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**Regulation by gut bacteria of immune response, *Bacillus thuringiensis*
susceptibility and *hemolin* expression in *Plodia interpunctella***

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Abstract

Plodia interpunctella (Hübner) is an important stored grain insect pest worldwide, and the first lepidopteran with reported resistance to *Bacillus thuringiensis* (*Bt*) toxins. Since gut bacteria may affect *Bt* insecticidal activity, we determined whether *P. interpunctella* lacking gut bacteria had differences in immune responses and susceptibility to a *Bt* formulation, Bactospeine. To clear the gut bacteria, third instar larvae were reared on artificial diet using antibiotics, or were from sterilized eggs and reared under sterile conditions, and larvae were fed diets with or without *Bt*. Mortality was significantly lower ($p < 0.05$) in bacteria-free larvae (either fed antibiotics in diet or from sterilized eggs and diet) treated with *Bt*, compared to larvae with gut bacteria and treated with *Bt*. The number of hemocytes was lower in control and *Bt*-treated larvae, but was significantly higher ($p < 0.001$) in larvae treated with antibiotics and *Bt*, and larvae from presterilized eggs and reared on sterile diet had the highest number of hemocytes. Phenoloxidase activity was significantly lower ($p < 0.05$) in *Bt*-treated larvae from presterilized eggs reared on antibiotics for 24 h or in larvae reared on antibiotic-treated diets prior to *Bt* introduction compared to those fed control diet. *Hemolin* gene expression was reduced in larvae fed *Bt* diets compared to control and was not detected in larvae treated with antibiotics. Larvae from sterilized eggs and fed sterile diet never reached the pupal stage. Therefore, the loss of gut bacteria in *P. interpunctella* larvae affected the host immune response and expression of the *hemolin* gene, and greatly reduced susceptibility to *Bt*.

Keywords: *Bt*-resistance; gut bacteria; hemocytes; phenoloxidase activity.

1. Introduction

The Indianmeal moth, *Plodia interpunctella* (Hübner) (Pyralidae), is an insect pest of stored grains and flour, and also attacks garlic bulbs and packaged goods. Every year, millions of dollars are lost worldwide due to *P. interpunctella* (Mohandass et al., 2007). Organic insecticides used to control this pest include the entomopathogenic bacteria *Bacillus thuringiensis* (*Bt*) Berliner.

Some studies have suggested that gut bacteria are important in the insect response to *Bt*. When gut bacteria were eliminated by antibiotic-supplied diets, susceptibility to *Bt* was significantly reduced in Lepidoptera species (Broderick et al., 2006, 2010). Their findings were in contrast to those of Frankenhuyzen et al. (2010) and Johnston and Crickmore (2009), who related changes in the susceptibility of insects to *Bt* that were independent from microbiota, but instead were associated with the effect of antibiotics in the insect. Recently, expression of an immunity gene was decreased by RNAi in *Spodoptera littoralis* Boisduval, with a concomitant increase in susceptibility to *Bt* that was linked to the proliferation of bacteria in the gut, contributing to septicemia (Caccia et al., 2016). *Bt* toxicity may depend on direct or indirect interaction between the entomopathogen and the intestinal microbial community, as bacteria are associated with growth and development, and may contribute to reproduction, digestion, nutrition, and pheromone production (Dillon and Dillon, 2004).

The pattern recognition receptors of the insects' immune system recognize bacterial peptidoglycans, resulting in signal transduction, which in turn results in induction of the immune response, such as the production of antimicrobial peptides (AMPs) (Kim et al., 2008). The humoral component of the immune response includes increased production of AMPs and induction of phenoloxidase (PO) cascade-mediated melanization, whereas the cellular response

involves phagocytosis by circulating hemocytes (Kim et al., 2008; Vizioli and Salzet, 2002), cell aggregation, nodulation, and encapsulation (Lavine and Strand, 2002). In addition, endosymbiotic bacteria were reported to regulate insect immune defense mechanisms, like melanization, against pathogens and parasites (Eleftherianos et al., 2013).

Knowledge of pest intestinal bacteria and how they affect *Bt* susceptibility, perhaps through an indirect immune response, could lead to new strategies for control. High expression of five different AMPs was reported in *Trichoplusia ni* Hübner strains with low susceptibility to *Bt* (Tamez-Guerra et al., 2008). However, these insects also had a decreased immune response (reduction in hemolymph protein concentrations, PO activity and the number of circulating hemocytes) after exposure to low *Bt* doses, and this may reflect a fitness cost associated with *Bt* resistance (Ericsson et al., 2009). In *P. interpunctella*, the expression of *hemolin* has been reported during development and was related to bacterial challenge (Aye et al., 2004). We found that *hemolin* transcript amplification was significantly different between *Bt*-susceptible and *Bt*-resistant *P. interpunctella* colonies (unpublished data). Hemolin also may play a role as a procoagulant (Alvarez-Flores et al., 2010), and is inducible by dsRNA injection, and affects insect development and immune response against viruses (Hirai et al., 2004).

To prolong the efficacy of *Bt* formulations in the field, it is important to understand the role of gut bacteria in *Bt* toxicity. The aim of the present study was to determine the relationship of *P. interpunctella* intestinal bacteria with susceptibility to *Bt* and various parameters of the immune response.

2. Materials and methods

All enzymes, substrates, and chemicals were obtained from Sigma-Aldrich Chemical, S. de RL de CV (Toluca de Lerdo, Estado de México, México), unless otherwise specified.

2.1 Bacterial formulation

Commercial *Bacillus thuringiensis* (Bt) var. *kurstaki* HD-1, Bactospeine® DF (Valent de México, Zapopan, Jalisco) containing Cry1Aa, Cry1Ab, Cry1Ac, Cry2A, and Cry2B protoxins and spores with 32,000 UI/mg potency, was used in this study.

2.2 Insect Colony

P. interpunctella larvae were obtained from stock cultures from the Center for Grain and Animal Health Research, Manhattan, KS, USA, and maintained at UANL. *P. interpunctella* larvae were reared on a cracked wheat diet (McGaughey and Beeman, 1988) at $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$, $55 \pm 10\%$ relative humidity, and 16h light/8h dark photoperiod. For gut bacterial assays, larvae were reared under normal artificial diet (Nuñez-Mejía et al., 2016) (Fig. S1), artificial diet (wheat agar, wheat germ, soy flour and sugar sterilized by autoclave, and vitamin solution sterilized by membrane filtration and added after artificial diet temperature was lower than 60°C), and/or antibiotic treated agar-based artificial diet, where antibiotic solution was sterilized by membrane filtration and added after artificial diet temperature was lower than 60°C . Either cereal or artificial diet treatments were assayed using individual tight-lid small plastic 47-mm diameter Petri dishes.

2.3 Gut bacteria eradication

Gut bacteria were eliminated using the technique described by Broderick *et al.* (2006, 2010); in brief, larvae were fed a sterile artificial diet containing an antibiotic solution of 500 µg/mL each rifampicin, gentamicin, penicillin, and streptomycin, which was mixed in the diet when it was liquid (at about 55 °C), and placed into Petri dishes to let it cool and solidify. The aerobic/facultative bacteria present in 3rd instar larvae gut were determined by the colony forming units (CFU) technique on LB agar medium, and analyzed by PCR using the universal *16S rRNA* gene primers for bacterial amplification, as described by Frank et al. (2008).

A second method was used to eliminate bacteria from the gut. Approximately, 100 µL volume of collected eggs were resuspended in 1.0 mL of 0.8% saline solution, and disinfected with 13% sodium hypochlorite solution (Cloralex™, Industrial Alen, Santa Catarina, N.L., MX), and diluted in distilled water to 5% vol:vol. Eggs were soaked in this solution for 15 sec, and then rinsed by three continuous washes in 0.8% saline solution. Eggs were carefully placed on sterile diet in Petri dishes. Experiments were performed under a laminar flow cabinet to avoid microbial contamination.

2.4 Gut bacteria identification

P. interpunctella larvae from various diet treatments were chilled to 4 °C in refrigerator for 20 min and surface sterilized in 5% commercial sodium hypochlorite diluted to a final 0.56% (Cloralex™, Industrial Allen, Santa Catarina, N.L., MX) for 15 sec. The guts of six larvae from

each treatment were pooled and total microbial DNA was extracted with the kit *Kit AxyPrepTM Multisource Genomic DNA Miniprep Kit* (Axygen, Union City, CA).

DNA extract was used as a template for PCR amplification of 16S rRNA genes using universal primers 27F 5'-AGA GTT TGA TCC TGG CTC AG-3', 1492 R 5'- TAC CTT GTT ACG ACT T-3' previously used by Frank et al. (2008). PCR was performed using Taq & GoTM Master Mix (Promega) for a 50 μ L volume, with 100 ng of template DNA, 2 μ M forward (27F) and reverse (1492R) primers. The thermal cycling conditions were an initial denaturation step at 94°C for 10 min, followed by 35 cycles of denaturation at 94°C for 40 sec, annealing at 60°C for 40 sec, and elongation at 72°C for 2 min, and a final extension at 72°C for 10 min.

Amplified PCR products were analyzed on 1.0 % agarose gel, purified with a Wizard® SV Gel and PCR Clean-Up System (Promega) and ligated into pGEM-T Easy (Promega) in competent *E. coli* TOP-10 cells. Detected plasmids were purified using the Wizard® Plus SV Minipreps DNA Purification System. Sanger sequencing was performed with standard vector M13F and M13R primers from the National Laboratory of Genomics for Biodiversity (LANGE BIO, Irapuato Mexico). Bacterial classification was performed using the Ribosomal Database Project (<http://rdp.cme.msu.edu/>; Wang et al., 2007).

2.5 Bioassays

The interaction of *Bt* (Bactospeine) and gut bacteria was evaluated using 3rd instar larvae. Treatments included 1) larvae fed artificial diet (control), 2) larvae exposed to artificial diet containing *Bt* at the LC₅₀ (541.4 UI/cm², *Bt*), 3) same as treatment 2, but using artificial diet with antibiotics (*Bt*+An), 4) larvae fed on diet with antibiotics for 24 h and subsequently exposed to

Bt-treatment in sterile diet with no antibiotics (*Bt*+An-SD), 5) larvae fed on diet with antibiotics (An), and 6) larvae fed on diet with antibiotics for 24 h and subsequently transferred to SD with no antibiotics (An-SD). *Bt* treatment was applied to the surface of the artificial diet, and if antibiotics were used in any of the six treatments, they were added to 6 mL of warm liquid artificial diet placed on 50 x 9 mm, tight-fit lid Falcon® sterile Petri dishes (VWR, Monterrey N.L. Mexico) (Tamez-Guerra et al., 2006). Treatments were incubated at $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$, $55 \pm 10\%$ relative humidity, and 16h light/8h dark photoperiod, until larvae from control treatment reached the pupa stage. Bioassays were done in triplicate, and percent mortality was recorded after four days.

2.6 PO activity

PO activity was determined according to Valadez-Lira et al. (2012). In brief, hemolymph was collected into 1.5-mL microcentrifuge tubes and frozen at 20°C for 5 min to lyse hemocytes and release the inner-cell plasma. Samples were then thawed and centrifuged at 5000 rpm for 1 min to separate the PO-containing plasma, and 5 μL of plasma sample were placed in a microplate well with 195 μL of 10 mM *L*-3,4-dihydroxy-phenylalanine (L-DOPA), diluted in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 , pH 7.4). Microplates were incubated in the dark at room temperature, and optical densities were read after 30 min at 490 nm, using a microplate reader DTX 880 Multimode Detector® (Beckman Coulter, Inc., Fullerton, CA, USA); 1 unit/ml PO activity was defined as the amount of enzyme resulting in an increase of 0.001 absorbance units/min (Baer et al., 2009).

2.7 Hemocyte count

The number of hemocytes per milliliter of hemolymph was determined using a hemocytometer. Hemolymph samples were collected by puncturing the larval proleg, and placed into 1.5 mL microcentrifuge tubes on ice (pooling three larvae samples for replicate), and then diluted 1:10 with ice-cooled PBS.

2.8 Hemolin transcript detection

Semi-quantitative RT-PCR was performed using the Improm-RT kit (Promega, Madison, Wisconsin, USA). PCR was conducted using GoTaq® Green Master Mix (Promega) and 1 μ M for each oligonucleotide. For the *hemolin* gene transcript amplification, the primers PiHemF 5'-AAA TTG GAG TGC CAG CAG TT-3' and PiHemR 5'-GGG GAG TTC CCC CAT AAA TA-3 were selected based on the forward and reverse sequences found in *P. interpunctella* AY771598. Quantification of *hemolin* transcript expression was normalized using the internal *rps7* rRNA gene (Fabrick et al., 2003), using as primers: 1RPS75 5'-ACA ACA AGA AGT CAA TCA TCA TCT A-3'; and 2RPS73 5'-TTG TGC TCA ATG GTG GTC TGT TG-3'. Ten microliters of the amplified sample were visualised in a 1% agarose gel and ethidium bromide stain under UV light, using a UVP trans-illuminator (VWR, Aurora, OH, USA). Semiquantitative expression was evaluated by measuring DNA band optical densities using the ImageJ version 1.46.

2.9 Statistical analysis

Results were expressed as mean $\pm$ SE of three replicate determinations from three independent experiments. Statistical significance was assessed by the ANOVA, $p < 0.05$, and pos-hoc Tukey, using SPSS 17 (SPSS, 2008). As dependent variables, mortality percentage, number of hemocytes, and PO activity data were analyzed. As independent variables, all treatments were compared (control, SD, *Bt*, An, *Bt* +An, An-SD, *Bt*+An-SD). In addition, the semi-quantitative expression of *hemolin* transcript was compared to that of the *rps7* internal gene among treatments. Significant statistical differences between the groups were $p \leq 0.05$, unless otherwise stated. The results were expressed as mean $\pm$ SE of three replicate determinations from three independent experiments.

3. Results

Since we wanted to use the LD₅₀ of the commercial product Bactoespeine against *P. interpunctella* in our bioassays, a dose response assay was conducted. The calculated LD₅₀ was 541.4 IU/cm² (C.L. = 432.65-692.35), and this amount was used in all subsequent bioassays.

A mix of antibiotics (rifampicin, gentamicin, penicillin, and streptomycin) was added to diets to eliminate *P. interpunctella* larval gut bacteria. Larvae were allowed to feed 24 h on diets containing the antibiotics cocktail, and guts were isolated from ice-anesthetized larvae. To confirm the eradication of gut bacteria, CFU assays and bacterial 16S rRNA PCR were performed with the extracted guts from 3rd instar larvae. Suppression of bacterial growth was observed at 250, 500, and 1000 μ g/mL in CFU assays (Table S1), and *16S rRNA* PCR analysis

indicated that bacteria were eliminated at doses equal to or greater than 100 µg/mL (Fig. S2). Similarly, absence of bacteria in larvae reared under aseptic conditions (after hypochlorite treatment of eggs and rearing hatching larvae under sterile conditions) was compared by CFU assays and bacterial 16S rRNA PCR, with similar results (data not shown).

Based on the sequences of 16S rRNA, *P. interpunctella* midgut bacteria were identified at the genus level using The Ribosomal Database Project (<http://rdp.cme.msu.edu/>; Wang et al., 2007). The results included sequences related to eight different genres (Table 1). The majority of the sequences corresponding to the phylum Proteobacteria (74.4%), Firmicutes (9%) and Actinobacteria (14%). From the Proteobacteria, 38% corresponded to the γ -proteobacteria, 20% to β -proteobacterias, and 17% to α -proteobacterias. Regarding the gut bacterial diversity classifiable at genera level, species of *Pseudomonas* were the major genera (34%), followed by *Achromobacter* (19%), *Methylobacterium* (16%), and *Propionibacterium* (12%).

3.1 *Bt* susceptibility after antibiotic exposure or sterile rearing conditions.

Bioassays of 3rd instar *P. interpunctella* larvae exposed to *Bt* at the LD₅₀ resulted in 60% mortality under expected confidence limits (Fig. 1A). However, treatment with the same *Bt* dose, but in diet mixed with antibiotics (*Bt*+An), resulted in less than 10% mortality. Significant differences ($p < 0.001$) in larvae mortality were observed between *Bt* treatment alone and unexposed control, as well as all treatments with the antibiotics mixture ($p < 0.05$). Larvae fed on antibiotic-treated diet, with or without *Bt* (An, *Bt*+An) or in presterilized rearing conditions (*Bt*+An-SD), resulted in significantly lower mortality compared with larvae under normal rearing

(*Bt*) conditions ($F= 20.3_{5,12}$; $P<0.001$, Tukey HSD, $\alpha = 0.05$), and mortality from these treatments were similar to that of the unexposed control. Furthermore, *Bt* susceptibility was significantly higher among normal rearing condition larvae (53.3% mortality), compared with larvae from hypochlorite-sterilized eggs and reared under sterile conditions (14.4% mortality, Fig. 1B).

3.2 Hemocyte count

Bt treatment of 3rd instar *P. interpunctella* larvae resulted in significantly lower counts of hemocytes from the hemolymph ($7.16 \text{ Hem} \times 10^6/\text{mL}$) compared with treatments that were designed to lower the amount of gut bacteria (An, An-SD, *Bt*+An, *Bt*+An-SD), and the count was slightly lower than that of the untreated control ($1.83 \text{ Hem} \times 10^7/\text{mL}$) (Fig. 2A). Overall, insects reared with antibiotics or under sterile rearing conditions had increased numbers of hemocytes compared to those on control diet. The most significantly ($p < 0.001$) high hemocyte count was detected in the hemolymph of *Bt*+An-treated larvae ($4.72 \text{ Hem} \times 10^7/\text{mL}$) ($F_{5,12} = 10.64$, $P < 0.001$). When *P. interpunctella* larvae were reared under sterile conditions (SD), larval hemolymph had higher numbers of hemocytes ($1.27 \text{ Hem} \times 10^8/\text{mL}$) than larvae reared under normal conditions ($3.83 \text{ Hem} \times 10^7/\text{mL}$) (Fig. 2B), or any other treatment (Fig. 2A).

3.3 Phenoloxidase activity

In contrast to the hemocyte count observations, the highest PO activity was observed in untreated control larvae and larvae reared on antibiotic treated diet, without or with *Bt* (An,

Bt+An) (Fig. 3A). *P. interpunctella* larvae reared under normal conditions had the highest PO activity (3.56 U/ μ L). PO activity in *Bt* treated larvae was statistically similar to that of larvae reared on sterile diet, and significantly less than that of the control and *Bt*+An larvae. The lowest PO activity was detected among *Bt*+An-SD treated larvae and was significantly different from all other treatments ($F_{5,12} = 20.35$, $p < 0.001$). PO activity in larvae fed control diet was significantly higher than that of larvae reared under sterile conditions and fed *Bt*-treated diet (*Bt*-SD) (1.07 U/ μ L) ($F_{2,6} = 7.089$; $P = 0.026$) (Fig. 3B).

3.4 *Hemolin* transcript detection

The effect of treatments on the expression of the *P. interpunctella* immune response gene *hemolin* was detected by RT-PCR. The relative density of the PCR product was calculated by dividing the *hemolin* density from each sample/treatment by the density of *rps7* (used as endogenous control gene and compared by densitometry) (Tan and Ng, 2008; Gassmann et al., 2009). *Hemolin* expression was the lowest in larvae fed on antibiotic-treated diet (An, An-SD) (Fig. 4A). Expression of *hemolin* was also suppressed in *Bt*-treated larvae, but was slightly increased in larvae fed *Bt* and the antibiotic mixture (*Bt*+An), and further increased when larvae were pretreated with the antibiotic mixture and then exposed to *Bt* (*Bt*+An-SD). Under sterile conditions, *hemolin* expression was decreased compared to control with or without *Bt* (Fig. 4B). However, these larvae did not molt to the pupal stage, and remained as 5th instar for more than one month.

4. Discussion

Since their discovery, bioinsecticides based on *Bt* have demonstrated targeted insect control with minimal environmental impact. Understanding the biology of insect pests, as well as interactions with pathogens and microbiota, can help to enhance their effect within these interactions. In the present study, methodology similar to previous studies was used to evaluate the effect of gut bacteria on the response to *Bt*. However, in addition to mortality; we also evaluated other bacterial responses in larvae, such as the number of hemocytes and PO activity in the larval hemolymph, as well as the relative expression of the *hemolin* gene.

All 3rd instar *P. interpunctella* larvae were fed a diet containing an antibiotic cocktail 24 h prior to *Bt* administration. Confirmation of the reduction of gut bacteria was evaluated by combining the CFU test with PCR amplification of 16S rRNA universal primers for bacteria. Regardless of the method of bacterial-clearing, larvae without gut bacteria were significantly less susceptible to *Bt*. Thus, removal of bacteria from the larval gut contributed to a significantly decreased susceptibility to *Bt*.

Streptomycin inhibited *Bt*-toxicity in *Bt*-resistant diamondback moth (*Plutella xylostella* L.), but not in *Bt*-susceptible insects, and this effect was only demonstrated with *Bt* subsp. *kurstaki*, but not with *Bt* subsp. *aizawai* (Liu et al., 1998). *Helicoverpa armigera* (Hübner) larvae feeding on a diet with the same antibiotic cocktail as reported by Broderick et al. (2006, 2010) did not develop resistance to Cry1Ab or Cry1Ac after transgenerational *Bt* exposure, but mortality was reduced 30% in the F3 generation of insects reared on antibiotics-free diet and exposed to *Bt* (Paramasiva et al., 2014). However, gut bacteria affected *Bt* Cry1Ac and formulation toxicity against *H. armigera* larvae (Visweshwar et al., 2015).

After exposing *P. interpunctella* to the antibiotics mixture and the LC₅₀ of the *Bt* formulation, larval mortality was significantly reduced compared with a control without antibiotics. However, the cellular immune response was affected by the antibiotic treatment, and the number of hemocytes in larval hemolymph from antibiotic treated larvae fed *Bt* was significantly higher compared with that of untreated control, in agreement with Frankenhuyzen et al. (2010). These changes were not solely related to *Bt* (spores and toxins present in Bactospine product), but by the combined *Bt* and antibiotic effect in the insect, since the number of hemocytes also was significantly higher than larvae fed *Bt* only. In addition, significantly higher numbers of hemocytes were also found in larvae hatching from sterilized eggs. The reduction of hemocytes after *Bt* exposure may be the result of micro-aggregations, or by an effect on hemocyte differentiation in response to *Bt*.

In contrast, larvae fed antibiotic-treated diet and reared under normal conditions (An and *Bt*+An treatments) resulted in PO activity levels similar to those of untreated control, whereas *Bt* alone and An-SD treatments resulted in reduced PO activity. Moreover, insects pretreated with antibiotics and then exposed to *Bt* (*Bt*+An-SD treatment) had the lowest PO activity among all treatments, which also was observed if eggs were presterilized and larvae were exposed to *Bt*. The contrasting effect observed between hemocytes (cellular response) and PO activity (humoral response) (James and Xu, 2012) may be attributed to the physiological cost of immune regulation, similar to that reported in other Lepidoptera exposed to *Bt* (Caron and Myers, 2008; Gassmann et al., 2009a, b), their toxins (Carrière et al., 2010; Tamez-Guerra, 2010) or nonpathogenic bacteria (Freitak et al., 2007).

Our results indicated that the expression of *hemolin* was lower among *Bt*-treated larvae compared with untreated control. Similarly, *hemolin* expression was also downregulated in

larvae exposed to antibiotics. These results were consistent with those reported by Wan et al. (2013) in *Bombyx mori* L., where *hemolin* expression was reduced by most Gram-positive (such as *Bt*) and negative bacteria (such as *Escherichia coli*). Bacterial infections and/or *Bt* exposure may cause differential interspecies responses. The ability of *Bt* to reduce the basal immune response in insects has been previously described (Dalhammar and Steiner, 1984; Fedhila et al., 2002); the effect has been related to an exoprotease A inhibitor, which degrades the antimicrobial peptides attacins and cecropins.

In *P. interpunctella* larvae reared from sterilized eggs, *hemolin* gene expression was not detected and larvae did not progress to pupae, but remained as 5th instar for more than one month. These data are in agreement with the observation that *hemolin* is involved in the metamorphosis process from larva to pupa (Aye et al., 2004; Bettencourt et al., 2000). Our data suggested that gut bacteria may influence the expression of *hemolin*. Yu and Kanost (1999) demonstrated that *hemolin* is primarily expressed in the fat body and gut epithelial tissue, and therefore expression may be influenced by gut bacteria (Freitak et al., 2007; Noverr and Huffnagle, 2004). In fact, bacteria, rather than viral infections, upregulate *hemolin* (Tereniu et al., 2005; Aye et al., 2004; Roxstrom-Lindquist et al., 2005).

It is important to note that the method of bacterial elimination (by larval feeding of antibiotics or by presterilization of eggs) resulted in different immune responses. The number of hemocytes was an order of magnitude higher in insects presterilized as eggs and reared on a sterile diet, compared to larvae fed antibiotic-treated diet. Conversely, the phenoloxidase activity was lower in presterilized insects reared on sterile diet compared to larvae fed antibiotic treated diet, especially when in addition larvae were exposed to *Bt*. We speculate that immune

responses, as well as other biological traits, are greatly altered in larvae deprived of gut bacteria from the embryo stage, and these observations require further study.

The gut bacteria of *P. interpunctella* do not resemble reports of bacterial communities in other lepidopteran species. For instance, in *Helicoverpa armigera* (Hübner) and *Spodoptera littoralis* (Boisduval), the highest bacterial percentage reported belonged to *Clostridium* and *Enterococcus* genus, with 42.2% and 42.3%, respectively (Tang *et al.*, 2012). In *Pieris rapae* L., most of detected bacteria belonged to *Methylobacteria* (Robinson *et al.*, 2010), found in our study in *P. interpunctella* was only 16% of total bacteria, whereas 9% of the sequenced clones were identified as *Propionibacterium acnes*. This bacterium has been also reported in *S. littoralis* and *H. armigera* (Tang *et al.*, 2012), and was also reported by Ranjith *et al.* (2016) as one of the largest species detected in *H. armigera*. *P. acnes* reportedly produce lactic acid, propionic acid and acetic acid from glucose (Piveteau, 1999). These compounds may reduce susceptibility to *Bt* by lowering the pH in the gut environment, affecting Cry toxin solubility, although this hypothesis remains to be tested.

In this study, the most abundant bacteria belonged to the *Pseudomonas* genus, with 35%, and phylogenetically identified as *Pseudomonas stutzeri*, species reported to have denitrifying activity and capable of carbon tetrachloride degradation after warehouse insecticide was used (Sepulveda-Torres *et al.*, 1999). This could explain how the bacterium colonized the gut of an insect pest of warehouse products. In addition, *P. stutzeri* has been isolated from the beetle *Costelytra zealandica* gut (Ray *et al.*, 2007) and identified by biochemical tests in the lepidopteran *Cydia pomonella* L. (Ertürk and Demirba 2006) as well as in *Rothschildia lebeau* pupae (Pinto-Tomás *et al.* 2007).

In summary, we found that *Bt* toxicity was significantly decreased when *P. interpunctella* larvae were deprived of gut bacteria. These observations have important implications in how *Bt* toxins kill lepidopteran larvae and may be exploited for modifications of *Bt* toxin pest management strategies. In addition, since the lack of gut bacteria inhibits *hemolin* expression and prevents the *P. interpunctella* pupation process, bacterial content may be manipulated to potentially increase toxicity in this important lepidopteran pest as a new method of pest control.

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5. References

- Alvarez-Flores, M.P., Furlin, D., Ramos, O.H., Balan, A., Konno, K., Chudzinski-Tavassi, A.M. 2011. Losac, the first hemolin that exhibits procogulant activity through selective factor X proteolytic activation. *J. Biol. Chem.* 286, 6918-6928. DOI: 10.1074/jbc.M110.167718
- Aye, T., Lee, K.Y., Kwon, Y.J. 2004. Cloning of immune protein hemolin cDNA from the Indian meal moth, *Plodia interpunctella*, and its high induction during development and by bacterial challenge. *Entomol. Res.* 34, 269–275. DOI:10.1111/j.1748-5967.2004.tb00122.x
- Bettencourt, R., Assefa-Redda, Y., Faye, I. 2000. The insect immune protein hemolin is expressed during oogenesis and embryogenesis. *Mechanisms of Development.* 95, 301–304. DOI:10.1016/S0925-4773(00)00359-2
- Baer, B., Eubel, H., Taylor, N.L., O'Toole, N., Millar, A.H. 2009. Insights into female sperm storage from the spermathecal fluid proteome of the honeybee *Apis mellifera*. *Genome Biol.* 10(6), R67. DOI:10.1186/gb-2009-10-6-r67
- Broderick, N.A., Raffa, K.F., Handelsman, J. 2006. Midgut bacteria required for *Bacillus thuringiensis* insecticidal activity. *PNAS-USA.* 103,15196–15199. DOI:10.1073/pnas.0604865103
- Broderick, NA, Raffa KF, Handelsman J. 2010. Chemical modulators of the innate immune response alter gypsy moth larval susceptibility to *Bacillus thuringiensis*. *BMC Microbiol.* 10(129). DOI: 10.1186/1471-2180-10-129
- Caccia, S., Di Lelio, I., La Stora, A., Marinelli, A., Varricchio, P., Franzetti, E., Banyuls, N., Tettamanti, G., Casartelli, M., Giordana, B., Ferre, J., Gigliotti, S., Ercolini, D., Pennacchio, F. 2016. Midgut microbiota and host immunocompetence underlie *Bacillus thuringiensis* killing mechanism. *PNAS*, 113, 9486-9491. DOI: <http://doi.org/10.1073/pnas.1521741113>

- Carrière, Y., Crowder, D.W., Tabashnik, B.E. 2010. Evolutionary ecology of insect adaptation to Bt crops. *Evol. Appl.* 3, 561-573. DOI: 10.1111/j.1752-4571.2010.00129.x
- Caron, V., Myers, J.H. 2008. Positive association between resistance to *Bacillus thuringiensis* and overwintering survival of cabbage loopers, *Trichoplusia ni* (Lepidoptera: Noctuidae). *Bull. Entomol. Res.* 98, 317-322. DOI:10.1017/S0007485307005597.
- Dalhammar, G., Steiner, H. 1984. Characterization of inhibitor A, a protease from *Bacillus thuringiensis* which degrades attacins and cecropins, two classes of antibacterial proteins in insects. *Eur. J. Biochem.* 139,247-252. DOI:10.1111/j.1432-1033.1984.tb08000.x
- Dillon, R.J., Dillon, V.M. 2004. The gut bacteria of insects: nonpathogenic interactions. *Annu. Rev. Entomol.* 49, 71–92. DOI: 10.1146/annurev.ento.49.061802.123416
- Eleftherianos, L., Atri, J., Accetta, J., Castillo, J.C. 2013. Endosymbiotic bacteria in insects: Guardians of the immune system? *Front. Physiol.* 4, 1–10.
<http://doi.org/10.3389/fphys.2013.00046>.
- Ericsson, J.D., Janmaat, A.F., Lowenberger, C., Myers, J.H. 2009. Is decreased generalized immunity a cost of Bt resistance in cabbage loopers *Trichoplusia ni*? *J. Invertebr. Pathol.* 100,61-67. DOI:10.1016/J.JIP.2008.10.007
- Ertürk, O., Demirba, Z. 2006. Studies on bacterial flora and biological control agent of *Cydia pomonella* L. (Lepidoptera: Tortricidae). *African J. Biotechnol.* 5, 2081-2085. DOI: 10.4314/ajb.v5i22.55972
- Fabrick, J. A., J. E. Baker, and M. R. Kanost. 2003. cDNA cloning, purification, properties, and function of a β -1,3-glucan recognition protein from a pyralid moth, *Plodia interpunctella*. *Insect Biochem. Mol. Biology.* 33,579-594

- Fedhila, S., Nel, P., Lereclus, D. 2002. The InhA2 metalloprotease of *Bacillus thuringiensis* strain 407 is required for pathogenicity in insects infected via the oral route. *J. Bacteriol.* 184,3296–3304. DOI:10.1128/JB.184.12.3296-3304.2002
- Frank, J.A., Reich, C.I., Sharma, S., Weisbaum, J.S., Wilson, B.A., Olsen, G.J. 2008. Critical evaluation of two primers commonly used for amplification of bacterial 16S rRNA genes. *Appl. Environ. Microbiol.* 74, 2461-2470. DOI:10.1128/AEM.02272-07
- Frankenhuyzen, K., Liu, Y., Tonon, A. 2010. Interactions between *Bacillus thuringiensis* subsp. *kurstaki* HD-1 and midgut bacteria in larvae of gypsy moth and spruce budworm. *J. Invertebr. Pathol.* 103,124–31. DOI:10.1016/j.jip.2009.12.008.
- Freitak, D., Wheat, C.W., Heckel, D.G., Vogel, H. 2007. Immune system responses and fitness costs associated with consumption of bacteria in larvae of *Trichoplusia ni*. *BMC Biol.* 5(56). DOI:10.1186/1741-7007-5-56
- Gassmann, M., Grenacher, B., Rohde, B., Vogel, J. 2009a. Quantifying western blots: pitfalls of densitometry. *Electrophoresis.* 30,1845-1855. DOI:10.1002/elps.200800720
- Gassmann, A.J., Fabrick, J.A., Sisterson, M.S., Hannon, E.R., Stock, S.P., Carrière, Y., Tabashnik, B.E. 2009b. Effects of pink bollworm resistance to *Bacillus thuringiensis* on phenoloxidase activity and susceptibility to entomopathogenic nematodes. *J. Econ. Entomol.* 102,1224-1232. DOI:http://dx.doi.org/10.1603/029.102.0348
- Hirai, M., Terenius, O., Li, W., Faye, I. 2004. Baculovirus and dsRNA induce *Hemolin*, but no antibacterial activity, in *Antheraea pernyi*. *Insect Molecular Biology*, 13(4): 399–405. DOI:10.1111/j.0962-1075.2004.00497.x
- James, R.R., Xu, J. 2012. Mechanisms by which pesticides affect insect immunity. *J. Invertebr. Pathol.* 109,175-182. DOI:10.1016/j.jip.2011.12.005.

- Johnston, P.R., Crickmore, N. 2009. Gut bacteria are not required for the insecticidal activity of *Bacillus thuringiensis* toward the tobacco hornworm, *Manduca sexta*. *Applied and Environmental Microbiology*, 75,5094–5099. <http://dx.doi.org/10.1128/AEM.00966-09>
- Kim, C-H., Park, J-W., Ha, N-C, Kang, H.J., Lee, B.L. 2008. Innate immune response in insects: recognition of bacterial peptidoglycan and amplification of its recognition signal. *BMB reports*, 41,93-101. DOI:10.5483/BMBRep.2008.41.2.093.
- Lavine, M.D., Strand, M.R. 2002. Insect hemocytes and their role in immunity. *Insect Biochem. Mol. Biol.* 32,1295-1309. DOI:10.1016/S0965-1748(02)00092-9
- Liu, Y.B., Tabashnik, B.E., Moar, W.J., Smith, R.A. 1998. Synergism between *Bacillus thuringiensis* spores and toxins against resistant and susceptible diamondback moths (*Plutella xylostella*). *Appl. Environ. Microbiol.* 64,1385–1389.
<http://aem.asm.org/content/64/4/1385.short>
- McGaughey, W.H., Beeman, R.W. 1988. Resistance to *Bacillus thuringiensis* in colonies of Indian meal moth and almond moth (Lepidoptera: Pyralidae). *J. Econ. Entomol.* 81,28-33. DOI:10.1093/jee/81.1.28
- Mohandass, S., Arthurb, F.H., Zhuc, K.Y., Throneb, J.E. 2007. Biology and management of *Plodia interpunctella* (Lepidoptera: Pyralidae) in stored products. *J. Econ. Entomol.* 43,302-311. DOI:10.1016/J.JSPR.2006.08.002
- Noverr, M.C., Huffnagle, G.B. 2004. Does the microbiota regulate immune responses outside the gut?. *Trends Microbiol.* 12,562-568. doi:10.1016/j.tim.2004.10.008
- Núñez-Mejía, G., Valadez-Lira, J.A., Gomez-Flores, R., Rodríguez-Padilla, C., Tamez-Guerra, P. 2016. *Trichoplusia ni* (Lepidoptera: Noctuidae) survival, immune response, and gut

- bacteria changes after exposure to *Azadirachta indica* (Sapindales: Meliaceae) volatiles. Flo. Entomol. 99,12-20. <http://dx.doi.org/10.1653/024.099.0104>
- Oppert, B., Kramer, K.J., Beeman, R.W., Johnson, D., McGaughey, W.H. 1997. Proteinase-mediated resistance to *Bacillus thuringiensis* insecticidal toxins. J. Biol. Chem. 272, 23473-23476. DOI:10.1074/jbc.272.38.23473
- Oppert, B, Kramer, K.J., Johnson, D.E., Upton, S.J., McGaughey, W.H. 1996. Luminal proteinases from *Plodia interpunctella* and the hydrolysis of *Bacillus thuringiensis* CryIA(c) protoxin. Insect Biochem. Mol. Biol. 26,571-583. DOI:10.1016/S0965-1748(96)00013-6
- Paramasiva, I, Sharma, H.C., Krishnayya, P.V. 2014. Antibiotics influence the toxicity of the delta endotoxins of *Bacillus thuringiensis* towards the cotton bollworm, *Helicoverpa armigera*. BMC Microbiol. 14(1),17. <http://doi.org/10.1186/1471-2180-14-200>
- Pinto-Tomás, A., Uribe-Lorío, L., Blanco, J., Fontecha, G., Rodríguez, C., Mora, M., Janzen, D., Chavarría, F., Díaz, J., Sittenfeld, A. 2007. Actividades enzimáticas en aislamientos bacterianos de tractos digestivos de larvas y del contenido de pupas de *Automeris zugana* y *Rothschildia lebeau* (Lepidoptera: Saturniidae). Rev. Biol. Trop. 55, 401-415. DOI: <http://dx.doi.org/10.15517/rbt.v55i2.6020>
- Piveteau, P. 1999. Metabolism of lactate and sugars by dairy propionibacteria: A review. Lait. 79, 23-41. DOI: 10.1051/lait:199912
- Ranjith, M.T., ManiChellappan, Harish, E.R., Girija D., Nazeem, P.A. 2016. Bacterial communities associated with the gut of tomato fruit borer, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) based on Illumina Next-Generation Sequencing. Journal of Asia-Pacific Entomology, (19)2: 333–340. <http://dx.doi.org/10.1016/j.aspen.2016.03.007>

- Ray, J.L., Andersen, H.K., Young, S., Nielsen, K.M., O'Callaghan, M. 2007. An assessment of the potential of herbivorous insect gut bacteria to develop competence for natural transformation. *Environ. Biosafety Res.* 6, 135–147. DOI:10.1051/eb:2007032
- Robinson, C.J., Schloss, P., Ramos, Y., Raffa, K.F., Handelsman, J. 2010. Robustness of the bacterial community in the cabbage white butterfly larval midgut. *Microbial Ecol.* 59, 199–211. DOI: 10.1007/s00248-009-9595-8
- Roxstrom-Lindquist, K, Assefaw-Redda Y, Rosinska K, Faye I. 2005. 20-Hydroxy-ecdysone indirectly regulates hemolin gene expression in *Hyalophora cecropia*. *Insect Mol. Biol.* 14, 645–652. DOI:10.1111/j.1365-2583.2005.00593.x
- Sepulveda-Torres, L., Rajendran, N. Dybas, M.J., Criddle, C.S. 1999. Generation and initial characterization of *Pseudomonas stutzeri* KC mutants with impaired ability to degrade carbon tetrachloride. *Arch. Microbiol.* 171, 424–429. DOI:10.1007/s002030050729
- SPSS. 2008. Version 17.0. SPSS Inc., an IBM Company. Chicago, Illinois
- Tamez-Guerra, P. 2010. A review of U.S. and Mexican cooperation to develop insect resistance management and monitoring methods for surveying transgenic crops expressing *Bacillus thuringiensis* proteins: 2003 to 2010. *Southwest. Entomol.* 35,373-382.
- Tamez-Guerra, P., Damas, G., Iracheta, M.M., Oppert, B., Gomez-Flores, R., Rodríguez-Padilla, C. 2006. Differences in susceptibility and physiological fitness of Mexican field *Trichoplusia ni* strains exposed to *Bacillus thuringiensis*. *J. Econ. Entomol.* 99,937-945. DOI:10.1093/jee/99.3.937
- Tamez-Guerra, P., Valadez-Lira, J.A., Alcocer-González, J.M., Oppert, B., Gomez-Flores, R., Tamez-Guerra, R.S., Rodríguez-Padilla, C. 2008. Detection of genes encoding

- antimicrobial peptides in Mexican strains of *Trichoplusia ni* (Hübner) exposed to *Bacillus thuringiensis*. J. Invertebr. Pathol. 98,218-227. DOI:10.1016/j.jip.2008.02.008
- Tan, H.Y., Ng, T.W. 2008. Accurate step wedge calibration for densitometry of electrophoresis gels. Optics Comm. 281,3013-3017. DOI:10.1016/j.optcom.2008.01.012
- Tang, X., Freitak, D., Vogel, H., Ping, L., Shao, Y., Cordero, E.A., Andersen, G., Westermann, M., Heckel, D.G., Boland, W. 2012. Complexity and variability of gut commensal microbiota in polyphagous lepidopteran larvae. PloS one, 7(7): e36978. <http://dx.doi.org/10.1371/journal.pone.0036978>
- Terenius, O., Popham, H.J.R., Shelby, K.S. 2009. Bacterial, but not baculoviral infections stimulate Hemolin expression in noctuid moths. Dev. Comp. Immunol. 33,1176–1185. DOI:10.1016/j.dci.2009.06.009.
- Valadez-Lira, J.A., Alcocer-Gonzalez, J.M., Damas, G., Nuñez-Mejía, G., Oppert, B., Rodriguez-Padilla, C., Tamez-Guerra, P. 2012. Comparative evaluation of phenoloxidase activity in different larval stages of four lepidopteran pests after exposure to *Bacillus thuringiensis*. J. Insect Sci. 12.80. DOI:10.1673/031.012.8001.
- Visweshwar, R., Sharma, H.C., Akbar, S.M.D., Sreeramulu, K. 2015. Elimination of gut microbes with antibiotics confers resistance to *Bacillus thuringiensis* toxin proteins in *Helicoverpa armigera* (Hübner). Appl. Biochem. Biotechnol. 177,1621–1637. DOI:10.1051/laib:199912
- Vizioli, J, Salzet, M. 2002. Antimicrobial peptides from animals: focus on invertebrates. Trends Pharmacol. Sci. 23,494-496. DOI:10.1016/S0165-6147(02)02105-3
- Wan, J., Zhou, X., Zhou, X. 2013. A review of innate immunity of silkworm, *Bombyx mori*. African J. Agr. Res. 8,2319–2325. DOI: 10.5897/AJARx11.079

- Wang, Q, G. M. Garrity, J. M. Tiedje, and J. R. Cole. 2007. Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73, 5261-5267. DOI: 10.1128/AEM.00062-07
- Yu, X.Q., Kanost, M.R. 1999. Developmental expression of *Manduca sexta* hemolin. *Arch. Insect Biochem. Physiol.* 42.198–212. DOI:10.1002/(SICI)1520-6327(199911)42:3<198::AID-ARCH4>3.0.CO;2-G

Table 1. Bacterial species identified in guts of *Plodia interpunctella* larvae feeding on artificial diet, identified at the genus level using 16S rRNA primers.¹

Treatments	Control	With antibiotics cocktail	Under sterile rearing conditions
Bacterial species detected	<i>Bacillus</i> sp.		
	<i>Methylobacterium</i> sp.		
	<i>Parapusillimonas</i> sp.		
	<i>Achromobacter</i> sp.	none detected	none detected
	<i>Stenotrophomonas</i> sp.		
	<i>Pseudomonas</i> sp.		
	<i>Propionibacterium</i> sp.		
	<i>Corynebacterium</i> sp.		

¹Bacterial classification was with the Ribosomal Database Project (<http://rdp.cme.msu.edu/>; Wang et al., 2007)

Figure legends

Figure 1. Mortality of 3rd instar *Plodia interpunctella* larvae fed on treated or untreated agar-based diet. A) Control: untreated larvae; *Bt*: larvae exposed to *Bt* only; *Bt*+*An*: larvae exposed to *Bt* and antibiotics; *Bt*+*An*-SD: larvae exposed to antibiotics for 24 h and subsequently exposed to *Bt* only; *An*: larvae exposed to antibiotics only; *An*-SD: larvae exposed to antibiotics for 24 h and subsequently transferred to untreated diet. B) Control: untreated larvae; *Bt*: larvae exposed to *Bt* only; *Bt*-SD: larvae from hypochlorite sterilized eggs and reared under sterile conditions and exposed to antibiotics for 24 h and subsequently exposed to *Bt* only. Bars indicate S.E., and different letters above each column indicate statistical significance by Tukey HSD, $\alpha = 0.05$.

Figure 2. Effects of different treatments on hemocytes from hemolymph of 3rd instar *Plodia interpunctella* larvae reared on agar-based diet. A) Larvae reared under normal conditions (see treatment information in Fig. 1A); B) Larvae reared under sterile (SD) conditions (see treatment information in Fig. 1B). Bars indicate S.E., and different letters above each column indicate significance by Tukey HSD, $\alpha = 0.05$.

Figure 3. Effects of different treatments on phenoloxidase activity (U/ μ L) from hemolymph of 3rd instar *Plodia interpunctella* larvae reared on agar-based diet. A) Under normal conditions (see treatments information in Fig. 1A); B) Under sterile (SD) conditions (see treatments information in Fig. 1B). Bars indicate S.E., and different letters above each column indicate significance by Tukey HSD, $\alpha = 0.05$.

Figure 4. Effects of different treatments on *hemolin* detection from the gut of *Plodia interpunctella* 3rd instar larvae. A) Larvae reared under normal conditions (see treatments information in Fig. 1A); B) Larvae reared under normal (*Bt*) or sterile (*Bt*-SD) conditions (see treatments detail in Fig. 1B).

Figure 1.

Figure 2

Figure 3

b

Figure 4

A

B