Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Intact gut microbiota modulates immunity and increases susceptibility to baculovirus in Spodoptera exigua

Loretta Mugo-Kamiri

lw.mugo@gmail.com

Institut de Recherche sur la Biologie de l'Insecte, IRBI, UMR 7261, CNRS - University of Tours

Marina Querejeta

Institut de Recherche sur la Biologie de l'Insecte, IRBI, UMR 7261, CNRS - University of Tours

Ben Raymond

University of Exeter

Elisabeth A. Herniou

Institut de Recherche sur la Biologie de l'Insecte, IRBI, UMR 7261, CNRS - University of Tours

Research Article

Keywords: Baculovirus, Gut immunity, Lepidoptera, Gut microbiota, Host-pathogen interactions

Posted Date: November 13th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11614639/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Additional Declarations: No competing interests reported.

Intact gut microbiota modulates immunity and increases susceptibility to baculovirus in *Spodoptera exigua*

Loretta Mugo-Kamiri[a,b*], Marina Querejeta[a,c], Ben Raymond[b], Elisabeth A. Herniou[a]

a) Institut de Recherche sur la Biologie de l'Insecte, IRBI, UMR 7261, CNRS - University of Tours, 37200 Tours, France.

b) Centre for Ecology and Conservation, Penryn Campus, Faculty of Environment, Science and Economy, University of Exeter, Cornwall, TR10 9FE, UK.

c) Ecologie et Dynamique Des Systèmes Anthropisés, EDYSAN, UMR 7058 CNRS - Université de Picardie Jules Verne, Amiens Cedex, France

*Address correspondence to Loretta Mugo-Kamiri, lw.mugo@gmail.com

Abstract

Growing evidence reveals that the gut microbiota is a key player in host-pathogen interactions, particularly for pathogens with a midgut stage such as baculoviruses. This study investigated how native gut microbiota and a strain of *Enterococcus mundtii* (Em) influence *Spodoptera exigua* survival, immune response and gut bacteria composition during infection with the entomopathogenic virus *Autographa californica nucleopolyhedrovirus*, AcMNPV. Our findings show that the presence of native gut microbiota increased host mortality during infection and was associated with a significant downregulation of a JAK-STAT signaling pathway gene. Supplementing the native microbiota with Em slightly elevated immune gene expression but did not improve larval survival. In contrast, suppressing the native gut microbiota with rifampicin significantly enhanced survival and the expression of the antimicrobial peptide gloverin. Em supplementation to the rifampicin treated larvae also upregulated gloverin but did not significantly boost survival. At 48 hours post-infection, Em supplementation, both as a main effect and in interaction with virus significantly altered gut bacterial community composition, with distinct indicator ASVs associated with Em-supplemented and non-supplemented communities. This study highlights that intact microbiomes do not always confer immune protection in insects. Microbiomes can persistently stimulate host immunity which can be energetically costly or harmful, hence the counterintuitive result that reducing microbial abundance using antibiotics can improve survival by resetting immune responses. Modulation of insect microbiomes could therefore be used as a tool to help protect beneficial mass-reared insects from specific immune challenges or to enhance the action of microbial biocontrol agents.

Key words: Baculovirus, Gut immunity, Lepidoptera, Gut microbiota, Host-pathogen interactions

1. Introduction

Microbiomes are ubiquitous and vital biological engines for their hosts, serving functions such as nutrient supplementation, detoxification and immune defense. However, not all microbiome – host interactions are beneficial [1]. Insect microbiomes range from highly specialized altruistic associations that the host cannot live without [2], to the dynamic, transient gut communities such as in Lepidoptera, that can also benefit immunological and metabolic functions but with much less fidelity [3]. Persistent defensive traits of symbionts typically result from tight coevolution between microbes and hosts. These require specialized adaptations that control vertical transmission of microbes, fostering alignment of the interests of host and microbes [4].

However, for the vast majority of host-microbe interactions, such as in Lepidoptera, microbes are sourced from the environment, housed in the gut, and change across diet, sex, and development stage [5] . Here, tight transmission controls do not exist, and there is potential for conflicts between the host and microbes [6]. Dynamic communities like those of caterpillars also require strict host control to keep a healthy balance of the ecosystem. This is achieved via physico-chemical factors such as high gut pH and the host immune system, the latter of which can come with fitness trade-offs that can influence downstream symbiont-derived functions such as pathogen defense.

Gut commensals stimulate and modulate host immunity in diverse and complex ways. In insects, commensals just like pathogens contain molecular signatures recognized by receptors on the gut epithelia, which trigger intracellular immune signaling pathways [7]. This initiates and sustains a continuous immune stimulation described as the basal sentinel immunity, which serves an immune priming role in hosts [8]. Numerous studies have highlighted the protective roles of intact microbial communities or individual symbiotic species against pathogens [9–12]. However, emphasis on beneficial microbial interactions may contribute to an incomplete understanding of host–microbiota–pathogen relationships, given growing evidence that commensal microbes can also increase host susceptibility to infection [13–16]. This underscores

the need for a more balanced understanding of the mechanisms underlying these contrasting outcomes, and clarity on the stability of microbiomes in the face of perturbations from pathogens.

Baculoviruses are insect pathogenic viruses recognized for their role in Integrated Pest Management (IPM) against lepidopteran pests of agriculture and forestry [17]. To initiate infection, baculovirus Occlusion Bodies (OBs) are ingested during feeding, and dissolve in the highly alkaline midgut to release Occlusion Derived Virions (ODVs) establishing the primary infection in the gut epithelia and producing Budded Viruses (BVs) [18]. BVs and some ODVs then penetrate other cells through the tracheal system triggering a systemic infection that eventually kills the host [18,19]. Baculoviruses therefore have an obligatory midgut stage in their pathogenesis and consequently, direct interaction with resident microbial communities in the lepidopteran gut that may prime the host's immune system. These viruses are also produced *in-vivo* using mass-reared caterpillars before being applied in agricultural fields for pest control [20]. Understanding baculovirus–gut microbiota interactions is therefore important not only for elucidating host–microbe–pathogen relationships, but also for optimizing baculovirus production and improving their efficacy as biocontrol agents.

Previous studies have demonstrated that a dynamic gut microbiome can either confer resistance or facilitate baculovirus infection in Lepidoptera [21–24] . Yet it remains unclear how perturbations such as different viral loads or antibiotics alter the gut community structure, and how these changes interact with host immunity to modulate virus infection. The Beet armyworm, *Spodoptera exigua* is a polyphagous pest of over 130 crop species and has developed resistance to almost all classes of insecticides [25–27], but baculoviruses remain a promising avenue for biocontrol [28–30]. In this study, we investigated how the native gut microbiome and a candidate probiotic strain, *Enterococcus mundtii* (Em), isolated from guts of a healthy and high performing colony of *S. exigua*, interacts with virus infection. We hypothesized that larvae harboring their native microbiome would exhibit reduced susceptibility to viral infection due to microbiome-mediated immune priming, and that virus infection would enrich for protective gut microbial taxa. Furthermore, we

expected that *E. mundtii* supplementation would confer probiotic benefits and act as a protective symbiont, further mitigating viral pathogenicity. Specifically, we studied the larvae survival rates during infection, gut immune gene expression and surveyed gut bacteria diversity and composition using 16S rRNA gene metabarcoding from extracted RNA to capture the metabolically active gut bacteria.

2. Materials and methods

2.1 Insect maintenance

Spodoptera exigua eggs were obtained from a laboratory colony maintained at the University of Valencia (Spain) [31]. This colony has been maintained for over 100 generations on a wheat germ based artificial diet as described in [32]. Fresh eggs were collected and shipped to our lab at ambient temperature and thereafter kept at 25°C for hatching. The neonates were used directly for experiments.

2.2 AcMNPV and *Enterococcus mundtii* strains

The polymorphic wild type *Autographa californica nucleopolyhedrovirus*, AcMNPV-WP10 isolate described in [33] was used in this study. *Enterococcus mundtii strain SeAI* isolated from third instar *S. exigua* larvae was used for bacteria supplementation [34]. The *E. mundtii* strain isolation and species identification details are described in supplementary material.

2.3 Experimental design

This study employed a 2 x 2 factorial experimental design that allowed the exploration of two factors: (1) the presence or absence of rifampicin antibiotic (effect of native gut bacteria) and (2) the presence or absence of *E. mundtii* on infection outcomes. The experiment had four treatment groups, **i)** No Antibiotic + no *E. mundtii* (Native) which served as the control group. **ii)** Rifampicin + no *E. mundtii* (Rifampicin) which assessed the effect of antibiotic treatment in the absence of *E. mundtii*. **iii)** No antibiotic + *E. mundtii* (Native + Em) which examined the effect of *E. mundtii* without the influence of antibiotics and **iv)** Rifampicin + *E.*

mundtii which investigated the combined effects of antibiotic treatment and *E. mundtii* introduction. These larvae were then challenged with two doses of AcMNPV corresponding to LC_{50} and LC_{75}.

Approximately 1200 neonates were maintained in groups of 80-100 larvae in 1-liter plastic boxes on autoclaved wheatgerm diet. Half of these were reared on diet amended with 250mg/L rifampicin antibiotic (Sigma-Aldrich, France) and the other half were fed diet without rifampicin. Larvae were grown in these conditions for 5 days after which early 3rd instar larvae were selected and transferred to individual 50mm single vent deep well Petri dishes with a 1 cm^3 diet cube. Larvae reared with antibiotics were further divided into two groups to assess how rifampicin affects virus infection with and without *E. mundtii* (Em) supplementation. Removal of the microbiota by rifampicin was confirmed by dissecting and plating pooled guts of 5 randomly selected larvae onto LB agar plates. Larvae that were reared without antibiotics were further divided into two groups to assess how native gut bacteria affects virus infection with and without *E. mundtii*.

2.4 Infection protocol

Bacteria inoculant was prepared by growing the *E. mundtii* strain *SeAI* in 5ml LB broth overnight at 30°C with shaking. Larvae were then fed a 1 cm^3 diet cube amended with 20µl of 10^7 cfu/ml overnight culture of *E. mundtii* and 0.45% sterile saline solution for the control group. Diet pieces were removed after 24 hrs and larvae were starved overnight before virus infection. For the virus infection, larvae from each of the antibiotic and *E. mundtii* combinations were droplet fed with two doses of AcMNPV, 10^5 OBs/ml and 10^4 OBs/ml in 10% sugar solution with 1% pink food dye to visualize the virus intake through the larval gut, following the protocol described in [35]. Mock-infected larvae were fed with a virus free inoculant of 10% sugar and 1% pink food dye. Only larvae that immediately fed on their droplet were kept in the study. After infection, larvae were fed *ad-libitum* on antibiotic free diet and mortality recorded daily.

2.5 Survival data analysis

Survival analysis was carried out using the *survival* and *survminer* packages in R [36,37]. Our data did not meet the assumption of constant hazard and thus a survival regression model was fitted assuming a Weibull distribution where hazard decreases with time. Virus dose was Log transformed and fitted as a covariate. Model fitting was done with all terms interacting and the non-significant terms were dropped until the best model fit was obtained based on Akaike Information Criterion (AIC).

2.6 Gut dissection, RNA extraction, metabarcoding library preparation and sequencing

The effect of antibiotic, Em supplementation and virus infection on the diversity and composition of the gut microbiota was assessed at 48 hours post infection (hpi), when infection was well established but most larvae not yet moribund. Approximately fifteen larvae per treatment were collected and euthanized on ice, surface sterilized in 70% ethanol, with 2 rinses in autoclaved DI water. Guts were dissected directly into DNA/RNA shield (Zymo Research) and kept at -20°C until nucleic acid extraction. RNA was extracted from guts using the *ZymoBIOMICS MagBead DNA/RNA* kit (Zymo Research) as per manufacturer's instructions. Template-free water blanks were included in the sample processing as negative controls to detect reagent contamination. Sample RNA (250ng per insect) was reverse transcribed to cDNA using QuantiTect Reverse Transcription kit (Qiagen). The 16S rRNA gene v3-v4 region metabarcoding library was prepared as previously described in [34]. For sequencing, pooled libraries were purified using MagSi-NGSprep Plus beads (Steinbrenner Laborsysteme GmbH, Wiesenbach, Germany) and the sequencing run was performed on an Illumina MiSeq using V2 chemistry (2 X 300 bp, 500 cycles) at the sequencing center of the Biozentrum of the Ludwig-Maximilian-University in Munich (Germany). Raw sequence data are available in the NCBI Sequence Read Archive under the BioProject ID PRJNA1457503 and metadata described in Supplementary Table 2.

2.7 Sequence Data Analysis

The quality of both forward and reverse reads were checked using Fast QC software (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Low-quality reads and adapters were removed using *cutadapt* software [38], and remaining reads merged using *PEAR* software with Phred score set at 30 [39]. Merged reads were quality filtered (*fastq_maxee* = 1) to discard reads that had more than 1 nucleotide error followed by a dereplication step to keep only unique sequences. A denoising step was then performed (min number of reads = 4) followed by indel removal (350-500bp) to obtain a *fasta* file of our biological communities as Amplicon Sequencing Variants (ASVs). Taxonomic classification was done using the *mothur* software [40]. The R package *decontam* was used to remove contaminating sequences based on the extraction control samples with threshold set at 0.1.

2.8 Biodiversity Analysis

To assess whether the sequencing depth was sufficient to capture the biodiversity in our samples, ASV richness rarefaction curves were computed in the R package *Vegan* [41]. Alpha diversity was calculated using the Shannon index in the R package *phyloseq* [42], with statistical differences between treatments tested using the Kruskal-Walis. Beta-diversity was quantified based on the Bray-Curtis dissimilarity. To determine if the community composition differed significantly among treatment groups, a non-permutational multivariate analysis of variance (PERMANOVA, 9999 permutations) on was computed using the function "adonis2" in the R package *vegan* [41]. Community composition was visualized using principal coordinate analysis (PCoA). Relative read abundance, which is the proportion of every read belonging to each ASV [43], was calculated using a customized R script based on the package *dplyr* and *ggplot2* [44,45]. Indicator species analysis was performed to identify ASVs significantly associated with treatment groups using the R package *indicspecies*[46] and significance was assessed by permutation testing with multiple-testing correction using the false discovery rate (FDR). ASVs with FDR-adjusted $P < 0.05$ were considered significant indicators.

2.9 qRT-PCR assay of immune gene expression

Quantitative RT-PCR was used to examine the expression of the immune genes PGRP, PPO, cecropin-b, JAK-STAT, gloverin, and defensin using cDNA synthesized above [47] (Supplementary table 1). Each qRT-PCR experiment consisted of four independent biological replicates with three technical replicates for each. PCR reactions of 13 µl had 3 µl cDNA (36ng), 6.25 µl MESA blue qPCR mastermix plus (2X) Sybr assay reagent for SYBR green (Eurogentec) and 1.25 µl of 3.125 pmol μl^{-1} of each primer and 2.55 µl of molecular grade water. Samples were run on a QuantStudio 6 Flex System (Thermo Fischer Scientific) using temperature cycling conditions of 20 seconds at 95°C followed by 40 cycles of: 95°C for 15 seconds, 60°C for 60 seconds. A final melt-curve step was included post-PCR to confirm the absence of non-specific amplification. Data were analyzed according to the ΔC_T method using the geometric mean of two housekeeping genes (RPL7A and ATP) for normalization as described previously [48]. Technical replicates resulting in a standard deviation greater than 0.5 were treated as outliers and omitted from the analysis to preserve samples with the least variability [49]. Statistical analysis using generalized linear models (GLMs) tested the effects of virus dose on gene expression within each treatment group followed by pairwise comparisons using estimated marginal means (EMMeans) in the R package *emmeans* [50] with p-value adjusted for multiple testing using the Benjamini-Hochberg method.

3. Results

3.1 Effect of native gut bacteria and *E. mundtii* supplementation on survival

After 5 days of rifampicin treatment, larvae did not have any detectable cultivable gut bacteria on LB plates hence colony forming units (cfu) data were not calculated, and only presence/absence was considered (Fig. 1a). Using a survival regression model, we found that rifampicin treatment as a main effect was associated with a significant increase in survival time represented by a positive coefficient (coeff = 0.5682, $p = 0.0031$). Moreover, there was significant rifampicin:virus interaction associated with an increase in survival time (coeff = 0.383, $p = 0.0256$) (Table 1), implying that reducing the native gut bacteria using rifampicin antibiotic

has a protective effect against virus infection (Fig. 1b). Em supplementation did not markedly alter survival in the virus infected samples when inoculated to both native and rifampicin groups (Fig.1b), indicating that Em does not provide pathogen protection. This was confirmed by fitting a survival regression model which showed that Em supplementation as a main effect and in interaction with virus decreased survival time as represented by the negative coefficients, although this was not significant (p = 0.0953 and 0.0943) (Table 1). We unexpectedly found that Em supplementation in the native group (native + Em) resulted in a high rate of mortality in the virus-free controls (mock infection), while supplementing Em to antibiotic-treated larvae did not lead to any mortality in virus-free controls (Fig.1b), highlighting that Em is likely parasitic to uninfected larvae but only in the presence of native gut bacteria.

3.2 Effect of virus infection on gut immune gene expression

Gut immune gene expression was evaluated at 48 hpi. In the native gut bacteria group, there was a significant downregulation of the JAK-STAT signaling pathway gene in the high virus dose relative to the control (GLM, p = 0.0235), while PPO, defensin, PGRP and gloverin showed reduced mean expression with virus treatment, although not statistically significant (Fig. 2). In the native group supplemented with Em there was no significant change in expression of all immune genes tested, although mean expression was slightly elevated in virus treated samples compared to controls.

In the rifampicin treated samples which were surviving virus infection better, only gloverin was significantly upregulated due to virus infection and substantially more in the low dose than the high dose (GLM, $p < 0.001$) (Fig. 2). Supplementing rifampicin group with Em also resulted in a significant upregulation of gloverin, which increased linearly with dose. Expression of the other genes in the rifampicin + Em trended upwards especially in the high dose relative to controls, but these contrasts were not significant.

3.3 Gut microbiota composition and diversity changes across treatments

We obtained an average of 32,000 reads per sample from 16S rRNA gene metabarcoding sequencing from 166 gut samples, from which we identified 2,376 Amplicon Single Variants (ASVs). Rarefaction curves

reached a plateau for each treatment indicating sequencing depth was sufficient to capture the microbial diversity (Supplementary Fig. 2). After removal of contaminants, pruning samples with less than 1000 reads and taxa with no reads, 127 samples and 1,161 ASVs remained for downstream analyses. However, in the rifampicin + *E. mundtii* + low-virus treatment only two biological replicates were retained after pruning due to majority of the samples containing low depth libraries. Consequently, microbial abundance patterns specific to this treatment combination were interpreted cautiously due to reduced replication.

Alpha diversity measured using the Shannon index remained relatively stable across treatment groups with no significant changes due to Em or virus infection at 48hpi ($p > 0.05$) (Fig. 3a). Beta diversity changes showed that community composition was significantly influenced by *E. mundtii* supplementation and Em:virus interaction**.** PERMANOVA based on Bray–Curtis dissimilarity showed a strong effect of *E. mundtii* treatment on community composition ($R^2 = 0.123$, $F = 17.50$, $P = 0.0001$), whereas virus dose alone had no significant effect ($R^2 = 0.024$, $F = 1.68$, $P = 0.100$) (Fig. 3b). A significant Em:virus interaction was detected ($R^2 = 0.035$, $F = 2.51$, $P = 0.0136$), indicating that the effect of virus infection on the microbial composition varied depending on presence or absence of *E. mundtii*. In contrast, rifampicin treatment and its interactions with *E. mundtii* or virus dose were not significant. Multivariate dispersion did not differ among treatment groups (PERMDISP, $P = 0.321$), indicating that the PERMANOVA results reflected true differences in community composition rather than unequal within-group dispersion.

Consistent with the significant effect of *E. mundtii* supplementation on overall microbial community composition, indicator species analysis identified several genus level ASVs associated with supplemented or non-supplemented communities (Fig. 3c). Two *Enterococcus* ASVs showed the strongest associations with *E. mundtii*-supplemented communities (indicator values = 0.989 and 0.980; FDR = 0.027), together with an ASV assigned to *Pluralibacter* (indicator value = 0.858; FDR = 0.040) (Fig 4c). Comparison of 16S rRNA sequences revealed that ASV uniq3 corresponding to *Enterococcus,* shared 100% sequence identity with the experimentally supplemented *E. mundtii* isolate, while uniq7 exhibited 99.77% identity. These findings

identify uniq3 as an exact 16S sequence match to the supplemented isolate and uniq7 as a closely related sequence variant indicating that microbiome sequencing strongly reflects the inoculated strain in this treatment. In contrast, ASVs assigned to *Halomonas*, *Streptococcus*, *Methylobacterium–Methylorubrum*, *Pseudomonas*, *Aeribacillus*, and *Georgenia* were associated with communities lacking *E. mundtii* supplementation (FDR < 0.05).

In both groups, indicator taxa spanned a wide range of relative abundances. *Enterococcus*, *Pluralibacter* and *Methylobacterium–Methylorubrum* had high relative read abundances (RRA) ranging from 4.0 - 32.5% while *Aeribacillus*, *Streptococcus*, *Georgenia*, and *Pseudomonas* occurred as rare taxa with RRA of <1%. These relative-abundance patterns varied across virus doses in a taxon-specific manner and no uniform dose-dependent trend was evident for majority of the taxa. The global relative abundance profiles at genus level across samples and treatment groups are shown in Supplementary Figure 3.

4. Discussion

While hosts harbor innate immune defenses against invading pathogens, associated microbiota can also play an important role in pathogen protection. Microbial communities may complement host immunity or serve as a primary line of defense by competitively excluding pathogens, producing antimicrobial compounds, modulating host immune responses, or potentially enriching protective taxa during infection [4,51,52]. Our findings, however, show that neither native resident microbiota nor supplementation by a potential probiotic isolate protects *S. exigua* from baculovirus infection, challenging the assumption that microbiomes are universally beneficial.

In our study, rifampicin treatment reduced cultivable gut bacteria to undetectable levels, markedly improved survival in both virus-infected and control larvae and resulted in an elevated immune response. These findings point to potential mechanisms by which an antibiotic-reduced microbiota mediates baculovirus resistance. The gut microbiota represents a variety of microbial relationships ranging from mutualistic to parasitic [53,54]. Rifampicin treatment likely suppresses parasitic bacteria and the overall metabolic and immunological demands imposed by the native microbiota. A less complex and less abundant microbial community decreases competition for host-derived nutrients, and the energetic costs of maintaining basal immune regulation at the gut interface. This reduction in microbiota-associated physiological demand may in turn permit the reallocation of host resources toward antiviral defense, and other processes that contribute to pathogen resistance and survival [55,56].

Consistent with this possibility, expression of the AMP gloverin was also markedly elevated in rifampicin-treated larvae compared to larvae having an intact microbiota reflecting enhanced immune activation when larvae are maintaining a reduced microbiota. Gloverin has been shown to have antiviral properties in several Lepidoptera species. In larvae of *Trichoplusia ni*, gloverin exhibited strong antiviral effects by binding to AcMNPV budded virus resulting in formation of membrane pores that disrupted the envelope protein and

blocked secondary infection [57]. Moreover, overexpression of gloverin 1 and 3 inhibited viral replication and thus increased *Hypantria cunea* resistance to *H. cunea* Nucleopolyherovirus (HcuNPV) [23].The antiviral action of gloverin was further confirmed in *Lymantria dispar* infected with LdMNPV where its suppression using RNA interference enhanced viral DNA replication while inducing gloverin suppressed viral replication, underscoring the importance of this AMP in baculovirus infections [58]. Taken together, these patterns suggest that a reduction in the resident microbiota facilitates a more robust immune response contributing to increased virus resistance, mediated by the AMP gloverin. This effect is however modulated by viral load since gloverin expression was higher at low virus dose compared to high dose, a trend also observed with defensin and PPO. This suggests that at higher viral loads either pathological effects dominate and impair immune upregulation or immune activation is attenuated allowing infection to proceed more effectively. The latter is highly plausible given that baculoviruses are known to manipulate host physiology and behavior, including mediating host protein synthesis shutdown which suppresses immune gene expression, to enhance virus infection [59–62].

This mechanism is further supported by patterns observed in larvae with an intact native microbiota. In this group, larvae failed to mount a notable immune response regardless of virus dose and most notably, our findings established that JAK-STAT signaling was significantly downregulated in the presence of native gut microbiota. The JAK-STAT pathway is an important antiviral defense pathway in Lepidoptera[63,64] , but how it interacts with the host gut microbiome has remained unclear [65]. Our study shows that virus-mediated immune suppression may be exacerbated in hosts maintaining a dynamic and metabolically costly microbiota [55] and such trade-offs could contribute to the higher susceptibility observed in the native group. Similarly, a dampened immune response was also observed in S. *exigua* 12 hours post injection with AcMNPV and 72 hours post oral infection with *Spodoptera exigua* nucleopolyhedrovirus, SeMNPV, in the presence of native microbiota, leading to an increase in gut microbial load and increased host susceptibility to virus [22,66].

The application of probiotic microorganisms is increasingly being adopted in insect mass-rearing industry to promote pathogen resistance and other developmental parameters, yet outcomes depend critically on the host and the microbial context [67]. While some studies have found *E. mundtii* to be beneficial for baculovirus resistance in *H. cunea* and *Helicoverpa armigera* [21,23,68], Em supplementation in our study acted as an ecological driver rather than a protective symbiont, significantly restructuring the gut microbiota composition and enriching taxa other than *Enterococcus*. The significant interaction between Em supplementation and virus dose observed here further suggests that the consequences of this restructuring depend on the infection status of the host. We also found that Em supplementation was parasitic in uninfected larvae with intact native microbiota while remaining neutral post-rifampicin, underscoring how the resident microbiome influences whether an inoculated strain becomes beneficial or antagonistic to the host, in line with context-dependent shifts along the parasite-mutualist continuum [53]. This aligns with reports of *E. mundtii* strains and other *Enterococcus* species being pathogenic in some contexts and beneficial in others [69–71]. These results reinforce that probiotic efficacy cannot be assumed without accounting for the host species and resident microbiota context.

In conclusion, while insects possess powerful innate immune systems to combat viral infections, the role of the gut microbiome remains critical to how insects respond to infection. This study underscores the importance of contextually considering gut microbiota composition in host immune response to baculoviruses as this carries significant implications for lepidopteran biocontrol applications. Additionally, the microbiome context may impact the efficiency of *in-vivo* production of baculoviruses, which relies on high mortality rates for optimal virus yields at the factory level. A better understanding of how the antiviral peptide gloverin modulates immune response could also provide new leverage for sustainable baculovirus production

Acknowledgement

This project was funded by the Marie Curie Action Horizon 2020 INSECTDOCTORS project, grant number 859850. The authors also acknowledge the University of Valencia for their donation of *Spodoptera exigua* eggs.

Conflict of Interest

The authors declare no conflict of interest.

References

1. Stevens, E. J., Bates, K. A. & King, K. C. Host microbiota can facilitate pathogen infection. *PLoS Pathog.* **17**, e1009514 (2021).

2. Douglas, A. E. Nutritional interactions in insect-microbial symbioses: Aphids and their symbiotic bacteria Buchnera. *Annu. Rev. Entomol.* **43**, 17–37 (1998).

3. Ravenscraft, A. & Coon, K. L. 40 On: Wed. *Annual Review of Entomology Annu. Rev. Entomol. 2026* **18**, 2025 (2026).

4. Ford, S. A. & King, K. C. Harnessing the Power of Defensive Microbes: Evolutionary Implications in Nature and Disease Control. *PLoS Pathog.* **12**, e1005465 (2016).

5. Shao, Y., Mason, C. J. & Felton, G. W. Toward an Integrated Understanding of the Lepidoptera Microbiome. *Annu. Rev. Entomol.* **69**, 117–137 (2024).

6. Douglas, A. E. Conflict, cheats and the persistence of symbioses. *New Phytologist* **177**, 849–858 (2008).

7. Khan, S. A., Kojour, M. A. M. & Han, Y. S. Recent trends in insect gut immunity. *Front. Immunol.* **14**, 1–21 (2023).

8. Ma, E. *et al.* Interaction of Viruses with the Insect Intestine. *Annual Review of Virology* vol. 8 115–131 Preprint at https://doi.org/10.1146/annurev-virology-091919-100543 (2021).

9. Shao, Y. *et al.* Symbiont-Derived Antimicrobials Contribute to the Control of the Lepidopteran Gut Microbiota. *Cell Chem. Biol.* **24**, 66–75 (2017).

10. Zhang, X. *et al.* The gut commensal bacterium Enterococcus faecalis LX10 contributes to defending against Nosema bombycis infection in Bombyx mori. *Pest Manag. Sci.* **78**, 2215–2227 (2022).

11. Upfold, J., Rejasse, A., Nielsen-Leroux, C., Jensen, A. B. & Sanchis-Borja, V. The immunostimulatory role of an Enterococcus-dominated gut microbiota in host protection against bacterial and fungal pathogens in Galleria mellonella larvae. *Frontiers in Insect Science* **3**, 1–15 (2023).

12. Gilliland, C. A., Mugo-Kamiri, L., Martin, S. & Vogel, K. J. Gut bacteria induce heterologous immune priming in Rhodnius prolixus encompassing both humoral and cellular immune responses. *PLoS Pathog.* **21**, e1012947 (2025).

13. Kuss, S. K. *et al.* Intestinal microbiota promote enteric virus replication and systemic pathogenesis. *Science (1979).* **334**, 249–252 (2011).

14. González, R. *et al.* Microbiome composition modulates the lethal outcome of Drosophila A virus infection. *Cellular and Molecular Life Sciences 2026 83:1* **83**, 61- (2026).

15. Sun, H., Longdon, B. & Raymond, B. Effect of the microbiome on pathogen susceptibility across four Drosophilidae species. *Insect Sci.* https://doi.org/10.1111/1744-7917.70275 (2026) doi:10.1111/1744-7917.70275.

16. Matthews, A. C., Mikonranta, L. & Raymond, B. Shifts along the parasite-mutualist continuum are opposed by fundamental trade-offs. *Proceedings of the Royal Society B: Biological Sciences* **286**, (2019).

17. Szewczyk, B., Hoyos-Carvajal, L., Paluszek, M., Skrzecz, I. & Lobo De Souza, M. Baculoviruses-re-emerging biopesticides. https://doi.org/10.1016/j.biotechadv.2005.09.001 (2005) doi:10.1016/j.biotechadv.2005.09.001.

18. Passarelli, A. L. Barriers to success: How baculoviruses establish efficient systemic infections. https://doi.org/10.1016/j.virol.2011.01.009 (2011) doi:10.1016/j.virol.2011.01.009.

19. Wang, M. & Hu, Z. Cross-talking between baculoviruses and host insects towards a successful infection. *Philosophical Transactions of the Royal Society B: Biological Sciences* **374**, (2019).

20. Beas-Catena, A., Sánchez-Mirón, A., García-Camacho, F., Contreras-Gómez, A. & Molina-Grima, E. BACULOVIRUS BIOPESTICIDES: AN OVERVIEW. *J. Anim. Plant Sci* **24**, 362–373 (2014).

21. Tian, Z. *et al.* The gut microbiome of Helicoverpa armigera enhances immune response to baculovirus infection via suppression of Duox-mediated reactive oxygen species. *Pest Manag. Sci.* **79**, 3611–3621 (2023).

22. Jakubowska, A. K., Vogel, H. & Herrero, S. Increase in Gut Microbiota after Immune Suppression in Baculovirus-infected Larvae. *PLoS Pathog.* **9**, e1003379 (2013).

23. Li, S., Yu, X., Fan, B. & Hao, D. A gut-isolated Enterococcus strain (HcM7) triggers the expression of antimicrobial peptides that aid resistance to nucleopolyhedrovirus infection of Hyphantria cunea larvae. *Pest Manag. Sci.* **79**, 3529–3537 (2023).

24. Donkersley, P., Rice, A., Graham, R. I. & Wilson, K. Gut microbial community supplementation and reduction modulates African armyworm susceptibility to a baculovirus. *FEMS Microbiol. Ecol.* **99**, 1–11 (2023).

25. Che, W., Shi, T., Wu, Y. & Yang, Y. Insecticide Resistance Status of Field Populations of Spodoptera exigua (Lepidoptera: Noctuidae) From China. *J. Econ. Entomol.* **106**, 1855–1862 (2013).

26. Hafeez, M. *et al.* Metabolic-based insecticide resistance mechanism and ecofriendly approaches for controlling of beet armyworm Spodoptera exigua: a review. *Environmental Science and Pollution Research* vol. 29 1746–1762 Preprint at https://doi.org/10.1007/s11356-021-16974-w (2022).

27. Ahmad, M. & Arif, I. M. Resistance of beet armyworm Spodoptera exigua (Lepidoptera: Noctuidae) to endosulfan, organophosphorus and pyrethroid insecticides in Pakistan. *Crop Protection* **29**, 1428–1433 (2010).

28. Smits, P. H. & Vlak, J. M. Biological activity of Spodoptera exigua nuclear polyhedrosis virus against S. exigua larvae. *J. Invertebr. Pathol.* **51**, 107–114 (1988).

29. Caballero, P., Muriloz, R., Munoz, D. & Williams, T. The nucleopolyhedrovirus of Spodoptera exigua (Lepidoptera: Noctuidae) as a biopesticide: analysis of recent advances in Spain. *Rev. Colomb. Entomol.* **35**, 105–115 (2009).

30. Bianchi, F. J. J. A., Vlak, J. M. & Van Der Werf, W. Evaluation of the control of beet armyworm, Spodoptera exigua, with baculoviruses in greenhouses using a process-based simulation model. *Biological Control* **24**, 277–284 (2002).

31. Mengual-Martí, A. *et al.* Impact of covert infections with an RNA virus on the susceptibility of Spodoptera exigua to natural enemies. *BioControl* **67**, 605–615 (2022).

32. Elvira, S., Gorría, N., Muñoz, D., Williams, T. & Caballero, P. A simplified low-cost diet for rearing Spodoptera exigua (Lepidoptera: Noctuidae) and its effect on S. exigua nucleopolyhedrovirus production. *J. Econ. Entomol.* **103**, 17–24 (2010).

33. Chateigner, A. *et al.* Ultra deep sequencing of a baculovirus population reveals widespread genomic variations. *Viruses* **7**, 3625–3646 (2015).

34. Mugo-Kamiri, L., Querejeta, M., Raymond, B. & Herniou, E. A. The effect of diet composition on the diversity of active gut bacteria and on the growth of Spodoptera exigua (Lepidoptera: Noctuidae). *Journal of Insect Science* **24**, (2024).

35. Van Houte, S., Ros, V. I. D., Mastenbroek, T. G., Vendrig, N. J. & Hoover, K. Protein Tyrosine Phosphatase-Induced Hyperactivity Is a Conserved Strategy of a Subset of BaculoViruses to Manipulate Lepidopteran Host Behavior. *PLoS One* **7**, 46933 (2012).

36. Therneau T. A Package for Survival Analysis in R. Preprint at (2022).

37. Alboukadel Kassambara, Kosinski, M. & Biecek, P. survminer: Drawing Survival Curves using 'ggplot2'. Preprint at https://cran.r-project.org/package=survminer (2021).

38. Martin, M. Cutadapt Removes Adapter Sequences From High-Throughput Sequencing Reads. *EMBnet. J.* **17**, (2011).

39. Zhang, J., Kobert, K., Flouri, T. & Stamatakis, A. PEAR: A fast and accurate Illumina Paired-End reAd mergeR. *Bioinformatics* **30**, 614–620 (2014).

40. Schloss, P. D. Reintroducing mothur:10YearsLater. (2020).

41. Oksanen, J. *et al.* Package 'vegan'. *Community ecology package, version* **2**, 1–295 (2013).

42. McMurdie, P. J. & Holmes, S. Phyloseq: a bioconductor package for handling and analysis of high-throughput phylogenetic sequence data. in *Biocomputing 2012* 235–246 (World Scientific, 2012).

43. Deagle, B. E. *et al.* Counting with DNA in metabarcoding studies: How should we convert sequence reads to dietary data? *Mol. Ecol.* **28**, 391–406 (2019).

44. Wickham, H., François, R., Henry, L. & Müller, K. *Dplyr: A Grammar of Data Manipulation.* (2021).

45. Wickham, H., Chang, W. & Wickham, M. H. Package 'ggplot2' Create Elegant Data Visualisations Using the Grammar of Graphics. (2016).

46. De Cáceres, M. & Legendre, P. Associations between species and groups of sites: Indices and statistical inference. *Ecology* **90**, 3566–3574 (2009).

47. Pascual, L. *et al.* The transcriptome of Spodoptera exigua larvae exposed to different types of microbes. *Insect Biochem. Mol. Biol.* **42**, 557–570 (2012).

48. Vandesompele, J., Preter, K. De, Poppe, B., Roy, N. Van & Paepe, A. De. Accurate normalization of real-time quantitative RT -PCR data by geometric averaging of multiple internal control genes. 1–12 (2002).

49. Maussion, G. *et al.* Auto-qPCR; a python-based web app for automated and reproducible analysis of qPCR data. *Sci. Rep.* **11**, 21293 (2021).

50. Lenth R & Piaskowski J. emmeans: Estimated Marginal Means, aka Least-Squares Means. R package version 2.0.3. *https://rvlenth.github.io/emmeans/.* (2026).

51. Gerardo, N. M. & Parker, B. J. Mechanisms of symbiont-conferred protection against natural enemies: an ecological and evolutionary framework. *Curr. Opin. Insect Sci.* **4**, 8–14 (2014).

52. King, K. C. Defensive symbionts. *Current Biology* **29**, R78–R80 (2019).

53. Drew, G. C., Stevens, E. J. & King, K. C. Microbial evolution and transitions along the parasite–mutualist continuum. *Nat. Rev. Microbiol.* **19**, 623–638 (2021).

54. Dillon, R. J. & Dillon, V. M. The Gut Bacteria of Insects: Nonpathogenic Interactions. *Annu. Rev. Entomol.* **49**, 71–92 (2004).

55. Gillingham, M. A. F., Prüter, H., Montero, B. K. & Kempenaers, B. The costs and benefits of a dynamic host microbiome. *Trends Ecol. Evol.* **40**, 255–272 (2025).

56. Krams, I. A. *et al.* Microbiome symbionts and diet diversity incur costs on the immune system of insect larvae. *Jorge Contreras-Gardun˜ o* **10**, 14 (2017).

57. Moreno-Habel, D. A. *et al.* Inactivation of the budded virus of Autographa californica M nucleopolyhedrovirus by gloverin. *J. Invertebr. Pathol.* **110**, 92–101 (2012).

58. Liu, L. & Wang, D. Four antimicrobial peptides of Asian gypsy moth respond to infection of its viral pathogen, nucleopolyhedrovirus (LdMNPV). *Pestic. Biochem. Physiol.* **190**, (2023).

59. Thiem, S. M. Baculovirus genes affecting host function. 111–126 (2009) doi:10.1007/s11626-008-9170-5.

60. Schultz, K. L. W. & Friesen, P. D. Baculovirus DNA Replication-Specific Expression Factors Trigger Apoptosis and Shutoff of Host Protein Synthesis during Infection ☋. **83**, 11123–11132 (2009).

61. Gasque, S. N., van Oers, M. M. & Ros, V. I. Where the baculoviruses lead, the caterpillars follow: baculovirus-induced alterations in caterpillar behaviour. *Curr. Opin. Insect Sci.* **33**, 30–36 (2019).

62. Li, Z. *et al.* Baculovirus manipulation of nutrient-based host choice via an infection responsive olfactory receptor in Spodoptera exigua. *Pestic. Biochem. Physiol.* **221**, 107156 (2026).

63. Zhang, X. *et al.* Identification, gene expression and immune function of the novel Bm-STAT gene in virus-infected Bombyx mori. *Gene* **577**, 82–88 (2016).

64. Liu, W. *et al.* Immune signaling pathways activated in response to different pathogenic micro-organisms in Bombyx mori. *Mol. Immunol.* **65**, 391–397 (2015).

65. Muhammad, A., Sun, C. & Shao, Y. The gut microbiome-immunity-virus axis in lepidopteran antiviral defense. *Curr. Opin. Insect Sci.* **78**, 101602 (2026).

66. Choi, J. Y. *et al.* Analysis of genes expression of Spodoptera exigua Larvae upon AcMNPV infection. *PLoS One* **7**, (2012).

67. Savio, C., Mugo-Kamiri, L. & Upfold, J. K. Bugs in Bugs: The Role of Probiotics and Prebiotics in Maintenance of Health in Mass-Reared Insects. *Insects* **13**, 376 (2022).

68. Chen, H., Hao, D., Chen, C., Sun, Y. & Yu, X. Effects of midgut bacteria in Hyphantria cunea (Lepidoptera: Erebidae) on nuclear polyhedrosis virus and Bacillus thuringiensis (Bacillales: Bacillaceae). *Journal of Insect Science* **23**, 1–1 (2023).

69. Deng, Y. *et al.* Symbiotic Enterococcus faecalis potentiates viral pathogenesis via fructose-1,6-bisphosphate-mediated insect gut epithelial damage. *npj Biofilms and Microbiomes 2025 11:1* **11**, 215- (2025).

70. Mason, C. J., Peiffer, M., Chen, B., Hoover, K. & Felton, G. W. Opposing Growth Responses of Lepidopteran Larvae to the Establishment of Gut Microbiota. *Microbiol. Spectr.* **10**, (2022).

71. Cappellozza, S. *et al.* Identification of Enterococcus mundtii as a pathogenic agent involved in the “flacherie” disease in Bombyx mori L. larvae reared on artificial diet. *J. Invertebr. Pathol.* **106**, 386–393 (2011).

72. Tamura, K., Stecher, G. & Kumar, S. MEGA11: Molecular Evolutionary Genetics Analysis Version 11. *Mol. Biol. Evol.* **38**, 3022–3027 (2021).

FIGURES

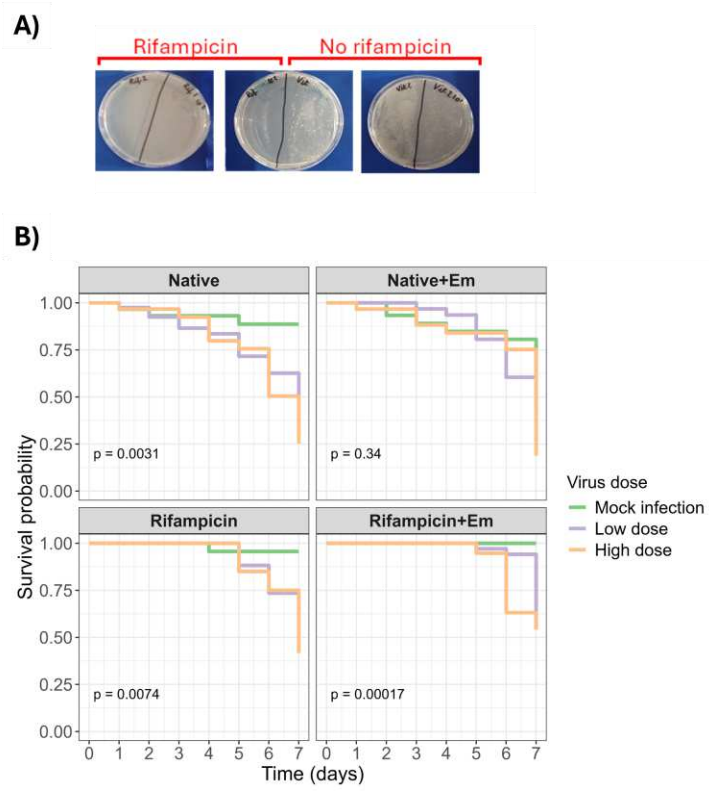


Figure 1: Effect of Rifampicin treatment on cultivable gut bacteria and survival curves of larvae infected with AcMNPV under different gut microbiota A) LB agar culture plates of larval guts showing rifampicin treated larvae had no detectable cultivable bacteria after 5 days of antibiotic treatment. **B)** Survival curves of larvae infected with AcMNPV under different gut microbiota conditions. Native: N = 99, Native+Em: N = 97, Rifampicin: N = 95, Rifampicin+Em: N = 95.

Table 1: Survival regression model summary statistics.

Treatment	Coefficients*	z-value	p-value
Rifampicin	0.5682	2.96	0.0031
Em	-0.2317	-1.67	0.0953
Virus	-0.0876	-3.09	0.0020
Rifampicin: virus	0.3833	-2.23	0.0256
Em: virus	-0.2644	1.67	0.0943
Scale = 0.252			
AIC = 703.612			

*Negative coefficients indicate a decrease in survival time while positive coefficients indicate an increase in survival time. The model did not include antibiotic: virus and antibiotic: Em interaction terms as excluding them resulted in a better fit (Lower AIC value). This model still retained some non-significant terms since removing them would have made the model worse by increasing the AIC value. A scale parameter of less than 1 indicates a decreasing hazard with time justifying the use of Weibull distribution. *P*-value threshold set at 0.05.

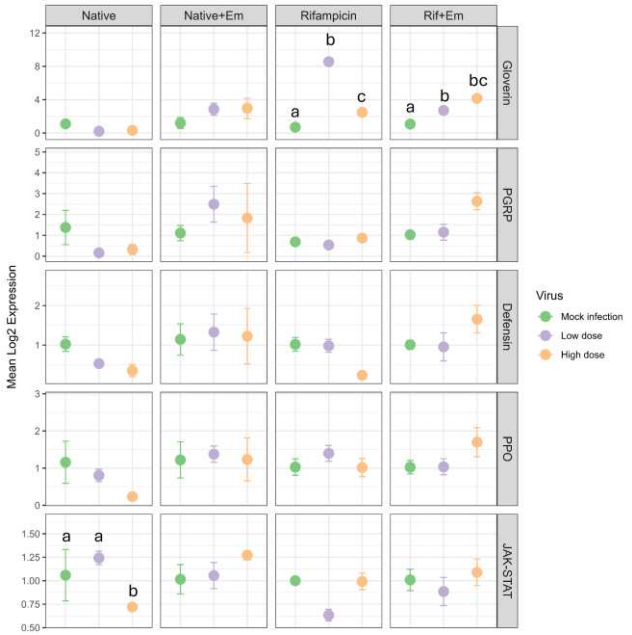


Figure 2: Gut immune gene expression of *S. exigua* during AcMNPV infection. Mean Log2 relative expression of immune genes tested (rows) in virus-infected larvae faceted by the different microbiota conditions (columns). Significant changes denoted by different letters. P-value threshold set at 0.05

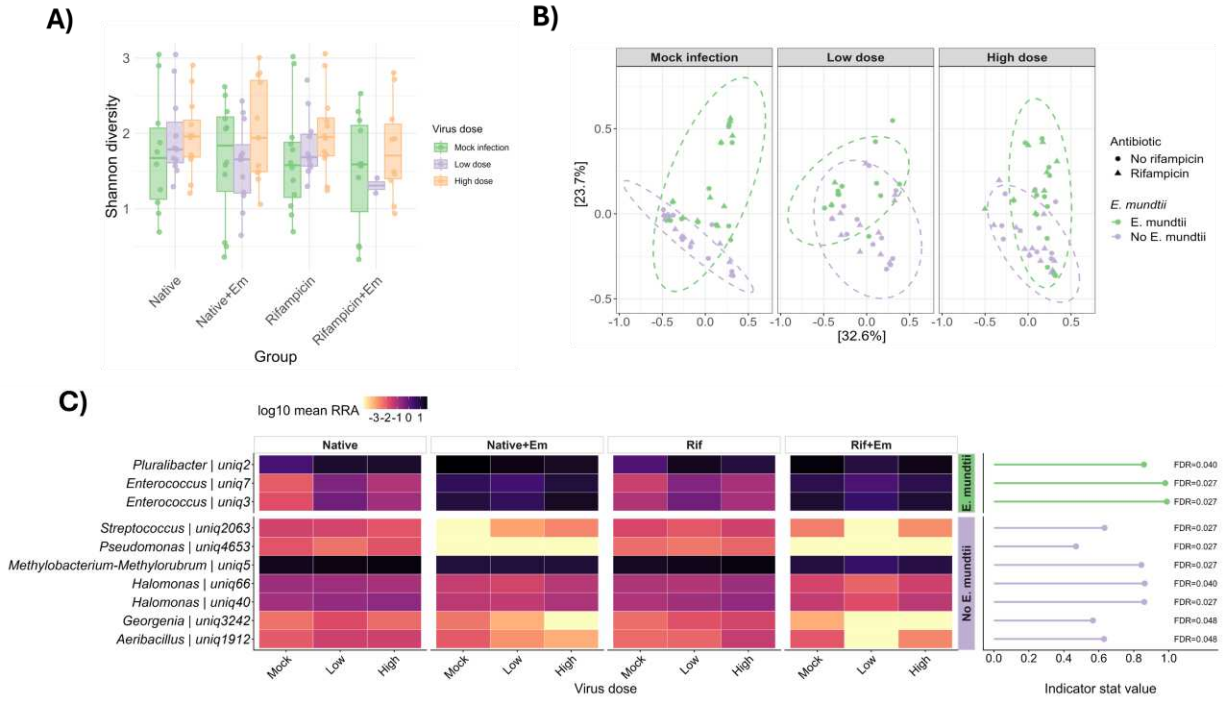


Figure 3: Effects of *E. mundtii* supplementation and virus infection on gut microbial diversity and community composition. A) Alpha diversity measured using Shannon index across treatment groups and virus doses. No significant differences in Shannon diversity were detected among treatments. **B)** Principal coordinates analysis (PCoA) of Bray–Curtis dissimilarities showing microbial community composition across *E. mundtii* supplementation, antibiotic treatment, and virus dose. Points represent individual samples, colors indicate *E. mundtii* supplementation, and shapes indicate antibiotic treatment; ellipses summarize the distribution of supplemented and non-supplemented communities within each virus-dose

treatment. **C)** Mean relative abundance of significant indicator ASVs across microbiota treatments and virus doses, displayed on a log10-transformed scale. ASVs are grouped according to their association with *E. mundtii*-supplemented or non-supplemented communities (rows). The adjacent lollipop plot shows indicator values, with larger values indicating stronger treatment associations; only ASVs significant after FDR correction (FDR < 0.05) are shown

Supplementary Material

Supplementary Table 1: Primer list of genes used for qRT-PCR

Target gene	Primer Name	Sequence 5' - 3'
Gloverin	G178-gloverin-q forward	GAAATGCTGGAAGAGGCAAG
	G179-gloverin-q reverse	ACCTCGGCCGAATAAAGAGT
Defensin	G174-defensin-q forward	GTTTGCCAGGAGCATTGTCT
	G175- defensin - q reverse	TAACGCAGATGCCGTAAGTG
JAK-STAT	A178-U12797-JAK-q forward	CGCCCTTACAGGATCATCTCA
	A179-U12797-JAK-q reverse	AGGCCGGATTCTAGGAGCTT
ATP synthase	G56-ATPsynthase- forward	GTTGCTGGTCTGGTGGGATT
	G57-ATPsynthase- reverse	AGGCCTCAGACACCATTGAAA
RpL7A	Ribosomal Protein L7A forward	TGAGCTTGTCCTCTTCCTGCCC
	Ribosomal Protein L7A reverse	GCTGCACGGTCGCCAGACTC
Peptidoglycan recognition protein (PGRP)	A152-c5919-pept-rec-q forward	GTAGTACCGGAGTGTGTTAGTGATGAG
	A153-c5919-pept-rec-q reverse	TTGTCCTATATCAGTGAATCCACGTT
Prophenoloxidase activating enzyme (PPO)	A150-c4232-PPOact-q forward	AGCTGTGCGGCCCAGAT
	A151-c4232-PPOact-q reverse	TCGACACCGCAACATTCACT

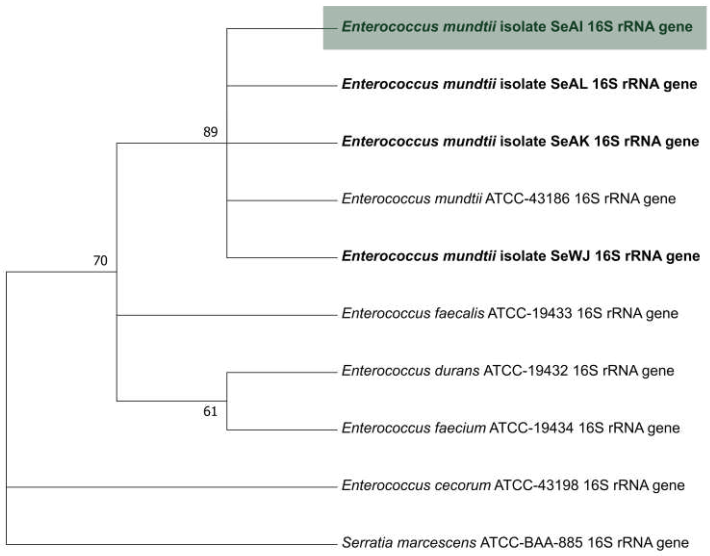
Supplementary Table 2:

Sample metadata with biosample and bioproject accession numbers from NCBI can be viewed here (https://docs.google.com/spreadsheets/d/17QWf0EmjebSvpmeO8jJDKH9sl1aYgQlFFnYPbs3rgX8/edit?gid=0#gid=0)

Enterococcus mundtii isolation and species phylogeny

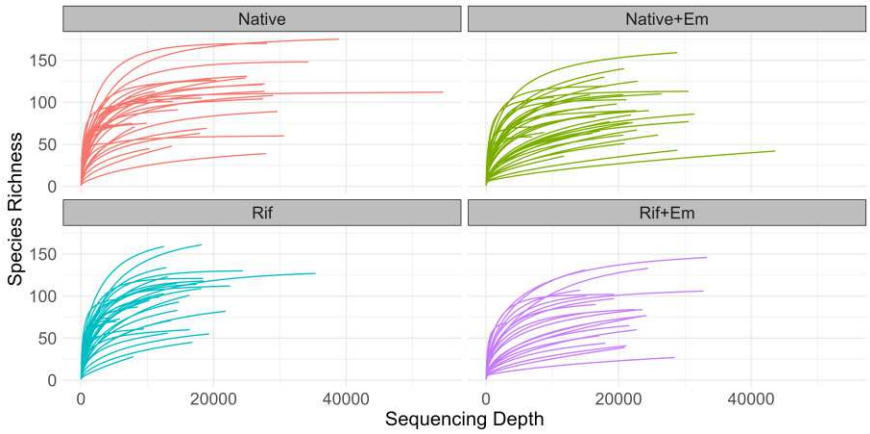
Pooled homogenized gut tissues from 10 third instar *S. exigua* larvae from a previous study [34] were recovered from glycerol stocks stored at -80°C. The homogenate was plated in Luria Bertani (LB) media and incubated for 48 hours at 30°C. Total of 9 distinct colonies based on morphological characterization were re-streaked to obtain pure colonies. Single pure colonies were grown in LB broth overnight at 30°C and the cells were used for molecular verification of the species. DNA was extracted using the QIAgen DNAeasy blood and tissue kit following manufacturer's instructions. PCR amplification was performed using the 16S primers 341F (5'-TACACGACGCTCTTCCGATCTTCATCATCCTAC-3') and 805R (5'-CAGACGTGTGCTCTTCCGATCACT ACH-3') with an initial denaturation at 95°C for 5 minutes, followed by 25 cycles of denaturation at 95°C for 40 seconds, annealing at 55°C for 2 minutes, primer elongation at 72°C for 1 minute and a final extension of 72°C for 7 minutes. Samples were sent for Sanger sequencing at Eurofins (Germany). A

Genbank BLAST search of the 16S rRNA gene sequences revealed that 4 of these isolates had > 99% sequence similarity to *Enterococcus mundtii*. A phylogenetic tree was constructed using the 16S rRNA gene sequences of these 4 isolates. Taxonomically related species were identified through the maximum likelihood approach in MEGA 11[72], revealing that the isolates were closely related to *Enterococcus mundtii* type strain (Supplementary Fig.1). *E. mundtii (*strain SeAI) was selected for further experiments (Supplementary Fig. 1). Bacteria inoculant for larval feeding was prepared by growing the *E. mundtii* strain SeAI in 5ml LB broth overnight at 30°C with shaking.

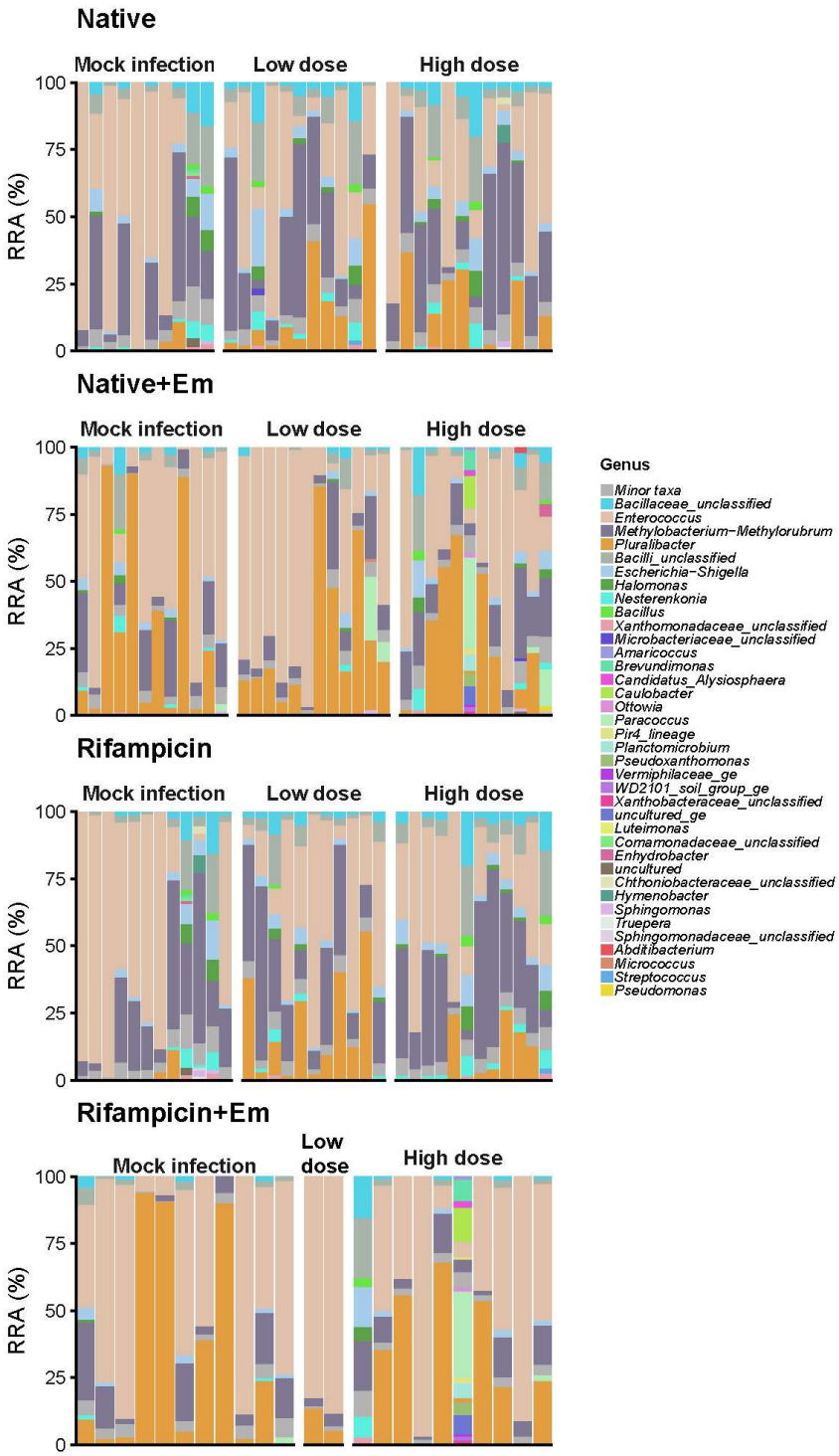


Supplementary Figure 1: Maximum Likelihood phylogenetic tree based on partial 16S rRNA gene confirming the four isolates belong to the species Enterococcus mundtii **(in bold).** Branch values represent the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test from 500 replicates. ATCC strains represent the reference type-strain species. Highlighted in green is the strain used in this study.

Rarefaction Curves



Supplementary Figure 2: Rarefaction curves of samples at treatment group levels reached a plateau showing that sequencing depth was sufficient to capture the microbial biodiversity present.



Supplementary Figure 3: Overall percentage relative read abundance of S. exigua larvae with intact native gut bacteria (Native), Native supplemented with *E. mundtii* (Native + Em), treateed with the antibiotic rifampicin to reduce the native gut microbiota and Rifampicin-treated larvae supplemented with *E. mundtii* and infected with two doses of AcMNPV baculovirus. Minor taxa represent the taxa with < 1% relative abundance.