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BRIEF REPORT

Toxicity assessment of *Bacillus thuringiensis* strains for the control of the lesser mealworm beetle *Alphitobius diaperinus* (Coleoptera: Tenebrionidae)

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Abstract This study addresses the pervasive challenge of lesser mealworm *Alphitobius diaperinus* (Coleoptera: Tenebrionidae) infestations in poultry farming. Our aim was to select toxic *Bacillus thuringiensis* strains against *A. diaperinus* larvae and determine the fraction(s) within the bacterial cultures harboring the active metabolites responsible for this insecticidal activity. Among the 41 strains evaluated, the Argentine strain INTA Mo4-4 showed the highest toxicity. Bioassays revealed that the main virulence factors reside within the spore-crystal pellet associated with the Cry protein. These results set the basis for the development of a targeted bioinsecticide. Further research is needed to elucidate the underlying mechanisms and validate these strategies in field trials. By harnessing *B. thuringiensis*-based bioinsecticides, we offer a sustainable solution to mitigate *A. diaperinus* infestations.
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PALABRAS CLAVE
Alphitobius diaperinus;
Bacillus thuringiensis;
Bioinsecticidas;
Granjas avícolas;
Control de plagas;
Actividad larvicida

Evaluación de la toxicidad de cepas *de Bacillus thuringiensis* para el control del escarabajo de la cama de pollos *Alphitobius diaperinus* (Coleoptera: Tenebrionidae)

Resumen Este estudio aborda el desafío generalizado de las infestaciones del escarabajo de la cama de pollos *Alphitobius diaperinus* (Coleoptera: Tenebrionidae) en la avicultura. Nuestro objetivo fue seleccionar cepas de *Bacillus thuringiensis* tóxicas para larvas de *A. diaperinus* y determinar las fracciónes de los cultivos bacterianos que albergan los metabolitos activos responsables de esta actividad insecticida. Entre las 41 cepas evaluadas, la cepa argentina INTA Mo4-4 mostró la mayor toxicidad. Los bioensayos efectuados revelaron que los principales

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factores de virulencia residen dentro del sedimento de la fracción esporas-cristales asociada a las proteínas Cry. Estos resultados sientan las bases para el desarrollo de un bioinsecticida dirigido. Se necesita más investigación para dilucidar los mecanismos subyacentes y validar estas estrategias en ensayos de campo. Los bioinsecticidas a base de *B. thuringiensis*, podrían ser una solución sostenible para mitigar las infestaciones por *A. diaperinus*.
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Poultry farming is a key player in global protein supply, though it faces many challenges including the lesser mealworm, *Alphitobius diaperinus* Panzer (Coleoptera), which is considered one of the most significant. This beetle belonging to the family Tenebrionidae is present in almost all poultry houses around the world and poses multiple threats to both poultry production and human health[11]. The impact of *A. diaperinus* is felt across different sectors of the chicken industry, significantly straining the finances of farmers as well as their operations.

At times, however, outbreaks escalate in severity over time as *A. diaperinus* populations grow because this insect has adapted and persists on poultry farms[11]. Its life cycle that features different developmental stages enables it to survive within various parts of chicken houses ranging from bedding material to feed storage areas[11]. Additionally, its resistance to harsh conditions and ability to exploit ecological niches within chicken housing contribute to its continuous presence and widespread[11].

Alphitobius diaperinus infestations have negative consequences. Additionally, it can also threaten biosecurity measures and result in operational inefficiency in addition to destroying facilities and equipment. This beetle is a great threat to the well-being and health of poultry[11]. Chicks infested with *A. diaperinus* suffer traumatic wounds, such as feather and skin lesions, which reduce productivity and increase susceptibility to infections. Furthermore, *A. diaperinus* helps transmit pathogens such as bacteria and parasites that serve as an accelerant in the spread of diseases among chicken stocks[11]. Moreover, this poses occupational risks for farm workers due to exposure to contaminated materials and beetle-related allergens in these poultry houses[11].

Thus, given the complexity of the *A. diaperinus* concern, it would be crucial to implement effective control measures to reduce the effect of its infestation on poultry farms. Chemical insecticides have been used as a traditional tool to control minor mealworm populations but are facing a dramatic decline in performance due to specific issues such as insect resistance, environmental concerns, and regulatory restrictions[9]. As a result, more attention has been directed towards alternative strategies for *A. diaperinus* control including the application of *Bacillus thuringiensis*-based bioinsecticides[1,6,10]. Unlike chemical insecticides, which non-specifically target a wide range of insects, *B. thuringiensis* provides a specific and environmentally sound approach to pest management.

Bacillus thuringiensis is a spore-forming Gram-positive bacterium that exists in various ecosystems, especially within soil environments[13]. This bacterium is known for its remarkable manufacturing capabilities regarding the synthesis of a variety of kinds of pesticidal proteins aimed at killing insects. Included among these proteins are Cry, Cyt, Vip, Vpa/Vpb and Mpp, all of which have high specificity against different orders of insects[2,3]. Traditionally, 84 serovars of *B. thuringiensis* have been identified based on the flagellar (H) antigen classification[13]. However, this method has become obsolete due to its limitations; it is unreliable for predicting insecticidal activity and requires a comprehensive panel of antibodies for accurate identification[13]. For example, *B. thuringiensis* serovar *morrisoni* encompasses diverse pathovars, some exhibiting specific activity against Lepidoptera, Coleoptera, or Diptera, while others show no insecticidal effect[13].

The toxicity of *B. thuringiensis* proteins, occurs after ingestion, targeting the epithelial lining of the midgut of insects. Some of these pesticidal proteins are secreted during the vegetative stage, while others are stored in crystalline inclusions, such as the parasporal crystal, which contains Cry and Cyt proteins, during sporulation[13]. The protoxins are released upon solubilization and consequently activated by midgut proteases. The protein crosses the peritrophic matrix and attaches to the receptors on the midgut epithelial membrane. The *B. thuringiensis* proteins make an oligomer which forms pores, normally consisting of four monomers of a toxin assembled into a pore-forming oligomer[3]. Upon insertion into the epithelial membrane, these pores disrupt its integrity, facilitating the invasion of gut bacteria into the hemolymph of the susceptible insect, leading to septicemia and mortality[13]. *Bacillus thuringiensis* remains harmless to humans, animals, plants, and beneficial insects.

The aims of our study were two: to select *B. thuringiensis* strains that exhibit maximum toxicity to *A. diaperinus* larvae and to determine the exact fraction of the bacterial cultures that harbor the active metabolite or metabolites responsible for this toxicity. Upon achieving these objectives, we aim to set a foundational framework for developing targeted and efficient bioinsecticide formulations aimed at mitigating lesser mealworm infestation in poultry farms.

In this work, a total of 41 different strains of *B. thuringiensis* were used. All these strains belong to the Bacterial Collection of the Instituto de Microbiología y Zoología Agrícola at the Instituto Nacional de Tecnología

Table 1 Toxicity assessment of whole cultures of Argentine and exotic *Bacillus thuringiensis* strains against second instar larvae of *Alphitobius diaperinus*.

Strain Argentine	Mortality[a] (%)	Source[b]	Strain Exotic	Mortality (%)	Source
INTA Mo1-12	0.0 ± 0.0 (2)	Stored product dust	svar. *aizawai* HD-133	0.0 ± 0.0 (2)	USDA
INTA H3-3	4.2 ± 2.1 (2)	Leaves	svar. *alesti* HD-4	6.5 ± 6.5 (2)	USDA
INTA H4-1	8.7 ± 4.4 (2)	Leaves	svar. *asturiensis* 4BQ1	0.0 ± 0.0 (2)	BGSC
INTA H4-3	2.2 ± 2.2 (2)	Leaves	svar. *canadienses* HD224	5.5 ± 5.1 (2)	BGSC
INTA H41-1	0.0 ± 0.0 (2)	Leaves	svar. *entomocidus* HD-110	4.3 ± 0.0 (2)	USDA
INTA Mo4-4	27.1 ± 4.0 (10)	Stored product dust	svar. *galleriae* T05001	2.8 ± 2.8 (2)	IP
INTA 7-3	4.3 ± 0.0 (2)	Soil	svar. *israelensis* IPS-82	0.0 ± 0.0 (2)	CINVESTAV
INTA 51-3	0.0 ± 0.0 (2)	Soil	svar. *israelensis* HD-567	0.0 ± 0.0 (2)	USDA
INTA Mo14-1	4.2 ± 2.1 (2)	Stored product dust	svar. *japonensis* 4AT1	0.0 ± 0.0 (2)	BGSC
INTA Mo14-4	2.2 ± 2.2 (2)	Stored product dust	svar. *kumamotoensis* 4W1	0.0 ± 0.0 (2)	BGSC
INTA TA21-2	0.0 ± 0.0 (2)	Spider web	svar. *kurstaki* HD-1	0.0 ± 0.0 (2)	USDA
INTA H48-5	0.0 ± 0.0 (2)	Leaves	svar. *kyushuensis* HD-541	9.4 ± 1.8 (2)	BGSC
INTA 56-4	0.0 ± 0.0 (2)	Soil	svar. *morrisoni tenebrionis* DSM2803	10.2 ± 2.1 (10)	CINVESTAV
INTA 272-1	0.0 ± 0.0 (2)	Soil	svar. *morrisoni* HD-12	2.1 ± 2.1 (2)	BGSC
INTA TA273-8	2.1 ± 0.0 (2)	Spider web	svar. *morrisoni* 4K3	2.1 ± 2.1 (2)	BGSC
INTA 33-5	0.0 ± 0.0 (2)	Soil	svar. *pakistani* 4P1	0.0 ± 0.0 (2)	BGSC
INTA 77-10	0.0 ± 0.0 (2)	Soil	svar. *roskildiensis* T45001	0.0 ± 0.0 (2)	BGSC
INTA Fr7-4	2.3 ± 0.0 (2)	Soil	svar. *thompsoni* HD-542	2.4 ± 2.4 (2)	USDA
			svar. *thuringiensis* HD-2	0.0 ± 0.0 (2)	USDA
			svar. *tochigiensis* 4Y1	2.1 ± 2.1 (2)	BGSC
			svar. *tolworthi* HD-125	8.7 ± 4.3 (2)	USDA
			svar. *toumanoffi* HD-201	6.7 ± 2.0 (2)	BGSC
			svar. *wuhanensis* HD-525	4.3 ± 0.0 (2)	USDA

[a] Mean ± SE. The number in parentheses indicates the number of repetitions assayed.
[b] USDA, United States Department of Agriculture (U.S.A.); BGSC, Bacillus Genetic Stock Center (U.S.A.); IP, Institut Pasteur (France); CINVESTAV, Centro de Investigación y Estudios Avanzados (Mexico).

Agropecuaria (IMYZA-INTA). Out of these strains, 18 were isolated from stored product dust, spider webs, leaves, and soils from various parts of Argentina. The remaining 23 strains comprised exotic strains generously supplied by several stock collections worldwide (Table 1).

To ensure standardized *B. thuringiensis* cultures for the experiments, we followed a previously described methodology[8]. This involved inoculating 50 μl of a highly concentrated spore stock suspension of each strain into 50 ml of BM medium, composed of 2.5 g NaCl, 1 g KH_2PO_4, 2.5 g K_2HPO_4, 0.25 g $MgSO_4{\cdot}7H_2O$, 0.1 g $MnSO_4{\cdot}H_2O$, 5 g glucose, 2.5 g starch, and 4 g yeast extract, in a total volume of 1 l of distilled water with a pH of 7.2.

The cultures were incubated at 29 °C in a rotary shaker at 250 r.p.m. for 72 h or until complete autolysis occurred. Twelve milliliters of whole cultures were preserved for preliminary bioassays. Subsequently, spore-crystal complexes were obtained by centrifuging the remaining culture at 12 000 *g* and 4 °C for 15 min, and the resulting pellets were then resuspended in 38 ml of distilled water. The supernatants were collected and filtered using a 0.22-μm pore-size cellulose acetate syringe filter (Micron Separation Inc.). These fractions and spore-crystal suspensions were kept at −20 °C until further use.

Bioassays were conducted against second instar larvae of *A. diaperinus* using the diet incorporation method[8]. Six milliliters of different *B. thuringiensis* preparations, including whole culture, resuspended spore-crystal pellet, or filtered supernatants, were incorporated into 34 ml of temperature-controlled (60 °C) artificial diet for *A. diaperinus* when required (133.3 g of balanced chicken starter feed, 2.5 g ascorbic acid, 1.25 g sorbic acid, 2.08 g nipagin, and 10 g agar, in a total volume of 1 l of distilled water). The volume of culture preparations used was the highest that could be added without altering the proportion of the diet components.

Four hundred microliters of these mixtures were added to each well of a 24-well plate (Nunc 143982). Natural mortality controls were prepared using sterile BM broth and distilled water. The larvae used in the bioassays were obtained from laboratory colonies maintained in the IMYZA-INTA facilities. Each assay used 24 second instar larvae of *A. diaperinus*, with a minimum of two replicates. Preliminary bioassays with most strains involved two replicates using whole cultures to identify non-active strains. Strains that exhibited mortality rates exceeding 10% in these initial tests were subjected to additional replicates to confirm reproducibility. Mortality was measured at the end of the incubation period, 7 d at 29 °C, when the larvae were not able to react to a soft touch; mortality was corrected with the Schneider-Orelli formula and compared to the controls, which were not treated[8]. The results were subjected to statistical analysis using the InfoStat program (Universidad Nacional de Córdoba, version 2020). Mortality data from the different treatments were presented as means ± standard error (SE). When applicable, an analysis

of variance (ANOVA), followed by Tukey's post hoc test, was performed, assuming equal variances. The level of significance was $p < 0.05$.

Among the 41 whole cultures of Argentine and exotic *B. thuringiensis* strains tested here, 40 exhibited null to low virulence against *A. diaperinus*, with mortality ranging from 0 to 27.1% (Table 1). Indeed, Argentine strain INTA Mo4-4 presented the highest mortality rate under the conditions tested, and thus, this strain was considered for further study (Table 1). It should be noted that INTA Mo4-4 caused mortality 2.7 times higher than that of the exotic *B. thuringiensis* svar. *morrisoni tenebrionis* DSM2803 strain ($F = 14.14$; $df = 19$; $p = 0.0014$), which is recognized worldwide for showing insecticidal activity against different coleopterans[14].

Our findings reveal significant disparities compared to those reported in the literature. While previous studies, such as that by Zanchi et al.[15], have shown that certain *B. thuringiensis* strains were highly toxic to Tenebrionidae species, including *A. diaperinus*, the present study showed null to low virulence among the evaluated strains. Moreover, Sallet[10] and Alencar[1] can be cited, where significant mortalities ranging from 30% to 61% were observed while assessing Brazilian strains of *B. thuringiensis* for *A. diaperinus*, with the most toxic strains identified as serovar *israelensis*. In the present study, none of the strains evaluated from this serovar revealed pathogenicity (Table 1).

Comparable results were reported by Koç et al.[6], who observed low larval mortality after four days of exposure to bioinsecticides based on highly concentrated spore and crystal suspensions of *B. thuringiensis* svars *kurstaki*, *aizawai*, and *israelensis*. Even after 14 days of exposure, these formulations induced only minimal larval mortality, a striking outcome considering that the strains used in these bioinsecticides are reported to exhibit significant activity against lepidopteran and dipteran larvae but not coleopterans[6].

Such important divergence in toxicity levels may result from differences in bioassay conditions, preparation methods, titer concentrations, or the specific populations of *A. diaperinus* evaluated. Further investigation is needed to clarify the basis of these divergent results.

The whole culture of *B. thuringiensis* INTA Mo4-4 was analyzed to evaluate the insecticidal metabolites present in the crystals and, potentially, those secreted to the culture medium during vegetative growth. The pellet, resuspended in water, caused a mortality rate of $16.2 \pm 2.8\%$, while that from the filtered supernatant, containing putative secreted insecticidal metabolites, was $6.2 \pm 2.1\%$. We conducted an ANOVA for comparing mortalities among the pellet, filtered supernatant, and the whole culture. The results revealed no significant differences among the three treatments ($F = 3.48$; $df = 14$; $p = 0.06$), inferring that both contribute equally to the virulence of INTA Mo4-4 against *A. diaperinus*. However, the pellet induced mortality 2.6 times higher than that of the supernatant and both were lower than that of the whole culture. This would point to the fact that the major insecticidal factors are mostly found in the pellet, attributed to the Cry proteins present in the crystals. These results agree with those found in other studies, showing that pesticidal proteins found in crystals are the main virulence factors of *B. thuringiensis* against *A. diaperinus*; these include coleoptericidal proteins such as Cry3Aa, Cry3Bb, Cry8Ca[7], and proteins with dual activity against Diptera and some coleopterans, including Cry4B, Cry10, Cry11A, and Cyt1A[10].

Some studies have also explored the potential of other microbial biocontrol agents for controlling *A. diaperinus*, showing promising results in both larval and adult stages. For instance, research on *Beauveria bassiana* showed mortality rates exceeding 40%[12], with methanolic extracts achieving up to 96% mortality[4]. These findings emphasize the insecticidal potential of fungal metabolites, such as cyclodepsipeptides and β-adenosine, although reduced susceptibility to extracts compared to insecticides was noted. Additionally, nematodes such as *Steinernema carpocapsae* and *S. feltiae* demonstrated high virulence, causing approximately 70% larval mortality under optimal conditions[5], although efficacy decreased at higher temperatures. Considering these observations, the combined use of *B. thuringiensis* with other microbial biocontrol agents for controlling *A. diaperinus* should not be discarded.

In summary, our study suggests that some Argentine and exotic *B. thuringiensis* strains, especially INTA Mo4-4, have potential for biological control of *A. diaperinus* infestation in poultry farms. After detecting strains that exhibited some level of toxicity to the larvae of *A. diaperinus*, INTA Mo4-4 showed the most significant and consistent mortality, making it a promising candidate for the development of bioinsecticides aimed at controlling these insects.

Furthermore, our study on various culture fractions of INTA Mo4-4 gave us insights into the main toxicity factors, suggesting that the spore-crystal pellet associated with Cry proteins is the main player in exerting insecticidal activity. It can be used for the development of bioinsecticides targeting populations of *A. diaperinus*.

Future studies should establish the exact mechanisms of virulence of the *B. thuringiensis* strains against *A. diaperinus*, especially the identification of the virulence factors involved in toxicity. Field studies are necessary to further confirm the efficiency of such biocontrol strategies under the conditions encountered in the field to make the adoption of this practice feasible in poultry farming operations.

By harnessing the potential of *B. thuringiensis*-based bioinsecticides, we offer a sustainable and environmentally friendly solution to poultry farmers to mitigate the economic and health impacts caused by *A. diaperinus* infestation. This, therefore, calls for cooperation among researchers, industry players, and policymakers to convert these scientific findings into practical tools that promote poultry health, welfare, and productivity in ways that guarantee the long-term sustainability of poultry production systems.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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