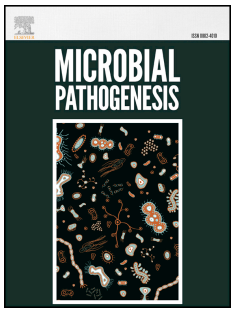

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PII: S0882-4010(22)00571-X

DOI: https://doi.org/10.1016/j.micpath.2022.105958

Reference: YMPAT 105958

To appear in: *Microbial Pathogenesis*

Received Date: 11 October 2022

Revised Date: 3 December 2022

Accepted Date: 21 December 2022

Please cite this article as: Grizanova EV, Krytsyna TI, Kalmykova GV, Sokolova E, Alikina T, Kabilov M, Coates CJ, Dubovskiy IM, Virulent and necrotrophic strategies of *Bacillus thuringiensis* in susceptible and resistant insects, *Galleria mellonella*, *Microbial Pathogenesis* (2023), doi: https://doi.org/10.1016/j.micpath.2022.105958.

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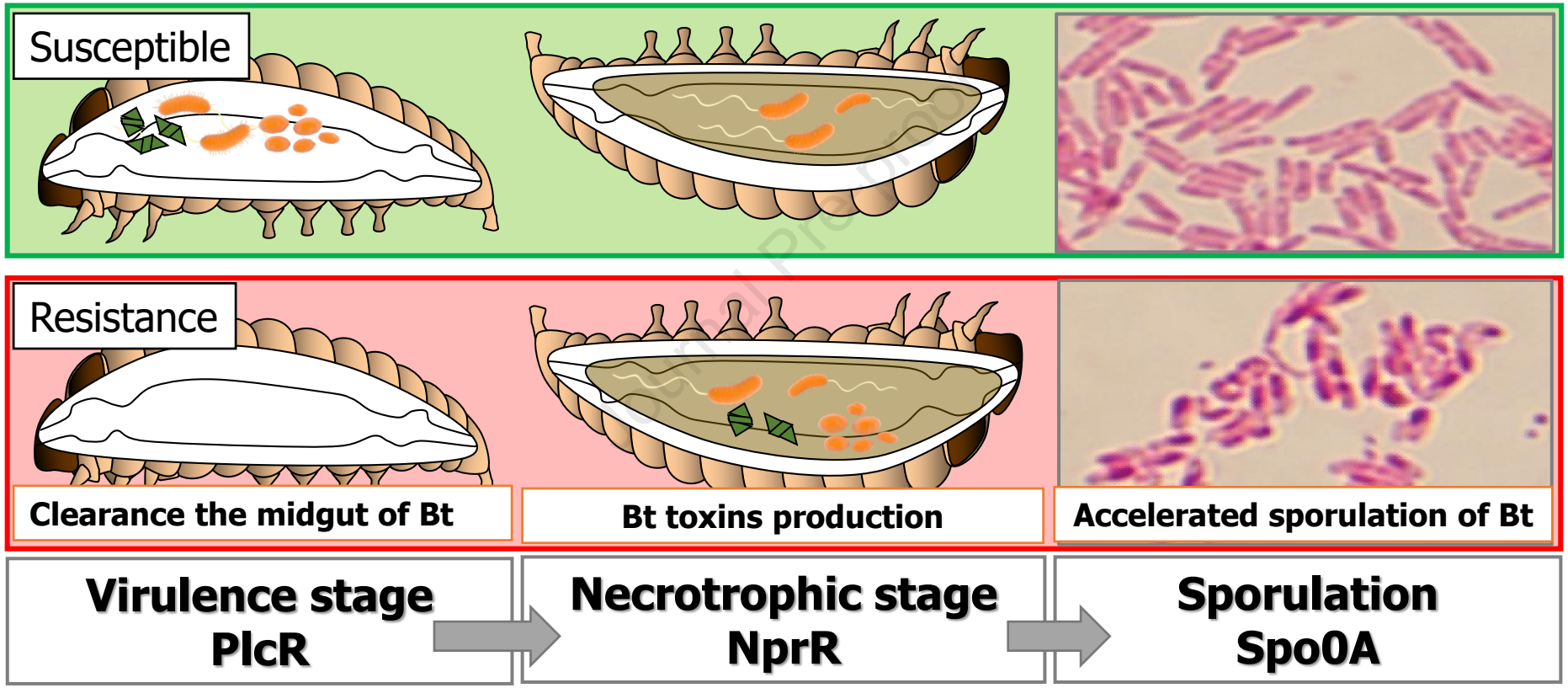

Virulent and necrotrophic strategies of *Bacillus thuringiensis* in susceptible and resistant insects, *Galleria mellonella*

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Abstract

Bacillus thuringiensis (*Bt*) is one of the most common entomopathogenic bacteria used as a biopesticide, and source of endotoxin genes for generating insect-resistant transgenic plants. The mechanisms underpinning an insect's susceptibility or resistance to *B. thuringiensis* are diverse. The bacterial lifecycle does not end with the death of a host, they continue to exploit the cadaver to reproduce and sporulate. Herein, we studied the progression of *B. thuringiensis* subsp. *galleriae* infection in two populations of wax moth larvae (*Galleria mellonella*) to gain further insight into the "arms race" between *B. thuringiensis* virulence and insect defences. Two doses of *B. thuringiensis* subsp. *galleriae* (spore and crystalline toxin mixtures) were administered orally to compare the responses of susceptible (S) and resistant to *Bt* (R) populations at ~30% mortality each. To investigate *B. thuringiensis*-insect antibiosis, we used a combination of *in vivo* infection trials, bacterial microbiome analysis, and RNAi targeting the antibacterial peptide gloverin. Within 48 hours post-inoculation, *B. thuringiensis*-resistant insects purged the midgut of bacteria, i.e., colony forming unit numbers fell below detectable levels. Second, *B. thuringiensis* rapidly modulated gene expression to initiate sporulation (linked to quorum sensing) when exposed to resistant insects in contrast to susceptible *G. mellonella*. We reinforce earlier findings that elevated levels of antimicrobial peptides, specifically gloverin, are found in the midgut of resistant insects, which is an evolutionary strategy to combat *B. thuringiensis* infection via its main portal of entry. A sub-population of highly virulent *B. thuringiensis* can survive the enhanced immune defences of resistant *G. mellonella* by disrupting the midgut microbiome and switching rapidly to a necrotrophic strategy, prior to sporulation in the cadaver.

Keywords: virulence factors; RNAi; gloverin; midgut microbiome; cadavers; innate immunity; necrobiology

Introduction

Bacillus thuringiensis (Bt) is one of the most common entomopathogenic bacteria used for biopesticides (natural alternatives to chemical pesticides) and as a source of endotoxin genes for developing transgenic plants with enhanced resistance toward insect pests [1,2]. Throughout the lifecycle of *Bt*, the bacterium produces a spectrum of major and minor virulence factors controlled by quorum sensing (QS) [3,4]. The main virulence factors are crystalline (Cry) toxins (endotoxin) produced as parasporal inclusions during sporulation, and are regulated by the sporulation factor Spo0A. Oral ingestion is the main route of bacterial entry into insects. Cry-toxins damage the midgut cells, making them leaky and resulting in septicemia and insect death. Additional virulence factors (cytotoxins, hemolysins, enterotoxins, phospholipase C) produced by vegetative cells are under the control of the transcription factor phospholipase C regulator (PlcR) in the infectious stage of *Bt*'s lifecycle. These proteins play key roles in nutrient acquisition (food supply) and virulence, cell protection and environment-sensing [4]. The necrotrophic regulator (NprR) regulon controls necrotrophic properties, including a suite of genes encoding for degradative enzymes (proteases, lipases, chitinases) and a lipopeptide (kurstakin) involved in biofilm formation, which allow bacteria to survive in the cadavers of septic insects [1]. Bacteria dwelling in the cadaver propagate until they exhaust all available organic material, then transition to sporulation. Virulent, necrotrophic and sporulation strategies during bacterial colonization of insects have been investigated with mutant strains of *B. cereus* and *Bt* deficient in genes associated with QS [4,5].

Larvae of the greater wax moth, *Galleria mellonella* (Lepidoptera, *Pyralidae*), represent an emerging *in vivo* model for studies of innate immunity and toxicology [6,7], virulence of zoonotic and human pathogens [8], and insect-entomopathogen interactions [9–12]. The population of *G. mellonella* selected for resistance to *Bt* subsp. *galleriae* under laboratory conditions has been studied for immuno-physiological adaptations and epigenetic mechanisms [13,14]. The mechanisms underpinning an insect's susceptibility or resistance to *Bt* are diverse. One of the most common insect resistance mechanism is mutation in the genes of receptors on the epithelial cells of the gut – disabling the Cry toxin mode of action [15]. Other ways of forming resistance are through the activation of

systemic and local immune responses [16–18]. Local immune responses and the production of reactive oxygen species (ROS) in the midgut represent the first line of defense against *Bt* infection, especially the synthesis of antimicrobial peptides (AMPs) [19,20]. The role of host microbiota in *Bt* pathogenicity is a controversial topic, however, their participation in septicemia during *Bt* infection is clear [19,21,22]. When Cry-toxins damage the midgut cells, the microbial consortium can switch from asymptomatic gut symbionts to haemocoelic pathogens, leaking into the haemolymph and causing septicemia, which enhances the virulence of *Bt* [23,24]. When *Bt* overwhelm the gut barrier and immune defences, the insect host will die, yet the bacteria continue to exploit the cadaver to further propagate and sporulate until all resources have been spent [25]. Little is known about Bt development and survival in the cadaver of a seemingly resistant host.

The overall aim of this study was to enhance our knowledge of the putative strategies used by *B. thuringiensis* subsp. *galleriae* when colonizing two distinct populations of *G. mellonella*, namely those considered susceptible or resistant to *B. thuringiensis* bacterial infection. To achieve this aim, we (1) monitored the expression of host immune factors and bacterial genes associated with virulence, necrotrophy and sporulation, and (2) profiled the midgut bacteriomes of infected insects and their cadavers. Additionally, we used RNAi targeting the antibacterial peptide, gloverin, to highlight its mechanistic role in driving antibacterial resistance in *G. mellonella* larvae.

2. Material and Methods

2.1. *Galleria mellonella* and *Bacillus thuringiensis* infection trials

Laboratory populations of *G. mellonella* from the Novosibirsk State Agricultural University (NASU) were reared in strict isolation at 28°C, 60% relative humidity, with a 12:12 hour light: dark cycle, and maintained using a honey-based diet as described in [26]. Selection of insects demonstrating resistance to *Bt* subsp. *galleriae* was performed over 40 generations as described previously by Dubovskiy et al. (2016) [13]. Full details of the selection process are provided in the online supplementary information (SI, Method 1).

The original *Bacillus thuringiensis* subsp. *galleria* strain GM-1 is maintained by the NASU [26], and was used to breed bacteria-resistant insects [as stated in 13], and for the infection trials of the current study. Fourth instar larvae of resistant ‘R’ and susceptible ‘S’ cohorts were starved for 2 h before exposure to *Bt*. Insects were infected

orally using gavage (force feeding) and a 30-gauge hypodermic needle attached to a syringe pump (KDS 100, KD Scientific). Two doses of *Bt* were used to achieve ~30% mortality in both populations of *G. mellonella* – 5×10^5 bacteria per 'S' larva and 2×10^6 bacteria per 'R' larva. Post-infection, larvae were placed in Petri dishes with continuous access to the artificial diet, and monitored for five days.

Independently, the midgut bacterial community and immune responses of S and R insects during *Bt* infection were compared at the same mortality level (i.e., LC_{30}). Moreover, uninfected and *Bt*-infected larvae from both S and R lines were collected at 48 hours post-exposure and dissected for midgut tissues to (1) profile the resident bacterial community (n = 3 per treatment; each representing a pool of 5 larvae), (2) quantify candidate gene expression in the midgut (n = 7 larvae per treatment; each representing a pool of 3 larvae), and (3) quantify *Bt* colony forming units (CFUs; (n = 9 larvae per treatment). The experimental sampling time was chosen carefully to reflect the acute stages of *Bt* infection and concomitant immune defences in living insects. Cadavers of R and S insects (infected and control) were collected at 3 hours *post-mortem* – representing the early stage(s) of *Bt* in a dead host – to (1) quantify gene expression of *Bt* (n = 5 per treatment; 1 cadaver per replicate), and (2) determine the bacterial community composition (n = 5 per treatment; 1 larva per replicate). Further details on the experimental design, bacterial culture technique and inoculation methods are provided in the online supplementary information (SI, Figure S1; Methods 2 and 3).

2.2. qRT–PCR analysis of insect gene expression in the midgut

Candidate gene expression levels (mRNAs) were quantified in the midgut tissues of both R and S larvae in the absence (control) and presence of *Bt* infection. Four key genes were investigated, three encoding the antimicrobial peptides galiomicin, gloverin, and cecropin D, and one for the insect metalloproteinase inhibitor (IMPI) [13]. Gene expression was measured quantitatively using reverse transcription (RT)-PCR based on normalised cDNA samples, the CFX96 Real-Time PCR detection system (Biorad) with BioMaster HS-qPCR SYBR Blue (2x) (Biolabmix, Russia) and two reference genes, 18S rRNA (AF286298) and Elongation Factor 1-alpha (EF1; AF423811). Further details of sample preparation, RNA extraction, cDNA synthesis and RT-PCR are provided in the online supplementary information (SI, Method 4).

2.3. qRT–PCR analysis of *Bt* gene expression in the cadavers of R and S insects

Differential expression levels of a suite of 18 genes – related to the virulent, necrotrophic and sporulation stages of *Bt* – were investigated in both S and R line insects. Oligonucleotides (PCR primers) were designed using the Primer Quest and Oligo Analyzer Tools available from Integrated DNA Technologies (https://eu.idtdna.com). A complete listed of candidate genes is provided in the online supplementary information (Table S2). Gene expression was measured by qRT-PCR as stated in section 2.2, but instead using mdh (CP010089.1) as a reference gene (SI, Methods 5).

2.4. RNA interference of the antibacterial peptide, gloverin, in *Galleria mellonella*

Insects were surface sterilized with 70% EtOH prior to intrahaemocoelic injection of dsRNA through the last left (ventral) pro-leg: either 5 µL dsGlo (treatment) suspended in DEPC-treated water (100 ng per larvae; n = 100), 5 µL dsLuc (negative control; 100 ng per larvae; n = 100), or with 5 µL of DEPC-treated water only (blank, n = 100) using a Hamilton microsyringe (Burkard, USA). Approximately 24 hours later, insects were starved (for 2 h) prior to inoculation with 2×10^4 (spores/crystal) *Bt* subsp. *galleriae* per larva via force feeding. Insect survival was recorded over five days. Further details of the gene targets and *in vitro* dsRNA synthesis are provided in the online supplementary information (SI, Method 6).

2.5. Microbiome analysis of the midgut and cadavers

Taxonomic profiles of bacteria from *Bt*-challenged S and R insects (*G. mellonella*) were gathered using 16S (V3-V4) rDNA meta-barcoding of tissues at 48-hours post-infection. Midgut tissues were dissected from surface-sterilized larvae (n = 3, each consisting of 5 pooled insects) and entire cadavers (n = 5, each consisting of 5 pooled insects) were snap-frozen in liquid nitrogen prior to gDNA extraction. DNA was isolated using the DNeasy Power Soil Kit (Qiagen, Hilden, Germany) and tissues were homogenized using a Tissue Lyser II (Qiagen) for 10 min at 30 Hz. The V3-V4 region of the 16S rRNA was amplified using established primers, 343F and 806R [27]. The 16S libraries were sequenced with 2×300 bp paired-ends reads on MiSeq (Illumina, San Diego, CA, USA) in SB RAS Genomics Core Facility (ICBFM SBRAS, Novosibirsk, Russia). The MiSeq data were deposited in GenBank under the accession number PRJNA832608. Further details on raw sequence analysis and filtering, bioinformatics and OTU analysis can be found in the online supplementary information (SI, Method 7).

2.6. Quantifying *Bt* spores and vegetative cells

Total numbers of *Bt* spores and vegetative cells were detrmined from CFUs in the midgut (n = 5) and larval cadavers (n = 5) by plating samples on a specilaist agar QMAFAnM (DIA-M, Russia) for 72 h at 28 °C. Inspection of colony morphology of *Bt* cells was carried out using compound microscopy and eoisin staining [2]. Full details of the characterization of *Bt* CFU contents are provided in the online supplementary information (SI, Method 8).

2.7. Data analyses

Data are presented as mean values ± standard error (SE) with all statistical analyses performed in GraphPad Prism v8 (San Diego, CA, USA). Data were checked for normality using the D'Agostino–Pearson omnibus test, and if non-normal, conservative non-parametric tests were applied. Kruskal–Wallis analysis (with Dunn's multiple comparisons) was used to determine differences between *Bt* virulence factor gene expression (qRT-PCR analysis), *Bt* CFU numbers in insects, and richness/evenness of the bacterial consortia. Midgut and cadaver microbiota from R and S larvae post *Bt* treatment were compared using two-way ANOVA with Tukey's multiple comparisons test (for abundance). Moreover, two-way ANOVA with Sidak's multiple comparisons test was used to asses differences in insect gene expression. Proportional hazards regression (Log-rank, Mantel–Cox tests) with Bonferroni correction was used for survival/mortality data.

3. Results

3.1. Susceptibility of insect lines to *Bt* infection

A tractable line of wax moths (*G. mellonella*) was selected for resistance to *B. thuringiensis* subsp. *galleriae* over 40 generations. By the 40th generation, and based on LC_{50} data, the resistance ratio (RR) to *Bt* for the resistant (R) larvae relative to a susceptible (S) larvae was 10. *Bacillus thuringiensis* spores and crystals contribute synergistically to *G. mellonella* mortality but the precise mechanism(s) remains unclear [27]. After 40 generations of selection, the RR may seem low at 10, but this could be due to the insects investing in defences that target spores and Cry toxins simultaneously, rather than spores or Cry-toxins alone (their combined effects are often overlooked). Mortality levels peaked at 60% for S larvae exposed to 2×10^6 bacteria, which is significantly higher than those infected with the lower dose of 5×10^5 (X^2 =

29.86; p < 0,0001) and uninfected (control; X^2 = 139.3; p < 0.0001) over the experimental period (SI Figure S2, Figure 1). A similar trend was observed for R larvae exposed to 2 × 10^6 and 5 × 10^5 bacteria (35% and 10% mortality respectively; X^2 = 53.62; p < 0.0001) and uninfected (control; X^2 = 78.57; p < 0.0001 for 2 × 10^6 and X^2 = 13.9; p = 0.0002 for 5 × 10^5). Moreover, S larvae were significantly more susceptible to either bacterial dose than R larvae (treatment with 2 × 10^6; X^2 = 40.43; p < 0.0001 and treatment with 5 × 10^5; X^2 = 69.35; p < 0.0001). The lower dose (5 × 10^5) of *Bt* was sufficient to kill ~36% of S larvae (X^2 = 65.74; p < 0.0001 vs uninfected (control)), whereas the higher dose (2 × 10^6) killed approximately the same number (~33%) of R larvae (Figure 1). These two doses of *Bt* subsp. *galleriae* (5 × 10^5, 2 × 10^6 spore and crystalline toxin /larva) were used for the remaining experiments to compare the responses of susceptible (S) and resistant (R) insect lines at a similar level of lethality (i.e., LC_{30}). Mortalities for uninfected (control) insects were negligible (<1.5%) for both insect lines.

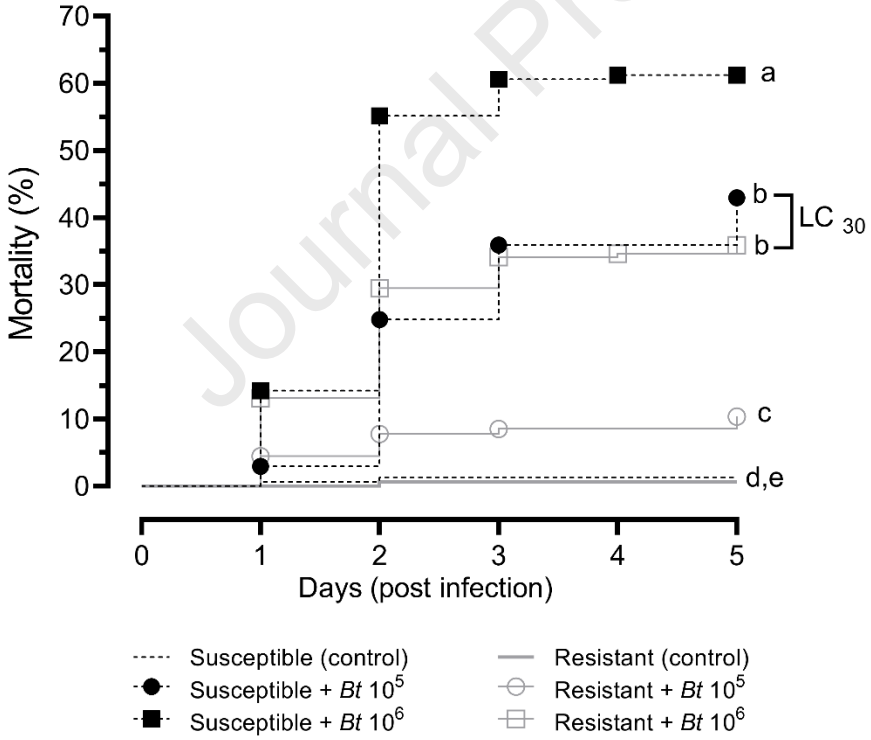

Figure 1. Mortality levels of susceptible (S) and resistant (R) *G. mellonella* line larvae following oral inoculation with *B. thuringiensis* subsp. *galleriae (Bt)*. The negative control consisted of administering PBS alone (no spores or toxins). Data were analyzed by comparing curves using Log-rank (Mantel-Cox) tests (n = 128-250 larvae per treatment). Survival of S and R lines was recorded over 5 days following exposure to the two different doses of *Bt*. Unshared letters represent significant differences (p < 0.01). LC_{30} values demonstrate similar mortality levels of S and R line larvae infected with 5 x 10^5 and 2 x 10^6 of *Bt*, respectively.

3.2. Expression of immune factors in the midgut of susceptible and resistant insects

Elevated transcript (mRNA) levels for genes encoding antimicrobial peptides (AMPs), notably >30-fold increase in gloverin, were detected in the midgut of uninfected larvae from the R line compared to the S line (Figure 2A). At 48 hours post infection (h.p.i.) with Bt, differential candidate gene expression for several immune factors were recorded (Figure 2B). Insect metalloproteinase inhibitor (IMPI), galiomicyn and cecropin D expression increased up to 10-fold for both S and R lines inoculated with *Bt*, whereas gloverin transcripts were upregulated almost 100-fold ($t = 3.184$; $df = 56.00$; $p < 0.01$) in the midgut of the R line and 10-fold in the S line (Figure 2B).

Treating R-line insects with dsRNA targeting gloverin, followed by a sub-lethal dose (2×10^4 per larva) of Bt decreased survival by 30%. The same dose of Bt led to <10% decline in insect survival levels when they had been injected with 100 ng of control dsRNA (luciferase), PBS or no injection – each of these were significantly different to the dsRNA gloverin treatment ($p < 0.01$ in all cases; Figure 2C; SI Table S1). In fact, gloverin-suppressed larvae were 3 to 3.7-fold more likely to die of bacteriosis.

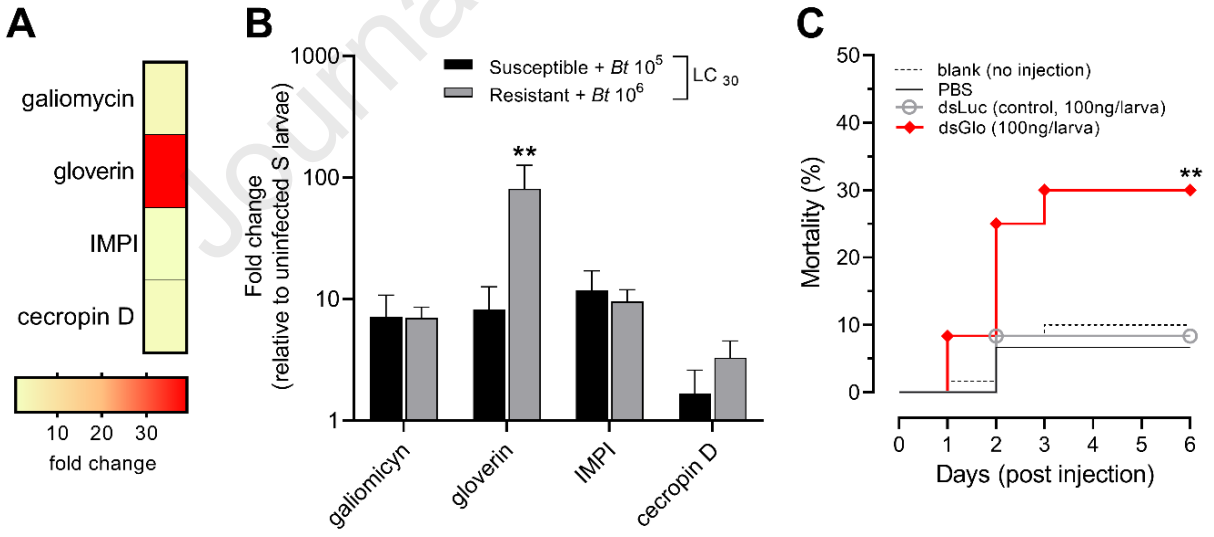

Figure 2. Gene expression (mRNAs) of immune factors in the midgut of susceptible (S) and resistant (R) *G. mellonella* when infected orally with *Bacillus thuringiensis*. **A)** Basal gene expression in uninfected R and S lines. **B)** Stimulated gene expression in R and S lines following oral inoculation with *Bt* (LC_{30}). Data are represented as fold changes relative to mRNA levels of the respective targets in S insects. Measurements were taken at 48 hours post-inoculation with *Bt*. Insect metalloproteinase inhibitor (IMPI), gloverin, galiomicyn and cecropin D. (** = $P < 0.01$; R versus S). **C)** RNAi-mediated knockdown of the antibacterial peptide, gloverin, in *G. mellonella* larvae. Survival of larvae (S line) infected with *Bt* (2×10^4 per larva) post injection with either PBS, control dsRNA (luciferase) or gloverin dsRNA. Asterisks

represent significant differences (** p < 0.01) between gloverin dsRNA (dsGlo) and other all other groups (at 6 days post injection).

3.3. Midgut microbiomes of susceptible and resistant insects infected with *Bacillus thuringiensis*

Classification of bacteria in the midgut of uninfected (control) wax moth larvae, S and R lines, based on 16S (V3/V4) rRNA meta-barcoding revealed communities dominated by only few taxa, with >98% represented by seven genera from six orders. Average relative abundances were calculated across all untreated, susceptible larvae: *Lactobacillales* (73 ± 11%), *Enterobacteriales* (8.2 ± 4.2%), *Oceanospirillales* (4.9 ± 1.4%), *Alteromonadales* (1.5 ± 0.7%), *Bacillales* (0.9 ± 0.6%), and *Pseudomonadales* (0.8 ± 0.4%) (Figure 3A). There were no gross differences in average relative abundances between untreated larvae from susceptible and resistant lines (Figure 3B). Oral infection of S larvae with *Bt* did not induce dramatic changes in the abundances of major midgut bacteria, *Enterococcus* and *Enterobacteriaceae* (Figure 3B). However, *Bt* infection in R larvae coincided with gross dysbiosis, such that *Enterococcus* levels in the midgut represented ~10% in contrast to ~86% in uninfected R larvae within 48 hours (Figure 3B; q = 19.07; df = 56; p < 0.0001). Some fluctuations in bacterial taxa were detected for *Enterobacteriaceae*, which increased in R larvae inoculated with *Bt* compared to uninfected (control) R group (Figure 3B; q = 20.83; df = 56; p < 0.0001). Midgut bacteria *Serratia, Halomonas* and *Shewanella,* of both insect lines remained stable in the absence/presence of *Bt*. *Bacillus thuringiensis* started to grow in the midgut of infected S insects, where they made-up ~22% compared to ~0.9% in uninfected S insects (q = 5.5; df = 56; p < 0.01). Interestingly, there was a significant reduction in bacterial diversity in the midgut of both the infected S (q = 6.98; df = 20; P < 0.01) and R (q = 4.96; df = 20; p < 0.05) lines compared to their uninfected counterparts (Figure 3C). Evenness indices of midgut bacteria from S and R lines were relatively unchanged during *Bt* infection (Figure 3D).

The total number of *Bt* in infected larvae was higher in S larvae, ~1 x10^5 colony forming units (Figure 4A). Vegetative cells in S larvae significantly outnumbered *Bt* spores (total *Bt* vs *Bt* spores; p = 0.0079), representing clear evidence of *Bt* germination in the midgut tissues of S larvae after an oral dose of the bacterium. *Bt* numbers were below detectable levels in the midgut of living R larvae (Figure 4A). Sporulation and crystal production of bacteria on specialist growth medium were lower for *Bt* collected from

cadavers of S larvae (1.2 ± 0.6% sporulated bacteria) compared to those from cadavers of the R-line (54.6 ±3.8% sporulated bacteria; p = 0.0079; SI Figure 3).

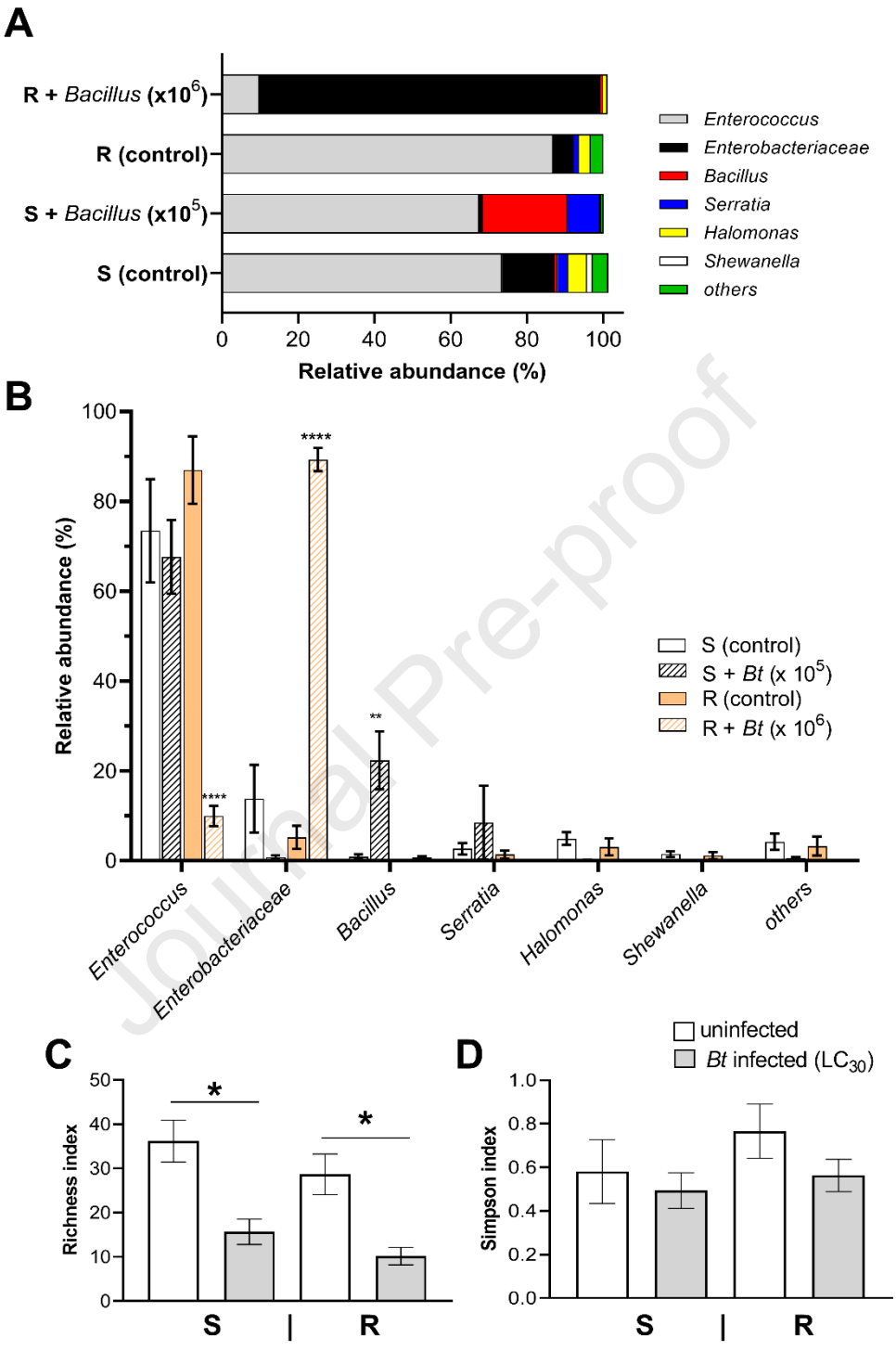

Figure 3. Bacterial microbiome (16S rRNA) profiles from the midgut of susceptible (S) and resistant (R) *Galleria mellonella* following infection (LC_{30}). DNA was extracted 48 hours post inoculation with *Bacillus thuringiensis*. (A) Bacterial abundances across biological replicates (n = 3 per treatment) according to genera. (B) Genus-level comparisons. (C) Diversity and (D) Evenness indices of bacterial communities in infected and uninfected larvae (R and S lines). Data represent mean ± SE. Asterisks denote significant differences: * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$ compared with other treatments within the same genus in (B), and compared with uninfected controls in (C).

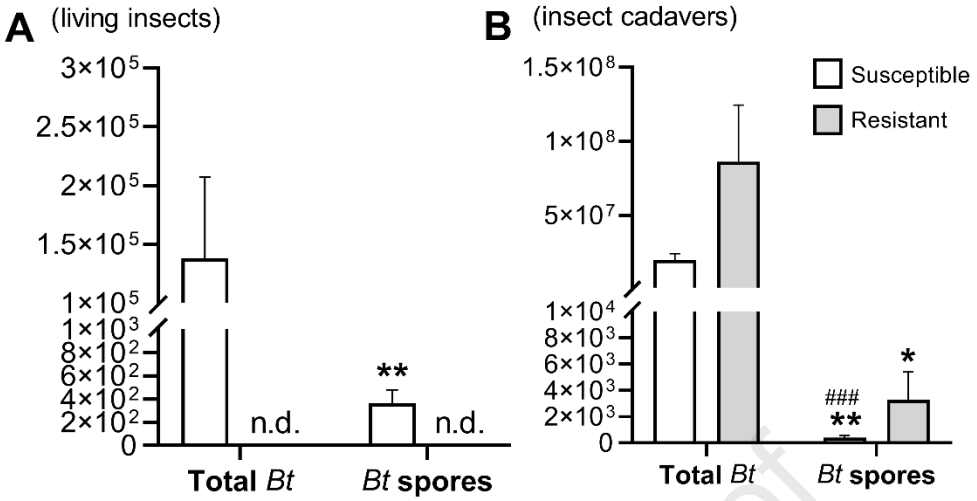

Figure 4. Colony forming units of *Bacillus thuringiensis* in living midgut tissues (A) and cadavers (B) of *Galleria mellonella* following infection (LC_{30}). Spores and vegetative cells of *Bt* (total *Bt*) and spores (*Bt* spores) were counted as CFUs per midgut of living insects (susceptible (S) and resistant (R) lines) at 48 hours post-infection and per cadaver at 3 hours *post-mortem*. No *Bt* were detected in uninfected (S and R) larvae. Data represent mean ± SE. Not detectable, n.d. Symbols (*, #) denote significant differences: * $p < 0.05$; ** $p < 0.01$; compared with total *Bt* in the same insect line (S or R); ### $p < 0.001$; compared with total *Bt* of the R line.

3.4. Microbiomes of insect cadavers after *Bacillus thuringiensis* infection

Taxonomic classification of bacteria taken from larval cadavers post *Bt* treatment (48 h.p.i.) were dominated by 4 genera (99.5%): *Bacillus, Enterobacteriaceae, Enterococcus* and *Serratia* (Figure 5A and 5B). *Bacillus* represented 70% of bacteria in cadavers of S larvae and the abundance of *Bacillus* was 2-fold higher when compared to the cadavers of R-line insect treatments ($t = 3.95$; $df = 40$; $p < 0.01$). *Enterobacteriaceae* represented 70% of bacteria in cadavers of R line cadavers, being significantly more than in S-line ($t = 4.43$; $df = 40$; $p < 0.01$) (Figure 5A and 5B). Again, richness and diversity indices of bacterial communities in cadavers of both insect lines remained unchanged (SI Figures S4, S5).

Total numbers of *Bt* (CFUs) in cadavers of infected larvae increased substantially in both lines when compared to living insects (Figure 4B). Vegetative cells of *Bt* in cadavers of susceptible and resistant lines following oral treatment with *Bt* (LC_{30}) were significantly higher than spores for the respective lines ($p = 0.004$ and $p = 0.04$, respectively; Figure 4B) and across both lines (S vs R line; $p = 0.005$).

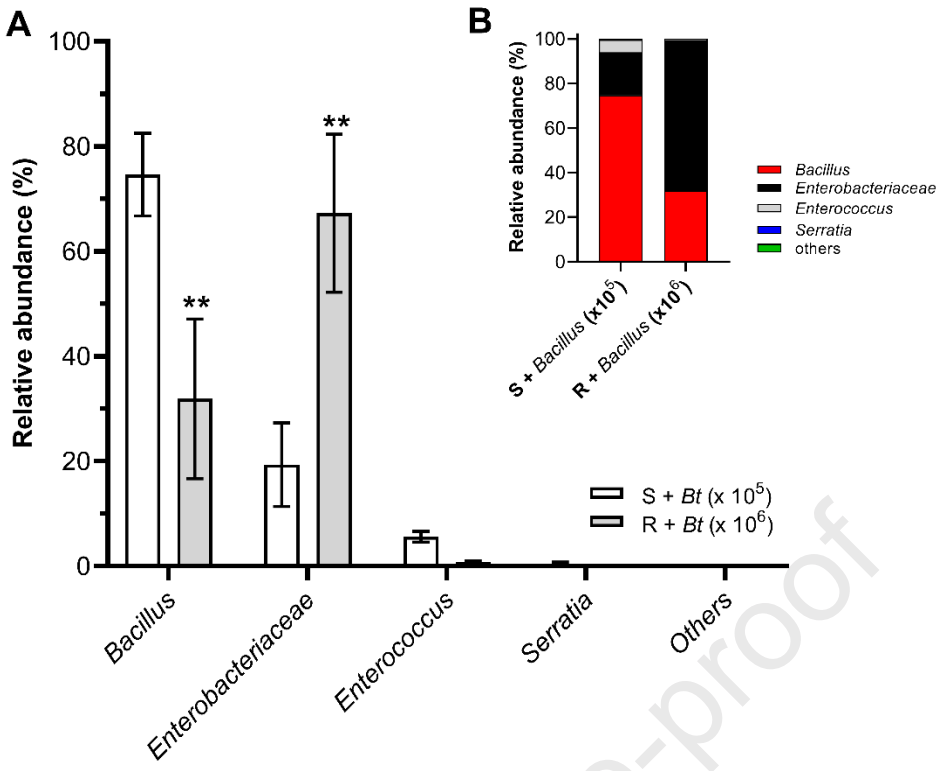

Figure 5. Bacterial microbiome (16S rRNA) profiles in the cadavers of susceptible (S) and resistant (R) *Galleria mellonella* following infection. **A**) Bacteria were classified according to genus and abundance across biological replicates (n = 5 per treatment). **B**) Bacterial genera of cadavers. DNA was extracted *post-mortem*. Data represent mean ± SE. Asterisks represent significant differences (** $p < 0.01$) when comparing cadavers of the S and R lines within the respective bacterial genus.

3.5. Expression of *Bacillus thuringiensis* genes in insect cadavers

Gene copy numbers (mRNA) for *Bt* factors related to virulence, coordination of necrotrophic development, and sporulation were measured in insect cadavers at 48 hours post inoculation (Figure 6A). Out of the 18 candidate genes measured, 14 were stimulated in *Bt*-infected S larvae compared to R larvae, with one gene (*entCW*) remaining unchanged, and three being down-regulated (Figure 6B). One such down-regulated gene, InhA2 (BC2984) – a member of the Immune Inhibitor A metalloprotease family – was decreased ~6-fold in cadavers of S larvae. The PI-specific phospholipase C (*plcA*), S8 family serine peptidase (*serP_S8*) and N-Acyl homoserine lactone hydrolase (*n-Ac*) levels were elevated also in S cadavers (~7, 36 and 26-fold, respectively).

Notably, four hemolysin genes (between 23 and 125-fold), four enterotoxin genes (between 1.7 and 10-fold) and three endotoxin-associated genes (between 11 and 174-fold) were significantly up-regulated in *Bt*-infected S cadavers (Figure 6B) relative to

their R counterparts – suggesting successful establishment of *Bt* in the S larvae prior to *in exitus*. Transcription regulator of virulence PlcR (*plcR*) increased by ~12-fold in cadavers of S larvae compared to R (Figure 6A). Expression of the necrotrophic regulator (*nprR*) and sporulation regulator (*spoOA*) were enhanced in both insect lines; ~7-fold (S) and 43-fold (R), and ~4 and 15-fold, respectively (Figure 7).

Necrotrophic regulator expression was significantly higher (6-fold; t = 8.533; df = 24.00; p < 0,0001) as was the sporulation regulator (3.5-fold; t = 2.421; df = 24.00; p < 0,05) for bacteria in cadavers of the R line over those in the S line (Figure 7).

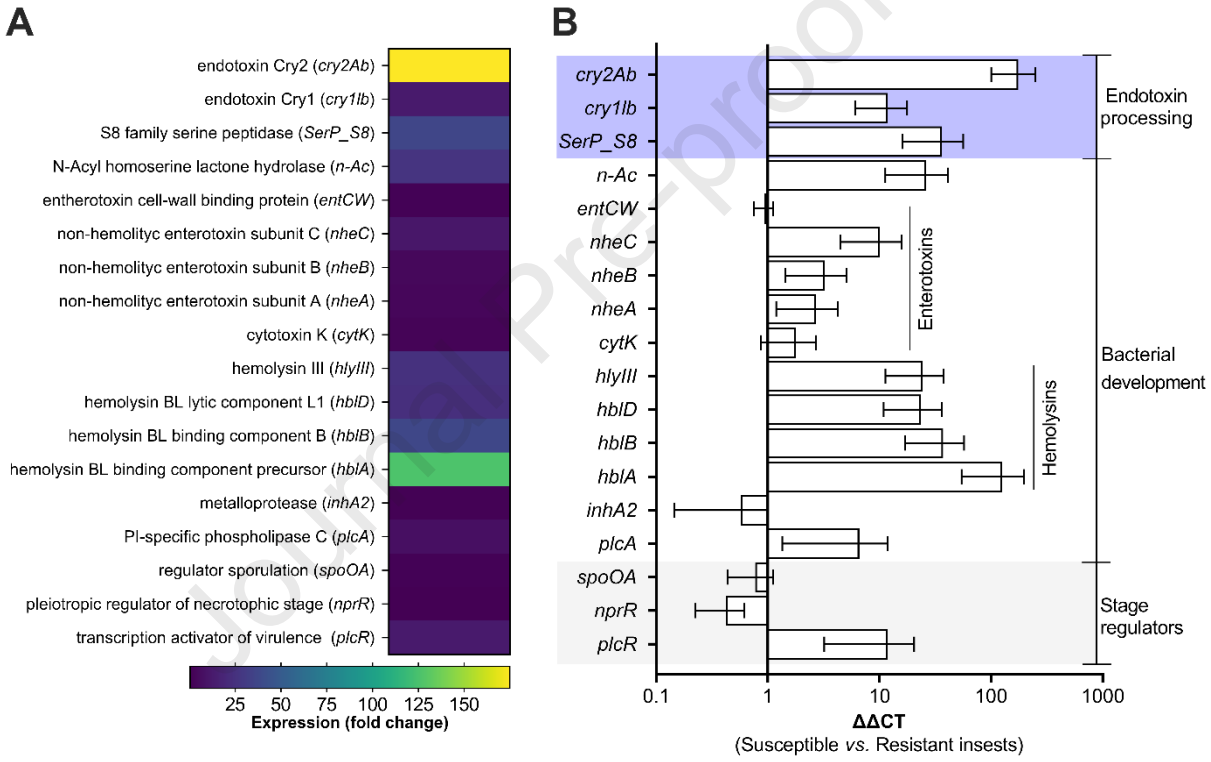

Figure 6. Differential gene expression of *Bacillus thuringiensis* in insect cadavers. A) mRNA levels of *Bt* genes quantified in susceptible (S) *G. mellonella* relative to the cadavers of resistant (R) *G. mellonella* 48 hours after bacterial inoculation (gene copies are displayed as a heat map with each gene function listed). (B) Up- and down-regulated *B. thuringiensis* genes in S when compared to the cadavers of R insects with functional annotation.

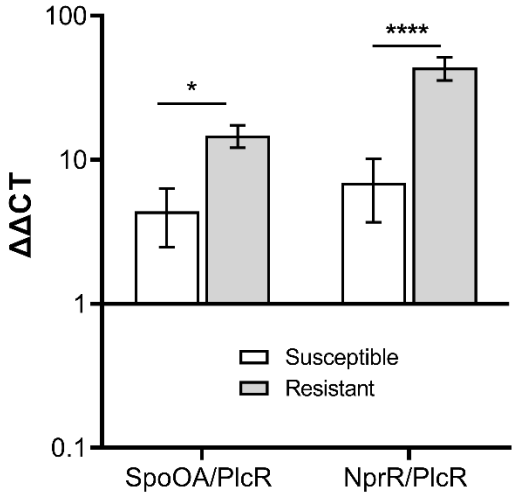

Figure 7. Differential gene expression of *Bacillus thuringiensis* developmental stage regulators. Gene copy numbers (mRNA levels) of 'transcription regulator of lifecycle' during sporulation (SpoOA/PlcR) and 'necrotrophic' (NprR/PlcR) stages in susceptible and resistant *G. mellonella* cadavers (* $p < 0.05$; **** $p < 0.0001$).

4. Discussion

Our aim was to gain insight into the putative strategies used by *B. thuringiensis* subsp. *galleriae* when colonizing two distinct populations of *G. mellonella* – those considered susceptible (S) to infection and those bred for resistance to *Bt* (R). Firstly, our data strengthen earlier findings that constitutively higher levels of AMPs, specifically the antibacterial peptide gloverin, in the midgut of R-line insects represents an evolutionary strategy "to be ready" or primed for infection through the gastrointestinal tract [13]. Hence, the midgut and cadavers of R line larvae are potentially harsh environments for *Bt* bacteria, and when they encounter a R host, *Bt* coordinates diverse transcriptional level changes in bacterial development, from virulence/colonization (e.g., toxins and lysins) to necrotrophy and expedited sporulation. This strategy permits *Bt* to maintain a sizable population in R insects compared to more susceptible ones, although *Bt* does require a significantly larger inoculum for an R insect to match the disease burden observed in an S host.

When faced with *Bt* infection, the levels of AMP genes in S and R insects increased substantially over uninfected insects. Of course, AMPs can kill or block bacterial growth and represent key components of insect humoral (immune) defenses [28,29]. Further support for this mechanism was provided by Orozco-Flores et al. and Krams et al. [19,30], elevated basal levels of immunity-related genes (Glo, Galio) could be exploited

as a prophylactic against opportunistic infections and as a mechanism in wax moth larvae that controls the resident gut symbionts. Interestingly, much higher levels of gloverin were detected in the midgut of R insects infected with *Bt* than S insects. RNAi-mediated suppression of gloverin in wax moth larvae resulted in enhanced susceptibility to *Bt* treatment. *Bt* infection arising in the insect midgut leads to local (midgut) and systemic (haemocoel) immune responses that are both needed to protect insects [20,31,32]. Selective RNAi-mediated silencing of an immune gene involved in the regulation of encapsulation and melanization reactions in *Spodoptera littoralis* reduced nodulation responses and significantly enhanced larval mortality triggered by *B. thuringiensis* [24]. Gloverin is an example of one AMP produced constitutively in insects and significantly increases in response to pathogen detection [33,34]. In previous studies, recombinant gloverins from the silkworm demonstrated antimicrobial activity against both Gram-positive and Gram-negative bacteria (*B. thuringiensis* subsp. *galleriae* and *S. marcescens*, respectively) [33,35]**.** Moreover, silencing of gloverin by RNAi in *S. exigua* larvae increased their susceptibility to *B. thuringiensis* [33]. Collectively, these findings point to a mechanistic role for gloverin in insect resistance to *Bacillus* spp.

The composition and activities of gut microbiota are linked to various physiologic processes in insects, such as nutrition, metabolic homeostasis, pathogen exclusion, and under some conditions, can trigger immune defences [36]. Johnston and Rolff (2015) demonstrated that *G. mellonella* immune factors (mostly lysozyme and AMPs) control the microbiota of adults to favor symbiont colonization [37]. We found that R-line *G. mellonella* have similar bacterial diversity and evenness indices in the midgut as the S line, which are restricted to certain taxa, e.g., *Entherococcus, Enterobacteraceae, Serratia*. The presence of *Serratia* in the midgut of both insect lines could be an additional factor promoting the infection process because *Serratia* and *Clostridium* species are known to switch from asymptomatic gut symbionts to haemocoelic pathogens in *S. littoralis* upon treatment with *Bacillus* Cry1Ca toxin [24]. Infection of R and S line *G. mellonella* with sufficient doses of *Bt* to achieve 30% mortality also led to similar bacterial profiles, and significant reductions of bacterial diversity when compared to uninfected insects. Reduced bacterial diversity in larvae may disrupt disease progression of *Bt* – the loss of gut bacteria in *P. interpunctella* modulated the insect's immune responses, thereby making them less susceptible to *Bt* [19]. Involvement of the host enteric flora in *Bt*-killing activity was shown in *S. littoralis* that were

immunosuppressed using RNAi [24]. The role of host microbiota in *Bt* pathogenicity is a controversial and understudied topic, however, their participation in septicemia during late-stage infection *Bt* is clear [19,22]. On one hand, the midgut microbiome could enable the establishment of *Bt* infection, and on the other, the resident microbes may protect the host from invader pathogens and opportunistic bacteria [24,28]. In the lepidopteran gut, *Enterococci* interact with pathogens through (i) competitive exclusion, (ii) attenuation by direct antagonism, or (iii) eliciting host immune responses and provide lepidopterans with protection against one of the most virulent entomopathogens, *Bt* [37]. The prevalence of the *Enterobacteraceae* (Gram-negative) and absence of *Bt* spores and vegetative cells in infected R larvae at 48 h.p.i. contrasts with S larvae where their midgut was replete with Gram-positive *Enterococcus* and *Bt* (spores and vegetative cells). Resistance mechanisms in R-line *G*. *mellonella* may involve clearing the midgut from Gram-positive bacteria. Notably, vegetative cells of *Bt* prevailed against spores in midguts of S larvae, which means *Bt* were germinating in the host. In contrast to S insects, R insects are able to purge the midgut of *Bt* during the first two days after infection. There is evidence that AMPs participate in cleansing the midgut of *Bt* [33,35]. Also, R line insects could protect themselves from *Bt* infection by activating tissue repair processes in the midgut to reinforce against Cry-toxin-associated damage, as well as elevated antioxidant activity that controls the level of reactive oxygen species generated during infection and directed toward the pathogen [13,40,41]. Thus, R-line insects are ‘ready for battle’ in the midgut.

If the R-line insects "lose the battle" in the midgut, then *Bt* will spill out into the haemocoel and reproduce, and together with the resident microbiota, will cause septicemia. Numbers of *Bt* in R larvae cadavers were suppressed compared to S larvae cadavers, although were not significantly different 3 hours *post-mortem*. *Bt* numbers did not dominant R line cadavers because the host immune system is likely more effective. Generally, richness and diversity indices of bacterial communities in uninfected cadavers for both insect lines were similar, and when considering *Bt* infected larvae, the richness index was significantly lower in cadavers from both lines also. Previously it was proposed that *Bt* uses a strict necrotrophic life cycle to colonize a wide variety of dead insects, and use the cadaver as a bioreactor to multiply and produce spores and toxins [25,42]

For S and R insect cadavers, expression levels of *Bt*'s necrotrophic stage regulon NprR were higher than the virulent stage regulon PlcR, likely because the main strategy of bacteria in a cadaver is to survive. Concurrently, *Bt* in the cadavers of S line insects also demonstrated elevated expression of regulon PlcR and decreased levels of SpoOA. *Bt* revealed significantly higher levels of expression of spore-forming SpoOA and necrotrophic NprR transcription activators in the cadavers of R line insects. In fact, the expression of SpoOA regulon depresses/inhibits PlcR expression [4,25]. Such differences in the expression of *Bt* life cycle regulators in R and S insects are likely the result of subpopulation differentiation as a strategy to survive [43]. The number of *Bt* spores were equal in cadavers of S and R insects, but *Bt* recovered from R insects grew and sporulated more rapidly on the selective medium. Hence, we consider that the *Bt* lifecycle is under prolonged pressure from defence mechanisms of the R host, and in response, a subpopulation of the bacteria continues to produce virulence factors in the cadavers of S insects such as the protein N-Acyl homoserine lactone hydrolase (*n-Ac*), which inactivates one of the QS components, namely N-acyl-homoserine lactone in Gram-negative bacteria responsible for a virulence state [44]. It is likely that *Bt* in the cadavers destabilize the midgut bacterial community, especially Gram-negative *Enterobacteriaceae* and *Serratia*, to reduce the competition for nutrients. Moreover, in the cadavers of the S line, elevated expression of enterotoxins and hemolysins were detected. Hemolysin BL (*hbl*) and non-hemolytic toxin (*nhe*) are pore forming cytolytic toxins and consist of three components (*hblB, hblL1, hblL2 and nheA, nheB, nheC*, respectively), altogether these are important for biological activity, i.e., osmotic lysis of the cells [45–49]. Hemolysin IV (or *cytK*) is a cytotoxic/necrotic protein that can destabilize cellular membranes by forming pores in the lipid bilayer [50]. Hemolysin III (*hlyIII)* and enterotoxin (*entCW/ entB*) are not so well characterized hemolytic toxins of *Bacillus* and do not depend on the PlcR regulon [51,52]. Elevated levels of genes encoding hemolysins and enterotoxins in the cadavers of S larvae allow *Bt* to compete with other bacteria and to access nutrients by degrading host tissues. In the present study, phospholipase C (*plcA*) expression was upregulated in the cadavers of S insects. Phospholipases produced by many of the *Bt* strains play important roles in virulence and can deform and rupture hemocytes when injected directly into the haemocoel of *G. mellonella* [53]. Other studies have reported on *Bt* using phospholipases for degradation of host tissue and to overcome the host defences [54,55]. At the necrotrophic stage of *Bt* development, elevated levels of metalloproteinase A2 (inhA2) were detected in cadavers of the R host. Metalloproteinase A2 contributes to *Bt* virulence by assisting the

bacterium in crossing the gut barrier into the haemocoel [5]. Members of the Immune Inhibitor A metalloprotease family, include inhA2 and inhA3, which help bacteria to resist insect immune defenses by degrading AMPs and hydrolysing various proteins and cellular components (e.g., fibronectin, collagens, laminin) [25,56,57]. We observed elevated levels of AMPs in infected R larvae, and coinciding with this, Bt gene copy numbers for various virulence factors, including proteinases, increased and remained at high levels *post-mortem*. Bacterial spores from the cadavers of R insects grew faster on selective growth medium and contained more crystals (Cry toxins) when compared to those spores isolated from S insects. A proportion of the *Bt* population in S line cadavers used both necrotrophic and sporulation stages, expressing higher levels of endotoxins (*cry1* and *cry2*) and serine peptidase s8 genes. Serine peptidase s8 participates in a wide range of biological process in the *Bt* lifecycle, like growth and cell differentiation, synthesis of spores and endotoxins, also post transcriptional modification of the sporulating cells to promote spore release [58,59].

5. Conclusion

Bacillus thuringiensis subsp. *galleriae* demonstrates plasticity at the transcriptional and cellular levels in susceptible or resistant *G. mellonella* to overcome the immune defences, and exploit the cadaver. In contrast to S insects, we propose bacteria switch more rapidly to quorum sensing (QS) and produce virulence factors in the resistant (R) insects – this would shorten the virulence stage of the *Bt* lifecycle because of the higher number of propagules used for infecting the insects and the antagonistic environment of the midgut (i.e., higher constitute levels of AMPs). Surviving R insects purged *Bt* from the midgut, and the insect population that perished (~30%) contained *Bt* subpopulations that were engaging in necrotrophy and sporulation. Support for this strategy was confirmed by the enhanced expression of NprR and SpoOA regulons, with reduced expression of PlcR in the cadavers of the R host, as well as decreased expression levels of virulence factors such as hemolysins and enterotoxins. Sequential passage, and isolation, of *B. thuringiensis* in *G. mellonella* could be used for developing highly virulent strains as biopesticides.

Data availability statemen

The raw data supporting the conclusions of this manuscript are held by the authors (ID and EG), and will be made available, without undue reservation, to any qualified researcher.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Acknowledgments

The authors acknowledge Nadejda Aculova from the Faculty of Physical Engineering, Novosibirsk State Technical University, Novosibirsk, Russia for the methodology support.

Funding/support

This research was supported in part by the Russian Science Foundation [Grant number 20-76-00025].

Appendix A. Online supplementary information

- Methods of Insect selection; Bacterial cultivation and infection; Midgut RNA extraction, cDNA synthesis and RT-PCR; RNA extraction, cDNA synthesis and RT-PCR of Bt; RNAi: Gene fragments and in vitro dsRNA synthesis; 16S (V3-V4) rRNA Bacterial Diversity Analysis of the Insect Midgut and Cadavers; Quantifying Bt CFUs.
- Figure S1: Experimental design. Full details of each protocol across each of the three experimental stages can be found in the main text, and online supplementary materials. Bt, Bacillus thuringiensis; Antimicrobial peptides (AMP), S susceptible; R, resistant; LC30, lethal concentration at 30%;
- Figure S2: Mortality of susceptible and resistant G. mellonella lines after oral inoculation (105 and 106) of *B. thuringiensis* subsp. *galleriae* (*Bt*). The negative control consisted of administering PBS alone (no spores or toxins). Unshared letters represent significant differences ($p < 0.01$; Log-rank (Mantel-Cox) tests; n = 128-250). Data represent day 5 post-inoculation;
- Figure S3: Figure S3. Sporulation and crystal production on selective growth medium. B. thuringiensis (Bt) were collected from cadavers of susceptible (A) and resistant (B) lines of G. mellonella cadavers (originally infected with Bt). Vegetative cells (vc), spores (sp) and crystals (cr) are indicated with arrows. Images were taken from samples 3 days post cultivation;
- Figure S4. Diversity and (d) Evenness indices of bacterial communities from the cadavers of susceptible (S) and resistant (R) *Galleria mellonella* following oral inoculation with *Bt*. Data represent Mean ±SE;
- Figure S5. Diversity (A) and evenness (B) indices of bacterial communities in the midguts and cadavers of susceptible and resistant *Galleria mellonella* following *Bt* treatment. Data represent Mean ±SE (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ compared with cadavers (S line); # $p < 0.05$; ## $p < 0.01$; #### $p < 0.0001$ compared with cadavers (R line)).
- Table S1: RNAi-mediated knockdown of insect Gloverin (Glo) in *Galleria mellonella* larvae.
- Table S2: Oligonucleotide primer sets targeting Bt genes.

Author Contributions

E.G. Conceptualization, investigation, methodology, writing - original draft, review and editing, funding acquisition. T.K. methodology, investigation, writing - review and editing.

G.K. methodology. M.K. software, formal analysis, resources. T.A. methodology, investigation. E.S. investigation. I.D. Conceptualization, software, formal analysis, writing—review and editing, visualization. C.C. software, formal analysis, visualization, writing - review and editing. All authors have read and agreed to the published version of the manuscript.

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Highlights

- Elevated basal and induced levels of AMP genes attend the evolution of insect resistance to Bt
- Survived Resistant insects can cleanse the midgut of Bt as compare with susceptible
- Bt infection in resistant insects coincided with gross dysbiosis in midgut microbiome
- Reductions in bacterial diversity of the midgut microbiome are detected in alive and dead susceptible and resistant insects post Bt infection
- Bt demonstrates different developmental strategies to survive in the cadavers of susceptible versus resistant hosts

Declaration of competing interest

The authors declare that they have no conflict of interest.