 h	1090.33 \pm 69.82	6.80 \pm 0.09	10.22 \pm 0.61	6.83 \pm 0.08 ^a
16	Cry1Aa+1Ac 270 mg/Kg, 24 h	1187.33 \pm 156.68	6.77 \pm 0.13	9.78 \pm 1.04	6.80 \pm 0.06 ^a
17	Cry1Aa+2Aa 270 mg/Kg, 24 h	942.67 \pm 44.98** ^a	7.00 \pm 0.11	11.78 \pm 1.17	6.88 \pm 0.07 ^a
18	Cry1Ab+1Ac 270 mg/Kg, 24 h	1171.67 \pm 60.58	6.78 \pm 0.16	11.16 \pm 1.45	6.54 \pm 0.10*
19	Cry1Ab+2Aa 270 mg/Kg, 24 h	1110.50 \pm 69.8	6.57 \pm 0.03 ^{b,d}	9.23 \pm 0.38 ^{b,d}	6.57 \pm 0.09
20	Cry1Ac+2Aa 270 mg/Kg, 24 h	1164.67 \pm 105.33	7.00 \pm 0.16	12.35 \pm 1.34	6.75 \pm 0.18
	P-values 24 hours	0.048	0.015	0.044	0.005
21	CP 27 mg/Kg, 72 h	1112.17 \pm 178.48	6.58 \pm 0.15	8.14 \pm 1.19	6.78 \pm 0.12
22	Cry1Aa 270 mg/Kg, 72 h	1152.33 \pm 96.43	6.65 \pm 0.10*	7.67 \pm 0.43**	6.78 \pm 0.15
23	Cry1Ab 270 mg/Kg, 72 h	928.67 \pm 92.55	6.93 \pm 0.22	11.83 \pm 1.55	6.58 \pm 0.16
24	Cry1Ac 270 mg/Kg, 72 h	1095.50 \pm 96.69	7.13 \pm 0.23	12.80 \pm 1.39	7.00 \pm 0.16
25	Cry2Aa 270 mg/Kg, 72 h	1196.00 \pm 67.53	7.16 \pm 0.21	12.28 \pm 2.12	7.04 \pm 0.18
	P-values 72 hours	0.454	0.077	0.010	0.316
26	CP 27 mg/Kg, 7 dias	1345.50 \pm 158.38	6.67 \pm 0.10	8.80 \pm 0.81*	6.72 \pm 0.09
27	Cry1Aa 270 mg/Kg, 7 dias	1002.50 \pm 101.89 ^a	7.23 \pm 0.18 ^a	12.07 \pm 0.96 ^a	7.05 \pm 0.22
28	Cry1Ab 270 mg/Kg, 7 dias	589.50 \pm 92.13 ^{b,b'}	7.47 \pm 0.34	15.03 \pm 2.23*	6.93 \pm 0.29
29	Cry1Ac 270 mg/Kg, 7 dias	1289.00 \pm 97.91	6.72 \pm 0.10	9.85 \pm 0.44	6.68 \pm 0.09
30	Cry2Aa 270 mg/Kg, 7 dias	1205.33 \pm 88.27	6.92 \pm 0.09	9.98 \pm 0.82	6.95 \pm 0.10
	P-values 7 dias	0.000	0.006	0.034	0.388
	Total P-values	0.005	0.002	0.002	0.020

The data correspond to the means and to the standard error of mean (SEM). Platelet indices: platelet count (PLT), mean platelet volume (MPV), platelet large cell ratio (P-LCR) and platelet distribution width (PDW); fl=fentoliters. P-values were generated by the Kruskal-Wallis test. Asterisks indicate significant ($p<0.05$) and highly significant ($p<0.01$) differences detected by the Mann Whitney U test in the comparisons with the negative control. The following symbols represent significant differences in respect to: ●=CP 24 h; ○=CP 72 h; †=dose-effect for the dose of 27 mg/Kg; ‡=dose-effect for the dose of 136 mg/Kg; a=Cry1Aa 270 mg/Kg, 24 h; a'=Cry1Aa 270 mg/Kg, 72 h; b=Cry1Ab 270 mg/Kg, 24 h; b'=Cry1Ab 270 mg/Kg, 72 h; d=Cry2Aa 270 mg/Kg, 24 h

Table 3: Results of platelet gram of Swiss albino mice treated with *Bt* spore-crystal Cry1Aa, Cry1Ab, Cry1Ac and Cry2Aa administered orally (*per os*, p.o.) 24 h, 72 h and 7 days before euthanasia. Control mice received filtered water (negative control) or cyclophosphamide (CP – positive control) at 27 mg/Kg.

compared to Cry1Ab+Cry2Aa ($p=0.024$). Similar results were observed for P-LCR, whose values were significantly higher for Cry1Ab ($p=0.048$) and Cry2Aa ($p=0.024$) in comparison to the binary combination Cry1Ab+Cry2Aa.

72 hours of exposure: Cry1Aa promoted significantly decreased MPV ($p=0.026$) and P-LCR ($p=0.002$) compared to negative control; for the latter, values presented for Cry1Aa were also lower in respect to those presented for treatments with Cry1Ab, Cry1Ac ($p=0.010$) and Cry2Aa ($p=0.017$).

7 days of exposure: Compared to negative control, CP caused a significant reduction in P-LCR ($p=0.041$), while Cry1Ab promoted a significant increase in its values ($p=0.048$). Cry1Ab also presented a reduced PLT number compared to Cry1Ac ($p=0.001$) and Cry2Aa ($p=0.003$), and increased MVP compared to Cry1Ac ($p=0.036$).

Micronucleus (MN) test (Table 4)

None of the tested Bt-toxins induced MN. Single doses of Cry1Aa 136 mg/Kg ($p=0.041$), Cry1Ab at 27 ($p=0.009$) and 136 mg/Kg ($p=0.026$), Cry1Ac 136 mg/Kg and Cry2Aa 27 mg/Kg ($p=0.009$ for both) significantly decreased cell proliferation in mice bone marrow (%PCE) compared with negative control. Binary combinations of Cry1Aa+Cry2Aa, Cry1Ab+Cry2Aa and Cry1Ac+Cry2Aa ($p=0.026$ for all) also decreased the %PCE index.

Colony Forming Units (CFU) (Table 5)

CFU varied with *Bt* strain. Thus, although animals received Cry1Aa, Cry1Ab, Cry1Ac or Cry2Aa at 27 mg/Kg, 136 mg/Kg or 270 mg/Kg, the number of viable *Bt* spore-crystals ingested by animals varied according to *Bt* strain.

Discussion

Since the late 1960's, spore-crystals from *B. thuringiensis* have been an important ally in combating insect pests in agriculture, against insect vectors of human diseases and in biological pest control, through their use as a foliar insecticide [1,31] and, more recently, by inserting δ -endotoxin genes in transgenic *Bt*-plants [5]. A major environmental advantage of microbial *Bt* preparations and of genetically engineered insect-resistant plants expressing genes encoding δ -endotoxins is the greater specificity of δ -endotoxins to target species, compared with use of many synthetic chemical insecticides. However, despite their more targeted specificity, there may still be insects and other non-target organisms potentially affected by the δ -endotoxins, and extended exposure might affect their populations [8]. Thus, the Brazilian Collegiate Board of Directors of the National Sanitary Surveillance Agency (ANVISA) N° 194/02 advocates evaluations of toxicity and pathogenicity of microbiological control agents (MCAs), given that little is known about their toxicological potential [32].

Group	Treatment	MN-NCE	Polychromatic erythrocytes (PCE)	
			MN-PCE	Cellular proliferation index (%PCE)
1	Filtered water	2.00 \pm 1.44	2.50 \pm 1.43	52.61 \pm 1.01
2	CP 27 mg/Kg	2.17 \pm 0.48	3.50 \pm 0.62	45.97 \pm 1.21 [~]
3	Cry1Aa 27 mg/Kg	0.40 \pm 0.25 [*]	3.00 \pm 0.55	48.55 \pm 1.54
4	Cry1Aa 136 mg/Kg	0.43 \pm 0.20 [*]	2.29 \pm 0.36	49.28 \pm 1.36 [~]
5	Cry1Aa 270 mg/Kg	1.00 \pm 0.37	3.67 \pm 0.56	52.90 \pm 1.57 [*]
6	Cry1Ab 27 mg/Kg	1.67 \pm 0.49	4.67 \pm 0.88	45.92 \pm 1.54 [~]
7	Cry1Ab 136 mg/Kg	1.50 \pm 0.43	3.33 \pm 0.33	47.78 \pm 1.28 [~]
8	Cry1Ab 270 mg/Kg	1.00 \pm 0.37	2.17 \pm 0.95	52.91 \pm 1.55 ^{*,†‡}
9	Cry1Ac 27 mg/Kg	1.33 \pm 0.21	3.33 \pm 0.76	49.49 \pm 2.14
10	Cry1Ac 136 mg/Kg	2.50 \pm 0.96	5.17 \pm 0.98	44.92 \pm 1.87 [~]
11	Cry1Ac 270 mg/Kg	2.17 \pm 0.40	4.00 \pm 0.73	48.69 \pm 1.73
12	Cry2Aa 27 mg/Kg	1.00 \pm 0.52	0.83 \pm 0.31 [*]	47.06 \pm 1.25 [~]
13	Cry2Aa 136 mg/Kg	0.50 \pm 0.22 [*]	2.00 \pm 0.45	47.93 \pm 1.89
14	Cry2Aa 270 mg/Kg	0.17 \pm 0.17 [*]	0.50 \pm 0.22 ^{*,†}	48.63 \pm 1.39
15	Cry1Aa+1Ab 270 mg/Kg	0.50 \pm 0.22 [*]	0.67 \pm 0.33	45.02 \pm 2.27 ^a
16	Cry1Aa+1Ac 270 mg/Kg	0.33 \pm 0.21 ^{*,c}	0.83 \pm 0.31 ^{*,a,c}	46.72 \pm 2.10
17	Cry1Aa+2Aa 270 mg/Kg	0.17 \pm 0.17 [*]	0.83 \pm 0.31 ^{*,a}	48.88 \pm 0.56 [~]
18	Cry1Ab+1Ac 270 mg/Kg	0.50 \pm 0.22 ^{*,c}	1.50 \pm 0.43 ^{*,c}	51.46 \pm 1.43 [*]
19	Cry1Ab+2Aa 270 mg/Kg	1.00 \pm 0.45	2.83 \pm 0.48 ^d	46.50 \pm 2.50 [~]
20	Cry1Ac+2Aa 270 mg/Kg	0.50 \pm 0.34 ^{*,c}	1.83 \pm 0.48 ^{c,d}	46.51 \pm 2.08 [~]
	P-values	0.001	0.000	0.026

The data correspond to the means and to the standard error of mean (SEM). MN-NCE and MN-PCE = micronucleus test results for normochromatic erythrocytes (NCE) and polychromatic erythrocytes (PCE), respectively. P-values were generated by the Kruskal-Wallis test. Asterisks indicate significant ($p<0.05$) and highly significant ($p<0.01$) differences detected by the Mann Whitney U test in the comparisons with the negative control. The following symbols represent significant differences in respect to: ●= CP; †=dose-effect for the dose of 27 mg/Kg; ‡=dose-effect for the dose of 136 mg/Kg; a=Cry1Aa 270 mg/Kg; c=Cry1Ac 27 mg/Kg; d=Cry2Aa 270 mg/Kg.

Table 4: Micronucleus evaluation of bone marrow cells from Swiss white mice treated with *Bt* spore-crystal Cry1Aa, Cry1Ab, Cry1Ac and Cry2Aa administered orally (*per os*, *p.o.*) 24 h before euthanasia. Control mice received filtered water (negative control) or cyclophosphamide (CP – positive control) at 27 mg/Kg.

	Cry1Aa	Cry1Ab	Cry1Ac	Cry2Aa
at 27 mg/Kg (cells/mL)	2 \times 10 ⁷	3 \times 10 ⁷	4 \times 10 ⁷	2 \times 10 ⁷
at 136 mg/Kg (cells/mL)	1 \times 10 ⁸	1 \times 10 ⁸	2 \times 10 ⁸	1 \times 10 ⁸
at 270 mg/Kg (cells/mL)	2 \times 10 ⁸	3 \times 10 ⁸	4 \times 10 ⁸	2 \times 10 ⁸

Table 5: Results of Colony Forming Units (CFU) for Cry1Aa, Cry1Ab, Cr1Ac and Cry2Aa and quantification of the viable *Bt* spore-crystals ingested by the animals at the different concentrations used (27 mg/Kg, 136 mg/Kg and 270 mg/Kg).

It has been reported that Cry toxins exert their toxicity when activated at alkaline pH of the digestive tract of susceptible larvae, and, because the physiology of the mammalian digestive system does not allow their activation, and no known specific receptors in mammalian intestinal cells have been reported, the toxicity these MCAs to mammals would be negligible [8,22,23]. However, our study demonstrated that *Bt* spore-crystals genetically modified to express individually Cry1Aa, Cry1Ab, Cry1Ac or Cry2A induced hematotoxicity, particularly to the erythroid lineage. This finding corroborates literature that demonstrated that alkali-solubilized *Bt* spore-crystals caused *in vitro* hemolysis in cell lines of rat, mouse, sheep, horse, and human erythrocytes and suggested that the plasma membrane of susceptible cells (erythrocytes, in this case) may be the primary target for these toxins [33].

It has been reported that strains of Cry toxins can be solubilized by alkaline buffer or a combination of alkaline buffer and reducing conditions, and that, although intravenous and subcutaneous administrations of 15-30 µg per gram body weight (0.4-0.9 mg per animal) of these alkali-soluble crystal proteins in Balb-C mice resulted in death, there were no toxic effects when orally administered [33]. In our study, lyophilized *Bt* spore-crystals resuspended in distilled water (and not in alkaline and/or reducing conditions) and orally administered at higher doses than the foregoing ones presented cytotoxic effects, particularly to the erythroid lineage of mice. Considering the increased risk of human and animal exposures to significant levels of these toxins, especially through diet, our results suggest that further evaluations are needed, with longer exposure of mammals to these diets, and involving clinical observations, before concluding that these microbiological control agents are safe to mammals. Cry1Ab induced microcytic hypochromic anemia in mice, even at the lowest tested dose of 27 mg/Kg, and this toxin has been detected in blood of non-pregnant women, pregnant women and their fetuses in Canada, supposedly exposed through diet [34]. These data, as well as increased bioavailability of these MCA in the environment, reinforce the need for more research, especially given that little is known about spore crystals' adverse effects on non-target species.

Because of its high mitotic index, hematopoietic tissue becomes the target of the adverse effects of many chemical substances entering the body. Some substances that act on bone marrow may have a selective effect, that is, they may be toxic to a given cell line [35]. Our study found selective cytotoxicity for the erythroid lineage and showed differences in the dose response curves of *Bt* spore-crystals genetically modified to express individually Cry1Aa, Cry1Ab, Cry1Ac or Cry2A. After 24 hours of exposure, Cry1Ac and Cry2Aa showed a non-monotonic dose response curve, where Cry2Aa presented a U-shaped dose response curve, with high responses at both low and high levels of contamination, while the Cry1Ac response curve was shaped like an inverted U with the greatest response in intermediate ranges. On the other hand, Cry1Ab seemed to have an effect similar to hormesis, where lower toxin doses (such as 27 and 136 mg/Kg) increased the body's tolerance for greater toxicity (such as 270 mg/Kg), while Cry1Aa seemed to maintain almost the same behavior, regardless of the dose. These differences among Cry toxins were already somewhat expected, since they have a defined spectrum of insecticidal activity, each of which is specific for some species belonging to a particular order of insects, according to their affinities for specific receptors on their target organisms [10,36]. Additionally, although results of CFU showed fewer viable spores/mL for Cry2Aa and Cry1Aa than for Cry1Ab and Cry1Ac, at 270 mg/mL the cytotoxic effects of Cry2Aa and Cry1Aa seemed higher compared to Cry1Ab and Cry1Ac.

Knowledge of the genotoxic potential of chemical industrial agents

or those naturally present in the environment is essential information for regulatory agencies, regarding the establishment of risk for humans [37]. Since micronuclei in interphase cells result from chromosomal breaks or chromosomal lagging, the MN test is most widely used for the detection of clastogenic and aneugenic agents [29,38,39]. For the fish species *Danio rerio*, it has been reported that Cry1Aa significantly increased the frequency of micronuclei in peripheral blood of erythrocytes, while Cry1Ab, Cry1Ac, and Cry2A did not present genotoxicity [40]. In our study, all evaluated spore-crystals in single or binary combinations were hematotoxic and cytotoxic to the mice bone marrow, but not genotoxic, and this could indicate differences among vertebrates.

In the biological control of pests, a combination of different mixtures of *Bt* spore-crystal strains is also used. Also, a second generation of *Bt*-plants expressing two different Cry toxins has been developed to avoid insect resistance. There are several examples of transgenic plants with dual *Bt* gene insertion, such as Bollgard® II RR Flex cotton (Cry1Ac+Cry2Ab) and maize (Cry1Ac+Cry2Ab) [41]. Consequently, various interactive processes may occur, such as additivity, synergism, potentiation or antagonism [41,42]. However, to date, there are few studies on the cytotoxicity of conjugated *Bt* toxins for different organisms, and no studies were found in the literature evaluating the potential toxic and genotoxic effects of binary combinations of Cry toxins for non-target organisms. Our results demonstrated that the binary combinations of Cry1Ac+Cry2Aa and Cry1Ab+Cry2Aa were also hematotoxic to the erythroid lineage in particular. Furthermore, these binary combinations and also Cry1Aa+Cry2Aa were cytotoxic to the bone marrow cells in that they reduced the %PCE.

Literature has shown that *Bt* toxins are generally nontoxic and do not bioaccumulate in fatty tissue or persist in the environment [23], but our study demonstrated that all Cry at 270 mg/Kg showed a more pronounced cytotoxic effect on the erythroid lineage from 72 hours of exposure onwards, and that these effects were more pronounced after 7 days of exposure. After 7 days of exposure, Cry1Ab was toxic showing alterations in the hematological parameters of the exposed mice. It is well known that processes or substances that cause damage in the hematopoietic stem cell or bone marrow stroma of mice can also cause a decrease in WBC count [43]. Indeed, Cry1Ab significantly decreased MCH, MCV, and RDW and also decreased the number of PLT, which was non-significant in relation to the negative control but was substantially lower than the reference values for mice (900-1600 × 10³/µL) [43], as well as significantly increased P-LCR and decreased lymphocyte number. The profile of observed cytotoxic effects of these Cry toxins can be related to their high concentrations and the exposure time. Such exposures at these high concentrations are not commonly found in the environment.

In mice, the inflammatory response is often associated with both increased lymphocytes and neutrophils, and small changes in the number of neutrophils may be biologically significant and reflected in the total leukocyte count [43]. In this context, our study showed a higher inflammatory response for Cry1Aa 270 mg/Kg after 72 hours of exposure and for Cry1Ac and Cry2Aa at 270 mg/Kg after 7 days of exposure. Immunophenotypic changes have been demonstrated in the intestine and peripheral sites of young and old mice after ingestion of *Bt* corn MON810 encoding the active form of Cry1Ab [44], and intragastric administration of Cry1Ac prototoxin has induced secretion of mucosal antibodies in mice [45]. Our results corroborate these findings for Cry1Ac and also demonstrate leukogenic activity for other spore-crystals not yet reported in the literature.

In conclusion, results showed that the *Bt* spore-crystals genetically modified to express individually Cry1Aa, Cry1Ab, Cry1Ac or Cry2A can cause some hematological risks to vertebrates, increasing their toxic effects with long-term exposure. Taking into account the increased risk of human and animal exposures to significant levels of these toxins, especially through diet, our results suggest that further studies are required to clarify the mechanism involved in the hematotoxicity found in mice, and to establish the toxicological risks to non-target organisms, especially mammals, before concluding that these microbiological control agents are safe for mammals.

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